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Assessing the Contribution Antiretroviral Therapy to Neuronal Damage and Death as a Mediator of Cognitive Decline in HIV-Associated Neurocognitive Disorders

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Abstract
The advent of combination antiretroviral therapy (ART) in 1996 revolutionized the treatment of HIV/AIDS and significantly decreased the incidence of HIV-associated neurocognitive disorders (HAND), a spectrum of HIV-related CNS dysfunctions ranging from mild cognitive deficits to severe dementia. Although the long-term prognosis for ART-treated, HIV-positive individuals continues to improve, the life-expectancy for this population remains 10-30 years less than that of uninfected individuals. Additionally, the clinical and pathologic presentation of HAND has evolved from a subacute, subcortical encephalitic condition, to a prolonged, cortical, neurodegenerative disease with pathological features that resemble those found in Alzheimer Disease (AD). The specific mechanisms driving these pathological changes remain unknown, although emerging evidence suggests that antiretroviral neurotoxicity may be a significant contributing factor. Here, we examined mechanisms by which antiretroviral drugs induce stress in neurons leading to changes in amyloid precursor protein (APP) processing. Utilizing in vitro models of acute ART exposure, we observed that HIV protease inhibitor (PI)-class ART drugs robustly active the unfolded protein response in primary neurons leading to translational de-repression of beta-site cleaving enzyme 1 (BACE1) by phosphorylated eIF2alpha; and augmented amyloidogenic cleave of APP. These results were corroborated in ART-treated, SIV-infected macaques were we saw increased hippocampal expression of BACE1 AND IN HAND patients where we also found similar increases in BACE1 expression in CA1 and CA3 hippocampal regions accompanied by accumulation of intraneuronal oligomeric Abeta;,. Finally, we demonstrate that inhibition of neuronal BACE1 activity in vitro protects cells from hydrogen peroxide and antiretroviral drug-mediated toxicity. From this body of work, we conclude that PI-class antiretroviral drugs play a prominent role in stress activation of CNS neurons leading to aberrant changes in APP processing and potentially contributing to neuronal damage and death in HAND. Lastly, we have identified BACE1 as an important adjunctive therapeutic target in the treatment of chronic cognitive decline in ART-mediated, HIV-positive individuals.

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ASSESSING THE CONTRIBUTION OF ANTIRETROVIRAL THERAPY TO NEURONAL DAMAGE AND DEATH AS A MEDIATOR OF COGNITIVE DECLINE IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

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ABSTRACT

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Kelly L. Jordan-Sciutto

The advent of combination antiretroviral therapy (ART) in 1996 revolutionized the treatment of HIV/AIDS and significantly decreased the incidence of HIV-associated neurocognitive disorders (HAND), a spectrum of HIV-related CNS dysfunctions ranging from mild cognitive deficits to severe dementia. Although the long-term prognosis for ART-treated, HIV-positive individuals continues to improve, the life-expectancy for this population remains 10-30 years less than that of uninfected individuals. Additionally, the clinical and pathologic presentation of HAND has evolved from a subacute, subcortical encephalitic condition, to a prolonged, cortical, neurodegenerative disease with pathological features that resemble those found in Alzheimer Disease (AD). The specific mechanisms driving these pathological changes remain unknown, although emerging evidence suggests that antiretroviral neurotoxicity may be a significant contributing factor. Here, we examined mechanisms by which antiretroviral drugs induce stress in neurons leading to changes in amyloid precursor protein (APP) processing. Utilizing in vitro models of acute ART exposure, we observed that HIV protease inhibitor (PI)-class ART drugs robustly activate the unfolded protein response in primary neurons leading to translational de-repression of beta-site cleaving enzyme 1 (BACE1) by phosphorylated eIF2α and augmented amyloidogenic cleave of APP. These results were corroborated in ART-treated, SIV-infected macaques where we saw increased hippocampal expression of BACE1 AND IN HAND patients where we also found similar increases in BACE1 expression in CA1 and CA3 hippocampal regions accompanied by accumulation of intraneuronal oligomeric Aβ. Finally, we demonstrate that inhibition of neuronal BACE1 activity in vitro protects cells from hydrogen peroxide and antiretroviral drug-mediated
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................... iii
ABSTRACT............................................................................................................................ iv
LIST OF TABLES................................................................................................................... ix
LIST OF FIGURES................................................................................................................ x

## CHAPTER 1: INTRODUCTION.............................................................................................. 1

1.1: HIV and the Neuropathogenesis of HAND................................................................. 1
1.2: Antiretroviral Therapy: Mechanisms of Action........................................................ 4
1.3: HIV and Aging in the Post-ART era.......................................................................... 7
1.4: The Unfolded Protein Response and Translational Protein Control...................... 14
1.5: Regulation and Function of BACE1 in Neurons....................................................... 22
1.6: Rationale and Hypothesis......................................................................................... 27
1.7: Bibliography............................................................................................................ 31

## CHAPTER 2: Current Understanding of HIV-Associated Neurocognitive Disorders

Pathogenesis......................................................................................................................... 48

2.1: Abstract.................................................................................................................... 49
2.2: Introduction............................................................................................................... 50
2.3: Role for Inflammation in Neuropathogenesis of HIV-Associated Neurocognitive Disorders........................................................................................................... 50
2.4: Association of HIV-1 clades/subtypes and Risk of HIV-Associated Neurocognitive Disorders........................................................................................................... 53
2.5: Association of Drugs of Abuse and Risk for HIV-Associated Neurocognitive Disorders........................................................................................................... 54
CHAPTER 5: Conclusions and Future Directions

5.1: Overview

5.2: Antiretrovirals and the Unfolded Protein Response

5.3: Mechanisms of BACE1 Regulation in Neurons

5.4: eIF2α Kinases as Therapeutic Targets in HAND

5.5: Considerations for in vivo Analysis of ART-associated CNS Damage

5.6: BACE1 Functions in Neurons: Implications for a Novel Therapeutic Target in HAND

5.7: References
LIST OF TABLES

CHAPTER 3
Table 1: Summary of human cases used for immunofluorescence staining in Figure 1.................................................................105

CHAPTER 5
Table 1: Summary of human samples from NNCT for post-mortem analysis.................................................................173
LIST OF FIGURES

CHAPTER 1

Figure 1: Phosphorylation of eIF2α by four kinases integrates cellular stress responses ................................................................. 30

CHAPTER 3

Figure 1: BACE1 and Aβ immunostaining are elevated in brains of HIV(+) cases ........................................................................ 106
Figure 2: Antiretroviral drugs administered to SIV(+) macaques induce CNS damage and elevated BACE1 ........................................................................ 108
Figure 3: Antiretroviral drugs activate the unfolded protein response and upregulate BACE1 expression in primary neurons ...................................................... 110
Figure 4: ARV drugs increase amyloidogenic APP processing in primary neurons .............................................................................. 112
Figure 5: Ritonavir upregulates neuronal BACE1 expression via translation-dependent control .................................................................................. 114
Figure 6: Ritonavir does not affect BACE1 protein stability in primary neurons ..................................................................................... 116
Figure 7: PERK regulates basal BACE1 expression in neurons ........................................................................................................ 117
Figure 8: HIV protease inhibitor-mediated BACE1 upregulation is PERK-dependent ............................................................................. 119

CHAPTER 4

Figure 1: BACE1 inhibition mitigates MAP2 and synapsin loss in primary neurons treated with hydrogen peroxide ................................................................. 146
Figure 2: BACE1 inhibition prevents MAP2 loss in ritonavir-treated neurons ....................................................................................... 147
Figure 3: Small molecule inhibitors of PKR and PERK block HIV protease inhibitor-mediated phosphorylation of eIF2α............................149

Figure 4: Pharmacological inhibition of PERK does not alleviate ritonavir-mediated neurotoxicity in primary neuroglial cultures.................................................................151

CHAPTER 5

Figure 1: Proposed mechanisms of antiretroviral drug-induced ER stress in neurons..175
CHAPTER 1

INTRODUCTION

1.1: HIV AND THE NEUROPATHOGENESIS OF HAND

Since its first clinical diagnosis in 1981, acquired immunodeficiency syndrome (AIDS) has become a global pandemic responsible for over 22 million deaths worldwide with an estimated sixteen thousand new human immunodeficiency virus (HIV) infections occurring daily (1). HIV, discovered in 1983 to be the causative agent of AIDS, is a retrovirus that primarily targets CD4$^+$ T cells and cells of the monocyte/macrophage lineage that express the CD4 receptor and one of the two required co-receptors, CXCR4 or CCR5. If untreated, HIV infection ends in the selective loss of the CD4$^+$ T cell population, leading to severe immunodeficiency and eventually death (2).

In addition to destruction of the immune system, HIV infection can also lead to a spectrum of central nervous system (CNS) complications, known collectively as HIV-associated neurocognitive disorders (HAND), in approximately 50% of infected individuals. According to the diagnostic criteria established for the assessment of neurocognitive impairment in HIV-infected individuals, the neurological deficits in HAND are divided into three diagnostic groups. This classification is based on the neuropsychological evaluation of multiple cognitive domains, including simple motor skills or sensory perceptual ability, complex perceptual motor skills, attention and working memory, learning and memory recall, verbal and language skills, abstraction and executive function. First, asymptomatic neurocognitive impairment (ANI) is defined as acquired impairment in at least two cognitive domains without a decline in activities of daily living (ADL), while mild to moderate neurocognitive impairment that affects ADL is termed minor neurocognitive disorder (MND). Finally, moderate to severe impairment in two or more domains with marked impact on ADL is defined as HIV-associated dementia (HAD).

Additionally, behavioral and emotional problems such as depression, psychosis, and anxiety are commonly observed in HAND patients. Since the multi-drug regimens that form the basis of antiretroviral therapy (ART) have become the mainstay for HIV treatment, the clinical
presentation and the course of HAND have become more unpredictable. The severity of deficits appears to fluctuate over time during the course of infection: some patients may experience continuing decline in cognitive abilities, while others show improvement of more severe neurocognitive impairments and exhibit only minor deficits for the remainder of the disease. While clinical studies clearly establish that suppression of viral replication below the level of detection is paramount to a more favorable outcome, the clinical course of HAND, once diagnosed, cannot be predicted successfully in most patients. Furthermore, while the prevalence of severe HAD has decreased in the post-ART era, the prevalence of ANI and MND, and HAND in general, has increased significantly due in part to the longer lifespan of HIV-infected individuals (3). Pathological studies have revealed that brains of ART-naïve HAND patients exhibit classic hallmarks of neuroinflammation including microglial nodules, multinucleated giant cells, astrogliosis, and myelin pallor (1). However, in the post-ART era, these hallmarks are not frequently found in post-mortem HAND brain tissue, although cyanptodendritic damage still persists (4-9). Similarly, while extensive neuronal death in the CNS of HAND patients has been mitigated in the post-ART era, there remains evidence for ongoing neuronal damage in the form of dendritic pruning, decreased synaptic density and loss of interneurons (10).

In the healthy brain, the blood brain barrier (BBB) and the blood-cerebrospinal fluid barriers are the first lines of defense against invading pathogens; however, HIV can circumvent these barriers and enter the CNS within 1-2 weeks after systemic infection. The mechanism(s) of HIV neuroinvasion remain a matter of speculation, however there is evidence that HIV can enter via transcytosis across endothelial cells or within infected monocytes (11, 12). The latter model was first proposed by Haase and colleagues who used visna virus to model HIV infection and proposed that HIV, and other lentiviruses, enter the CNS as passengers in infected monocytes (13-15). According to this “Trojan Horse” model, supported by a multitude of in vitro and in vivo studies, the infected monocytes traverse the BBB and differentiate into macrophages, thus constituting the viral reservoirs in the CNS (15-18). Direct contact of the infected cells with neighboring, resident immune cells of the CNS, including perivascular macrophages, astrocytes,
and microglia, leads to their subsequent infection and activation. This population of infected immune cells then releases a variety of neurotoxic molecules, including proinflammatory cytokines, chemokines, reactive oxygen species (ROS), quinolinic and arachidonic acid, glutamate, and nitric oxide, leading to activation of uninfected immune cells and eventual neuronal damage and dysfunction observed in HAND.

Importantly, neurons themselves are not a source of productive infection in the CNS, as they lack the CD4 receptor, rather HIV replication occurs primarily in monocyte-derived perivascular macrophages and microglia (18). However, given the extensive evidence of neuronal damage in HAND, other mechanisms have been implicated in HIV-mediated neurodegeneration. The first hypothesis, known as the indirect or bystander effect hypothesis, maintains that neuronal damage is mediated largely by the immune response mounted by infected and uninfected non-neuronal cells against HIV infection, as described previously. Chronic neuroinflammmation then leads to an excitotoxic environment within the CNS, chronic activation of neuronal N-methyl-D-aspartate (NMDA) receptors and a toxic accumulation of intraneuronal Ca^{2+} leading to free radical production, oxidative stress, and activation of Ca^{2+}-dependent death proteases, such as calpains and caspases. Alternatively, the direct injury hypothesis holds that HIV viral proteins, such as gp120, Tat, and Vpr, released from infected monocytes and microglia, interact directly with neurons through a variety of mechanisms, including interaction with chemokine receptors, leading to neuronal damage and apoptosis. Despite several lines of in vitro data supporting this hypothesis, in vivo studies suggest that markers of neuroinflammation continue to persist in post-ART HAND despite suppression of HIV replication in the CSF to levels below the limit of detection. These findings suggest that indirect mechanisms of neuronal toxicity may be the major contributors to the underlying neuropathology of HAND in the post-ART era.
1.2: ANTIRETROVIRAL THERAPY: MECHANISMS OF ACTION

Initial quantitative studies of in vivo HIV-1 dynamics and virion clearance rates using novel antiretroviral drugs yielded several critical findings: 1) viremia is sustained by continuous rounds of replication, infection, and rapid cell turnover, 2) mutations in the HIV-1 genome likely occur at every position multiple times per day accounting for the failure of previously-used monotherapy, 3) when new cycles of infection are halted by antiretroviral drugs, plasma virus levels fall by as much as 99% within weeks after treatment, and 4) effective treatment of HIV must employ the use of multiple, potent antiretroviral drugs to force the viral genome to mutate at several distinct positions simultaneously (19, 20). From these landmark studies came the advent of combinatorial highly active antiretroviral therapy (HAART), now simply termed ART, which is the mainstay treatment for HIV, leading to suppression of viral replication to below the limits of detection, reconstitution of the immune system, and increased long-term survival rates in HIV-positive individuals (21).

The United States Panel on Antiretroviral Guidelines for Adults and Adolescents currently recommends the initiation of ART when CD4 cell counts are below 350 /mm$^3$ peripheral blood or in those with AIDS-defining illnesses irrespective of CD4 levels (22, 23). Initial ART regimens typically include the use of two nucleoside reverse transcriptase inhibitors (NRTI) combined with either a non-nucleoside reverse transcriptase inhibitor (nNRTI), HIV protease inhibitor (PI), or integrase inhibitor. The 24 FDA-approved antiretroviral drugs currently available fall into six broad categories based on which step of the HIV viral replication cycle they inhibit: entry, reverse transcription, integration, transcription, virus assembly and production, and protease processing (24). The first step in HIV replication involves viral entry into helper T lymphocytes and monocytes via engagement of the viral envelope glycoprotein, gp120, with the CD4 receptor followed by binding to either the CXCR4 or CCR5 co-receptors (25). These interactions induce a conformational change in the HIV envelope, leading to gp41-mediated fusion with the host cell membrane (26). This process, which lasts approximately 1 hour, is targeted by entry inhibitors, divided further into attachment inhibitors, chemokine receptor antagonists, and fusion inhibitors.
Currently, two entry inhibitors, maraviroc and enfuvirtide, are approved for use in humans, however, antibody-based drugs directed at gp120 and CD4 are in development and show promising clinical results (24, 27).

Viral entry and fusion is followed by uncoating of the viral core and intracellular injection of the single-stranded RNA genome along with various viral enzymes, including reverse transcriptase (RT), which generates double-stranded, complimentary proviral DNA from viral RNA over a period of 10 hours (24, 28). RT was the first HIV-1 enzyme targeted for antiviral treatment with the advent of zidovudine (AZT) in 1987, which comprised HIV monotherapy until combination ART in 1996 (29-31). HIV-1 RT has since become a major area of drug development, which includes nucleoside and non-nucleoside reverse transcriptase inhibitors that encompass 12 FDA-approved drugs, accounting for half of all approved antiretroviral drugs. NRTIs are administered as prodrugs that must be phosphorylated in three steps by cellular thymidine kinases to become actively antiviral in vivo (32-35). Following activation, NRTIs serve as structural analogs of native deoxynucleotides needed for reverse transcription, but lack the 3'-hydroxyl group on the deoxyribosyl moiety which prevents formation of a 5'-3' phosphodiester bond between incorporated NRTIs and incoming native nucleosides (23, 36). The net result is early chain termination of the growing viral DNA chain. nNRTIs also prevent production of proviral DNA from viral RNA but do so by binding to an allosteric, hydrophobic site proximal to the active site, thereby changing its conformation and inhibiting its catalytic activity (37-39). Unlike NRTIs, nNRTIs do not require phosphorylation to become active in vivo and they do not inhibit the RT activity of other lentiviruses. One advantage of nNRTIs compared to NRTIs is their ability to evade pyrophosphorolysis as a viral resistance mechanism, which selectively removes NRTIs from the 3' end of the nascent chain (24, 38). However, viral resistance to nNRTIs has emerged in the form of amino acid substitutions in the allosteric binding site that prevents nNRTIs from binding (38, 40, 41).
Reverse transcription and generation of proviral DNA leads to assembly of the pre-integration complex which is transported into the nucleus of the host cell and integrated in the host genome, a process that is catalyzed by the integrase enzyme and occurs within roughly 20 hours of initial cellular infection (42). Integration occurs in three sequential steps: assembly of the viral DNA, removal of 2-3 nucleotides from one or both 3’ ends of the viral DNA, and finally strand transfer in which viral DNA is ligated to the host genome (43). Integrase inhibitors represent the newest class of antiretrovirals, with two FDA-approved drugs, raltegravir and dolutegravir, that specifically inhibit strand transfer by preventing binding of the pre-integration complex to the host DNA (44). These drugs have proven to be very efficacious in preventing viral replication in randomized clinical trials, and in some cases outperformed more widely-used drugs, such as efavirenz (45).

Integration is a necessary precursor to transcription of viral genes leading to the production and assembly of mature infectious HIV-1 particles. HIV transcription is unique in that it requires binding of a regulatory protein, Tat, to the HIV RNA element (TAR) for transcript elongation, making it a promising therapeutic target (24, 46). However, efforts to design small molecule inhibitors against this process have proved to be inefficacious in clinical trials. Similarly, while HIV assembly and maturation has become an area of increasing interest for drug discovery, inhibitors, such as betulinic acid, have not shown evidence of sufficient in vivo antiviral activity to be considered viable for use in humans (47).

The final step in the 24 hour intracellular HIV-1 replication cycle is proteolytic cleavage of the viral gag and gag-pol polyprotein precursors by the HIV-1 aspartyl protease enzyme at the inner surface of the host cell plasma membrane leading to generation of mature virions (37, 48). Targeting the last essential enzyme in the viral replication cycle, HIV protease inhibitors (PIs) constitute some of the most potent antiretroviral agents developed to date. The 10 approved PIs all share similar peptidomimetic structural features and bind to the active site of the HIV protease inhibiting its ability to cleave viral polyprotein precursors (49). Given the large size and
lipophilicity of PIs, co-administration of a boosting agent, ritonavir, is typically recommended to enhance bioavailability via reduction of CYP450-mediated inactivation in the liver during first-pass metabolism (50, 51).

Despite the remarkable achievement of ART in transforming HIV-1 disease into a manageable, chronic disorder, numerous issues remain including treatment failure, poor adherence to life-long therapy due to drug toxicity and pill burden, and sub-optimal pharmacokinetics of ART in many patients (52). Fixed-dose combination antiretrovirals, such as Kaletra (lopinavir/ritonavir) have been developed to improve regimen adherence and reduce pill burden for patients, while efforts to enhance the pharmacokinetic profile of ART drugs include the use of nanoformulated ART (NanoART). Recently, Gendelman and colleagues developed a long-acting, folic-acid coated, NanoART formulation that improved targeting of ART to HIV-infected macrophages while increasing plasma and tissue drug concentrations by five-fold up to two weeks after a single dose in mice (53, 54). More importantly, these NanoART formulations were also shown to improve biodistribution of ART to viral reservoirs in the CNS, leading to speculation that nano-targeting of ART could alleviate the growing problem of HAND persistence in the post-ART era (55-57). However, the toxic side effects of many ART drugs has led to questions regarding the role of antiretrovirals in the persistence of HAND among the aging HIV-positive population in the developing world.

1.3: HIV AND AGING IN THE POST-ART ERA

The widespread use of ART has drastically decreased the incidence of AIDS-related complications and improved the long-term prognosis of HIV-positive individuals. As of 2011, 30% of the HIV-positive population in the United States was over the age of 50, and by 2015 it is estimated that more than 50% will be over the age of 50 (58, 59). Despite this remarkable development, the life expectancy for ART-treated, HIV-positive individuals remains 10-30 years less than that of uninfected individuals (60). Given the rapid global expansion of this population,
it has become increasingly important to understand the risk factors that lie at the intersection of HIV, ART, and aging.

Older HIV-positive patients, including those treated with ART, are at increased risk for systemic diseases including atherosclerosis, liver and kidney failure, cancer, and osteoporosis (60, 61). The aging brain may also be more vulnerable to the effects of HIV as older adults display an increased susceptibility to HAND, and emerging evidence suggests an increased prevalence of neurodegenerative diseases, including Alzheimer and Parkinson, in this patient population (62, 63). It remains unclear if the increased prevalence of HAND is a result of HIV and related comorbidities, including hypertension, insulin resistance, and lipodystrophy, or other confounding factors such as immunosenescence and ART toxicities, all of which are likely to impact CNS disease progression in older HIV-positive individuals (64, 65).

Antiretroviral therapy effectively limits HIV disease progression, maintains patients in a state of partial immune competence, and arrests subjects in a pre-symptomatic state (66). However, despite the ability of ART to reduce plasma HIV RNA to undetectable levels, HIV-positive individuals remain at higher risk for opportunistic infections and premature death (3, 67). Thus, ART may reduce, but does not appear to eliminate, premature and/or accelerated aging in HIV-infected individuals. This may be attributed to many factors including drug toxicity and slower immune recovery following ART initiation in older patients, compared to younger adults (68). Furthermore, advanced age has been linked to decreased production of T cells, B cells, and cytokines, as well as to chronic immune activation, the latter of which may be linked to the breakdown of gut-associated lymphoid tissue (GALT) and to the elevated levels of systemic lipopolysaccharide (LPS) (69, 70).

Older patients also display a dampened recovery of CD4 cells following treatment with ART, which may increase their risk for systemic diseases ranging from heart disease to cancer (70). Thus, it is not surprising that advanced age at seroconversion and/or onset of ART treatment is considered a major risk factor for severe HIV disease (71, 72). Gotez et. al.
performed a retrospective study on HIV-positive patients receiving ART treatment at the Veteran’s Administration Greater Los Angeles Medical Center between 1996 and 1999, and found that for every 10 years of additional age at the onset of ART treatment, the rate of CD4 cell replenishment decreased by 35 cells per microliter of blood (73). Despite the obvious benefits of beginning ART treatment in asymptomatic HIV-positive individuals, there remain significant concerns for initiating drug therapy sooner than necessary and how this may negatively impact drug toxicity, long-term patient outcome, and the evolution drug resistant strains of HIV (72, 74). Based on the updated recommendations for treatment initiation, all HIV-infected individuals will be put on an ART regimen upon diagnosis, and the impact of this approach on older patients will be revealing.

Lower CD4 count, in addition to advanced age, also places older patients at a nearly four-fold higher risk for liver-related mortality compared to younger patients (75). This risk is exacerbated by other factors commonly afflicting ART-treated, HIV-positive individuals including diabetes, alcohol abuse, as well as antiretroviral and cholesterol drug toxicity (75). Among all non-AIDS-related complications, liver disease is the primary cause of death in HIV-positive patients (75). In addition, older HIV-positive individuals are at increased risk for frailty, bone loss, and non-AIDS related cancers (76, 77). It remains unclear if HIV itself places older individuals at higher risk for heart disease compared to older, HIV-negative individuals, though specific classes of antiretrovirals, especially protease inhibitors, have been linked to atherosclerosis (69, 78, 79).

The CNS is particularly susceptible to the synergistic neurodegenerative effects of HIV and aging. Several studies have demonstrated that, compared to younger (age 20 to 39 years) cohorts, older HIV-positive individuals (age >50 years) display decreased neurocognitive functioning in several areas including memory, psychomotor speed, and executive functions (59, 80, 81). The persistence of HAND in individuals with an undetectable viral load and CD4 cell counts greater than 200/µL is not well understood and may be a result of aging-associated processes rendering the cells of the CNS more vulnerable.
Several recent neuroimaging studies have begun to address the structural, physiological, and functional changes in the CNS in the context of HIV and aging. Six MRI investigations that assessed the structural changes in the brains of older HIV-positive individuals between 1998 and 2012 found evidence of premature or accelerated aging characterized by significant brain atrophy in the basal ganglia, cerebellum, and frontal and temporal brain regions, when compared to seronegative controls (64). However, several diffusion tensor imaging (DTI) studies found only normal, age-dependent changes in mean diffusion and fractional anisotropy, which reflects the directionality of water diffusion in the brain, and is greater along organized white matter tracts, but decreased in pathologically damages, disorganized tracts (64, 82-84).

Other studies have employed proton magnetic resonance spectroscopy (MRS) to assess changes in brain metabolite levels that are indicative of neuronal damage and death or glial activation. Ernst and Chang demonstrated a five-fold acceleration of aging effects in a relatively young (mean age 36 years) ART-naive, HIV-positive cohort, as compared to HIV-negative controls, as reflected by increased levels of glial activation markers, myoinositol (MI), and choline compounds (CHO) and a decrease in the neuronal marker, N-acetylaspartate (NAA) (85). A recent multicenter MRS study of slightly older (ages 30-70), ART-treated HIV-positive individuals demonstrated elevated MI and CHO in all brain regions of patients with asymptomatic or mild neurocognitive impairment, but decreased levels of MI in those with dementia, which the authors interpreted as premature microglial senescence (64, 86). In addition, this study found an age-dependent decrease in NAA in frontal white matter, but only in patients with HAD (87). Thus, while ART-naive, HIV-positive patients show evidence of increased, age-dependent glial activation and neuronal damage leading to accelerated aging, ART-treated individuals show only signs of premature aging.

As discussed previously, the clinical and pathological hallmarks of post-ART HAND differ from those in the pre-ART era. While HAND presented as a subcortical dementia afflicting the basal ganglia and white matter, many post-ART studies suggest the focus of the inflammation
has shifted primarily to the hippocampus, even in effectively treated patients (88-90). Furthermore, there is emerging evidence that pathologic similarities exist between HAND and some common neurodegenerative disorders such as Alzheimer disease, which is characterized by the presence of extracellular beta amyloid (Aβ) plaque deposits and intracellular neurofibrillary tangles composed of hyperphosphorylated Tau (61, 66, 91, 92). In vitro work involving the viral protein Tat has demonstrated the ability of this viral protein to inhibit the activity of the Aβ-degrading enzyme, Neprilysin, and bind to the receptor for advanced glycation end products (RAGE), all of which may promote Aβ accumulation in the CNS. Indeed, some individuals with HAND display CSF levels of Aβ42 comparable to those observed in AD patients (61, 93, 94). Among the studies underlining the similarities between HAND and AD, Esiri et al. were the first to report a predisposition to plaque formation in the brains of pre-ART, HIV-positive individuals (95). Such pathological changes have since been observed in HIV-positive patients despite successful virologic control with ART, suggesting that antiretrovirals either cannot achieve therapeutic concentrations within the brain parenchyma, allowing for ongoing viral replication and neuroinflammation, or may have toxic effects that could facilitate neurodegeneration (96, 97). To address the latter concern, several reports investigated differences in either phospho-Tau or Aβ in ART-naïve vs. ART-treated individuals. Two groups independently reported elevated Aβ deposition/accumulation in the hippocampus of ART-treated individuals compared to pre-ART patients, while Bell and colleagues reported only increased hyperphosphorylated Tau, but no Aβ deposition in the hippocampus and entorhinal cortex of HIV-positive individuals (61, 90, 92, 98). To date, no group has reported concomitant phospho-Tau accumulation and Aβ plaque deposition in the same brain samples from HIV-positive cohorts. Although differences in patient age and the antibodies used to detect Aβ may account for the varied outcomes of these reports, studies utilizing the amyloid-binding, carbon 11-labeled Pittsburgh compound B (11C-PiB) and PET imaging found that irrespective of neurocognitive impairment, HIV-positive individuals showed no increase in 11C-PiB levels, highlighting a potential key difference between Aβ metabolism in HAND vs. AD despite some overlapping pathological features (91, 99).
Importantly, the aforementioned studies highlight the potentially underappreciated concern of antiretroviral-associated neurotoxicity and its effect on neuropsychological outcomes in long-term ART-treated patients. ART drugs have been linked to wide-ranging, peripheral metabolic and neural disturbances that could themselves influence the progression of HAND and foretell potential mechanisms of toxicity in the CNS (78, 100-111). Specifically, PIs have been associated with ER stress and the development of premature atherosclerosis as well as metabolic syndrome, characterized by lipohypertrophy, hypercholesterolemia, and insulin resistance (78, 112-114). Yet, the most serious limitation of ART drugs, specifically NRTIs, is mitochondrial dysfunction and oxidative stress, manifested clinically as peripheral neuropathy and myopathy (100, 115). NRTI toxicity is specific to myocytes and peripheral neurons due to the presence of thymidine kinase isoforms in these cell types capable of phosphorylating the prodrugs to their active form. Following phosphorylation, NRTIs are sequestered by DNA polymerase γ and are either incorporated into mitochondrial DNA (mtDNA), causing permanent damage, or terminate the growing DNA chain, depleting mtDNA-encoded enzymes necessary for the electron transport chain (ETC). Decreased mtDNA results in the loss of mitochondrial membrane potential, decreased oxidative phosphorylation, increased reliance on glycolysis for ATP production, and lactic acidosis. While direct CNS effects of ART are poorly understood, Schweinsburg et. al. demonstrated an association between NRTIs and decreased levels of frontal white matter NAA, which they attributed to NRTI-mediated mitochondrial dysfunction and depletion of cellular respiration (102). Recently, a cross-sectional analysis of 540 HIV-infected individuals in Amsterdam found that cumulative use of high dose ritonavir (> 400 mg/24 hours) was associated with an increased risk factor for age-associated non-communicable comorbidities, potentially due to the known effects of ritonavir on endothelial dysfunction and induction of premature cellular senescence (116).

Confounding the issue of direct CNS toxicity of antiretroviral medications is the variability in BBB permeability amongst different drug classes as determined by various physicochemical properties such as plasma protein binding, lipophilicity, molecular size, and interaction with drug
transporters in the BBB, such as P-glycoprotein (P-gp) and multidrug resistance associated proteins. Based on these properties, a scoring system, called the CNS penetrance effectiveness (CPE) score, was developed to estimate the efficacy with which various ART drugs would reach therapeutic concentrations in the brain (117). ART drugs with poorer CNS-penetrating properties, such as nelfinavir and saquinavir, are given a score of 0, while those with intermediate penetrance are given a score of 0.5, and others with high penetrance, such as AZT, receive a score of 1. However, the accuracy of this scoring system has recently been called into question based on a number of experimental and clinical studies that refute these assumptions. For example, based on the CPE scoring system many PIs, which are targets of P-gp transporters, have high molecular weights (>500 Daltons) and are highly protein bound (up to 99%) in blood, are estimated to achieve sub-therapeutic concentrations in the brain based on CSF levels measured in humans (118, 119). Yet, a recent pharmacokinetic analysis of ART administration in guinea pigs showed significantly higher uptake of [³H] ritonavir into brain parenchyma compared to CSF, suggesting that clinical estimates of CNS penetrance for some antiretrovirals may be lower than actual drug concentrations in the brain (50, 120, 121).

Although it is widely held that ART regimens with higher CNS penetrance generally confer improved neuropsychological outcomes in HIV-positive individuals, numerous clinical studies have suggested these regimens may negatively impact cognition. In a prospective study, Marra et. al. found a significant association between highly CNS-penetrant ART regimens and worse neurocognitive and motor performance 24 and 52 weeks after beginning or changing therapy, despite decreased CSF HIV RNA in a small HIV-positive cohort (122). In addition, Robertson et. al. reported an improvement in neuropsychological outcomes in a cohort of patients with interrupted drug treatment (123). Studies using SCID mice, which display neuropathological hallmarks similar to those associated with HIV, showed reduced viral load and astrogliosis following administration of ART, but no improvement in cognitive dysfunction (124). Altogether, these studies reinforce an emerging hypothesis that chronic use of antiretroviral drugs may be contributing to the rising prevalence of HAND in the aging HIV-positive population.
In contrast, numerous clinical studies have demonstrated beneficial effects on neurocognitive functioning by ART regimens with high CPE scores (125, 126). A cross-sectional study of 2636 adults from the AIDS Clinical Trials Group Longitudinal Linked Randomized Trials (ALLRT) cohort on effective ART for at least 6 weeks showed better neurocognitive performance in individuals receiving ART medications with higher CPE scores, although in some cases, participants required more than 3 antiretrovirals to treat HIV in the CNS. (127). Letendre et. al. demonstrated improvements in cognition over a 15-week period in patients beginning ART with higher CPE scores versus those beginning ART with lower CPE scores (128). Another recent investigation utilized MRS imaging to investigate the effect of multiple ART regimens with various CPE scores on changes in brain NAA metabolite levels. Over 48 weeks, HIV-positive, ART-naïve individuals receiving regimens with the highest CPE scores displayed the greatest increases in NAA levels and showed the most improvement in a battery of neuropsychological tests (129). These studies suggest that ART medications with higher CNS penetrance may have a neuroprotective effect, at least initially, in successfully treated HIV-positive adults. Importantly, these studies assess the effects of ART only after initiation of therapy, but given the well-characterized toxicities of ART medications in the periphery and the potential impact of related co-morbidities on brain pathology, the long-term synergistic effects of aging and ART warrant a careful longitudinal examination of the safety profile of these drugs in the CNS.

1.4: THE UNFOLDED PROTEIN RESPONSE AND TRANSLATIONAL PROTEIN CONTROL

The unanticipated persistence and evolution of HAND in the post-ART era has led to a concerted effort to understand the underlying molecular pathways affected in chronic HIV-1 disease in the CNS. As discussed previously, there is extensive evidence for chronic neuroinflammation, oxidative stress, and accumulation of neurodegenerative proteins in brains of ART-medicated, HIV-positive individuals. Common among these various conditions is their ability to induce ER stress and activate the unfolded protein response (UPR), a ubiquitous cellular response mechanism conserved from yeast to humans (130). While the UPR has long been implicated in
many neurodegenerative diseases, such as Parkinson and Alzheimer disease, Lindl et. al. were the first to show evidence of ER stress activation in HAND, as evidenced by increased CNS expression of the ER-resident molecular chaperone, BiP, ATF-6β, and phosphorylated eIF2α (p-eIF2α) (131-138). These findings suggest that chronic UPR activation, which has been linked to long-term memory loss and neurodegeneration, may play an important role in the pathogenesis of HAND (139, 140).

In eukaryotes the UPR is comprised of three major pathways, each under the control of an upstream effector protein, PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), located in the ER membrane (141, 142). In the absence of ER stress, each of the effector proteins is kept inactive via interaction at its luminal domain with the molecular chaperone, BiP/GRP78 (Immunoglobulin heavy-chain binding protein/glucose-regulated protein 78), which serves as the main regulator of the UPR (143). Accumulation of misfolded proteins and exposed hydrophobic residues in the ER disrupts the interaction between BiP and PERK, ATF6, and IRE1α, permitting their subsequent activation (144). The most evolutionarily conserved pathway of the UPR, IRE1α is a 100 kDa type I transmembrane protein that homo-oligomerizes after dissociation from BiP and activates both its kinase and endoribonuclease activity via autophosphorylation of its C-terminal kinase domain (145). IRE1α regulates the expression of numerous molecular chaperones and protein degradation genes via use of its endoribonuclease activity, which is utilized to non-conventionally splice a 26-nucleotide intron from XBP1-mRNA (146-148). The resulting 376 amino acid spliced XBP1 acts as a potent transcription factor that enhances expression of many genes including P58IPK, which inhibits PERK activity (149). If transient UPR activation is unable to restore cellular homeostasis, sustained IRE1α activation leads to autophagy and later apoptosis through its regulation of B-cell lymphoma 2 (Bcl2) expression and activity (142, 150).

ATF6 activation is unique among the three branches of the UPR in that it does not involve dimerization and autophosphorylation, but rather translocation to the Golgi and sequential
regulated intramembrane proteolysis in two steps by serine protease site-1 protease (S1P), which cleaves at the luminal domain, followed by N-terminal cleavage by metalloprotease site-2 protease (S2P) (141, 147). The resulting 50 kDa cleaved ATF6 product constitutes the transcriptional domain, which translocates to the nucleus and activates transcription of a cohort of genes with ATF/cAMP and ER stress response elements (ERSE) in their promoters (151). These genes include molecular chaperones, such as BiP, transcription factors, and ER-associated degradation (ERAD) machinery (142). The ATF6 transcriptional profile is ultimately a cytoprotective, pro-survival program designed to aid cells in adapting to sub-toxic accumulation of misfolded proteins.

Under mild stress conditions, PERK, a serine-threonine kinase, is the first initiator protein to be activated in the UPR, although following sustained ER stress PERK can initiate pro-death signaling, similar to IRE1α. Following dissociation from BiP, PERK dimerizes and activates its kinase domain via autophosphorylation. This phosphorylation event results in a subsequent, PERK-mediated phosphorylation of the eukaryotic translation initiation factor 2 (eIF2) at Ser51 of the α subunit and global repression of cap-dependent protein translation (152, 153). eIF2 is composed of three subunits, α, β, and γ, and when bound to GTP and Met-tRNA, comprises the ternary complex that binds to the 40S ribosomal subunit to form part of the 43S pre-initiation complex (154, 155). After binding to the eIF4F cap recognition complex at the 5’ end of mRNA, the pre-initiation complex scans the 5’ leader sequence for an AUG start codon. Once an AUG is recognized within a favorable sequence for translation initiation, GTP is hydrolyzed releasing eIF2 from the pre-initiation complex allowing the 60S ribosomal subunit to bind the mRNA and begin the elongation phase of translation (156). Phosphorylation of eIF2α inhibits this process by blocking the dissociation of eIF2 from eIF2B, a guanine nucleotide exchange factor that is needed for the exchange of GDP for GTP on the γ subunit of eIF2 (157). This converts eIF2 from a substrate into a competitive inhibitor of eIF2B, slowing the exchange rate of GDP for GTP and limiting the availability of competent ternary complex to form the pre-initiation complex (158). Repression of global protein synthesis ultimately reduces influx of new mRNAs into the ER,
allowing folding enzymes and molecular chaperones to reduce the burden of misfolded proteins and prevent cellular toxicity.

Importantly, eIF2α is phosphorylated by numerous kinases that respond to a wide variety of stress factors, highlighting its importance in cellular adaptation and protein translational control (Figure 1). The first eIF2α kinase identified was heme-regulated inhibitor of translation (HRI) kinase, which serves the critical function of balancing the amount of globin production with the amount of heme available by inhibiting protein translation in reticulocytes in response to low heme levels (159-162). HRI binds heme at its N-terminus which triggers the formation of stable HRI dimers (162). When heme levels are low, HRI dimers undergo autophosphorylation, similar to PERK, activating its kinase domain and leading to eIF2α phosphorylation and inhibition of global protein synthesis (160). HRI knockout mice display hyper-sensitivity to heme deficiency but no other physiological abnormalities (163-165). HRI is not thought to play a significant role in neuronal p-eIF2α levels given that it is primarily expressed in erythroid cells and has no known roles in other cell types.

Double-stranded RNA-dependent protein kinase (PKR) is a cytosolic, constitutively-expressed, mammalian kinase that was discovered after the initial observation that extracts from vaccina virus-infected cells treated with interferon showed enhanced sensitivity to translational inhibition after addition to a cell-free system of exogenous mRNAs or synthetic double-stranded RNA (dsRNA) (166-168). Follow-up studies revealed that activation of PKR kinase is stimulated through binding of dsRNA at its N-terminal double-stranded RNA-binding domains (DSRBs), leading to dimerization and autophosphorylation of its kinase domain (167). Downstream phosphorylation of eIF2α limits translation of viral mRNAs while enhancing the expression of antiviral proteins (159, 169, 170). PKR can also be activated by a variety of dsRNA-independent stress conditions including type 1 interferons, oxidative stress, and ER stress via interaction with the PKR Activator (PACT) (171). PACT is phosphorylated under various stress conditions and utilizes one of its three DSRBs to interact with and activate PKR (172). Overexpression of PACT
sensitizes cells to viral infection and other stresses while knockdown of PACT mitigates stress-induced PKR activation and promotes clonal cell growth. Following activation by PACT, PKR phosphorylates eIF2α and a variety of other targets, including signal transducers and activators of transcription (STAT), interferon regulatory factor 1 (IRF-1), Jun-N terminal protein kinase (JNK) and p53, which may mediate some of the observed tumor suppressor activity of PKR (167, 173, 174). Overexpression of PKR leads to potent activation of apoptosis, likely as a protective response against the spread of viral infection. However, despite the ability of PKR to regulate cell survival and death, PKR knockout mice develop normally and display no phenotypic abnormalities (175). Several studies have suggested that PKR may play a key role in AD pathogenesis based on observations that phospho-PKR is upregulated in the AD brain and correlates with increased levels of the beta-site APP cleaving enzyme 1 (BACE1) (176-178).

General control non-derepressible 2 (GCN2) was originally identified in yeast as a gene that senses and responds to amino acid deprivation, although in mammals it appears to be sensitive to other stressors including viral infection and UV irradiation (179, 180). GCN2 is activated when uncharged tRNAs bind to its histidyl-tRNA synthetase (HisRS)-related domain inducing dimerization and autophosphorylation to activate its kinase domain (172). GCN2-mediated phosphorylation of eIF2α results in translational upregulation of activating transcription factor 4 (ATF4), which stimulates the transcription of numerous genes involved in the amino acid biosynthetic pathway (181, 182). GCN2 can sense infection by RNA viruses, such as Sindbis virus and Semliki Forest virus, by binding to viral RNA at its HisRS-related domain and inhibiting translation via eIF2α phosphorylation (159, 183). One recent study also demonstrated that GCN2 inhibits replication of HIV in vitro and in vivo and is directly cleaved by the HIV-1 protease (184). Given its prominent expression in the brain and interaction with HIV, GCN2 may provide a mechanistic link between HIV infection in the CNS and UPR activation observed in HAND.

PERK was the final eIF2α kinase to be identified although it is the most physiologically important for control of protein translation in mammals. In addition to alleviating ER stress, PERK
plays a critical role in limiting oxidative stress via activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that regulates the expression of a battery of genes involved in the antioxidant response, including detoxifying enzymes such as glutathione S-transferase A2 (GSTA2) and NADPH quinone oxidoreductase (NQO1) (185). Normally, Nrf2 is kept inactive in the cytoplasm via interaction with and constitutive targeting for degradation by Kelch-like ECH-associated protein 1 (KEAP1) (186). Following phosphorylation by PERK, Nrf2 dissociates from KEAP1, migrates to the nucleus and activates transcription of genes with an antioxidant response element (ARE) in the promoter (187). The importance of this link between ER stress and Nrf2 activation is highlighted by the fact that cells lacking Nrf2 are hyper-sensitive to compounds that activate ER and oxidative stress (188). PERK also plays an important physiological role in secretory cell types, such as pancreatic β-cells, which are frequently under a large biosynthetic load in response to varying demands for insulin production. Missense mutations in the PERK gene have been linked to Wolcott-Rallison syndrome in humans, which is characterized by permanent neonatal diabetes, growth retardation, pancreatic and skeletal system deficits, and in some cases mental retardation (189, 190). Similar phenotypic abnormalities have also been observed in PERK -/- mice. Forebrain-specific ablation of PERK in mice results in impairments in cognitive and information processing, suggesting that PERK plays a key role in cognition in the adult brain. However, aberrant PERK signaling is also implicated in neurodegenerative disorders, such as AD, where it is known to be upregulated early in disease and may contribute to increased amyloidogenic processing of APP via upregulation of BACE1 (133, 191-201). Similarly, genetic and pharmacologic disruption of PERK activity in the CNS has been shown to have protective effects in mice inoculated with prions and in an AD mouse model (197, 202). Altogether, these studies suggest that eIF2α phosphorylation by HRI, PKR, GCN2, and PERK is a critical cellular and physiological mechanism of protein translational control that protects cells against a wide variety of stressors, but can lead to pathogenic conditions when activated in a chronic, uncontrolled fashion, as in AD and other neurodegenerative diseases.
Importantly, eIF2-mediated repression of protein translation is not universal; a subset of genes involved in redox homeostasis, protein refolding and cell survival, such as ATF4, are translationally upregulated in response to ER stress. These mRNAs escape translational repression through a variety of mechanisms including the presence of internal ribosome entry sites (IRESs) within the 5' untranslated region (UTR). Originally discovered in piconavirus RNAs, IRESs are cis-acting elements found in the 5' UTR of many viruses and up to 3-5% of eukaryotic mRNAs (203). IRESs directly recruit the small ribosomal subunit in a cap-independent manner to internal codons with the help of trans-acting cellular proteins known as IRES trans-acting factors (ITAFs) which can confer tissue-specific regulation of protein translation (204, 205). Although the regulation and function of cellular IRESs remains unclear, it appears that many genes with IRESs encode for proteins involved in cell differentiation, growth, and apoptosis (206). Although IRES-dependent translation also requires active ternary complex for initiation, some evidence suggests that eIF2 phosphorylation augments IRES-mediated translation of some genes, including vascular endothelial growth factor (VEGF), but not others (BiP) (206). Currently, the specific role of p-eIF2α in cap-independent translation remains unknown.

A second method of evading translational repression, known as leaky ribosome scanning (LRS), occurs when one or more upstream open reading frames (uORFs) in the 5' UTR of an mRNA are bypassed by the 40S ribosomal subunit allowing for translation to begin at a distal AUG sequence. Typically, LRS occurs when a particular upstream AUG is located in a suboptimal nucleotide context to initiate translation. AUG initiators that differ drastically from the optimal context 5'-CCRCCAUGG-3' for initiation are likely to allow for stronger LRS, compared to those that differ only slightly (207). The use of alternative codons due to weak LRS is emerging as a common mechanism by which cells can derive several protein isoforms from a single mature transcript, as has been characterized for histone H4 and C/EBP mRNA (207-209).

The most well characterized mechanism of cap-dependent escape from translational repression, and the most relevant for ER stress-regulated genes, such as ATF4, is the utilization
of small (~30 codons) uORFs in the 5' UTR of gene transcripts (172, 181). When eIF2α phosphorylation is low and eIF2-GTP levels are high, scanning ribosomes will translate the first uORF it encounters assuming the AUG is in an optimal context for initiation. If a transcript contains uORFs upstream of the authentic start codon, as with ATF4, most of the ribosomes will initiate translation at the proximal uORFs, which decreases the likelihood that scanning ribosomes will re-initiate in time to begin translation at the authentic AUG start codon, resulting in suppressed protein translation. However, when p-eIF2α levels are high and eIF2-GTP is limiting, scanning ribosomes will still initiate translation at the first uORF, but are more likely to scan through subsequent uORFs and re-initiate at the authentic start codon due to decreased availability of the pre-initiation complex. Thus, decreased availability of the ternary complex/pre-initiation complex simultaneously reduces the translation rate of most cellular mRNAs that contain one start codon, while increasing the efficiency of translation in mRNAs with multiple uORFs. This mechanism was first characterized in the yeast homologue of ATF4, GCN4, which contains four uORFs and has subsequently been established for a variety of genes involved in stress response, survival, and apoptosis (152, 172, 206, 210-212). It is estimated that 35-49% of human gene transcripts contain uORFs, indicating that protein translational control via p-eIF2α is likely a widespread mechanism of regulation involved in many cellular functions and may be a rich area of investigation for therapeutic intervention in the pathophysiology of human disease (213).

Many genes encoding for growth factors, transcription factors, and proto-oncogenes also utilize long, GC-rich 5' UTRs to regulate protein translation levels through formation of extensive secondary structures, which inhibit the helicase activity of eIF4A and promote dissociation of ribosomes from the mRNA prior to initiating translation at the first start codon (214). Secondary structures in GC-rich 5' UTRs, typically hairpins, are the result of complementary base-pairing and strong hydrogen bond formation between guanosines and cytosines. The position and stability of the secondary structures determines how effectively they inhibit translation of a transcript. For example, a hairpin with a free energy of -30 kcal/mol located near the 5’ cap is
sufficient to prevent binding of the pre-initiation complex, while hairpins located further down the 5’ UTR require free energies of at least -50 kcal/mol to inhibit translation (214-216). The endogenous mechanism(s) by which cells overcome translational block by secondary structure is unclear, however, overexpression of eIF4A and eIF4B can partially overcome the free energy requirements needed for unwinding (214, 217). Notably, many transcripts utilize secondary structure in conjunction with uORFs, IRESs, or trans-acting factors, such as RNA binding proteins to further suppress protein translation. One of the most well characterized examples of this is found in the mRNA of transforming growth factor-beta 1 (TGF-β1), which controls cell growth and proliferation and is stimulated by growth factors and cytokine signaling (218). The 5’ UTR OF TGF-β1, which is 867 nucleotides in length, forms stable stem loops which utilize the RNA-binding protein, Y-box protein-1 (YB-1), to cooperatively facilitate formation of duplex structures that significantly downregulate translation of TGF-β1 under normal conditions in renal proximal tubular cells (214, 218-220). In the presence of platelet-derived growth factor, YB-1 dissociates from the 5’ UTR and the translational block is overcome (218). In summary, eukaryotic cells have evolved eloquent mechanisms to regulate protein translation of critical gene transcripts allowing for rapid cellular adaption to ever-changing inter- and intracellular conditions.

1.5: REGULATION AND FUNCTION OF BACE1 IN NEURONS

Alzheimer disease (AD) is a debilitating neurodegenerative condition characterized by progressive memory loss and impaired cognition over a period of 15-20 years. AD remains the leading cause of dementia in the elderly, affecting over 5.4 million people in the United States and an estimated 26.6 million people worldwide (221). Although patients with AD often follow a stereotypic pattern of short-term memory loss followed by impairment in other cognitive domains, such as language and reasoning, a definitive diagnosis of AD can be made only after post-mortem analysis of brain tissue in patients suffering from dementia. The pathological hallmarks of AD include intraneuronal neurofibrillary tangles (NFTs), composed of hyperphosphorylated Tau, and extracellular senile plaques composed primarily of 38 to 42 amino acid-length Aβ peptides. Although most AD is sporadic, approximately 5% of cases result from genetic
mutations that invariably affect production of Aβ, supporting the idea that Aβ accumulation and deposition is a critical early event in AD pathogenesis. This theory, known as the amyloid cascade hypothesis, posits that Aβ dysregulation precedes and contributes to NFT formation and the ensuing loss of synapses and neurons that underlie dementia (222). Despite overwhelming genetic and pathophysiological evidence that Aβ accumulation is critical in AD development, the number of NTFs, and not senile plaques, correlates with the degree of dementia in patients. These observations suggest that senile plaques themselves are not toxic, but rather an attempt to sequester and segregate toxic Aβ oligomers and aggregates that induce neuronal death. Under pathological conditions, Aβ monomers can undergo rapid aggregation to form oligomeric species that slowly transition into the large, insoluble fibrils that comprise senile plaques (223, 224). A growing body of work suggests that Aβ oligomers are the toxic intermediates responsible for neuronal death in AD, while the fibrils found in plaques are relatively inert (225).

Aβ is produced constitutively from the sequential endoproteolysis of the amyloid precursor protein (APP), a ubiquitously expressed type I transmembrane protein of unknown function found at highest levels in the CNS. During amyloidogenic processing, APP localized in endosomes or the trans-golgi network (TGN) is first cleaved at the N-terminus by β-secretase yielding a large, secreted fragment, sAPPβ, and a small, membrane-bound C-terminal fragment, C99, that contains the Aβ domain. C99 is then cleaved by a second protease complex, γ-secretase, which is comprised of four transmembrane proteins: presenilin 1/2 (active site), Aph-1, PEN-2, and nicastrin. The γ-secretase cleavage, which also occurs in the TGN or endosome compartments, releases the APP intracellular domain (AICD) fragment and Aβ peptides of either 40 or 42 amino acids in length, which are exocytosed out of the cell. While Aβ42 peptides constitute only a small fraction of the total Aβ produced, they are known to be more highly amyloidogenic and are more likely to contribute to fibril formation in the CNS. Alternatively, APP can be cleaved by α-secretase (primarily by ADAM10, a matrix metalloprotease) at the plasma membrane yielding sAPPα and C83 fragments and, after sequential cleavage by γ-secretase, a non-toxic p3 peptide plus AICD. Importantly, α-secretase cleavage of APP occurs within the Aβ
domain, preventing the production of any Aβ peptides and thus constituting the non-amyloidogenic pathway of APP metabolism. Although Aβ is produced during normal cellular conditions, its physiological function remains elusive. Additionally, it is unclear when normal production of Aβ becomes pathogenic, although familial AD (FAD) mutations that lead to early-onset AD typically result in enhanced processing of APP through the amyloidogenic pathway, indicating that cleavage of APP is normally skewed heavily towards α-secretase and disruption of this homeostatic balance can lead to pathology. Other FAD mutations linked to γ-secretase shift the ratio of Aβ40:Aβ42 production towards increased Aβ42, triggering enhanced fibrillogenesis and plaque deposition (226). Importantly, overexpression of APP, even in the absence of FAD mutations that alter APP processing, is sufficient to cause amyloid pathology, which is best exemplified in trisomy 21, wherein patients harbor three copies of the APP gene and develop early onset memory loss accompanied by senile plaque and NFT formation in the CNS (227, 228).

Following the characterization of APP processing in the early 1990s, intensive efforts were directed towards identifying the enzyme responsible for β-secretase activity in neurons. Extensive biochemical analysis revealed that β-secretase was insensitive to pepstatin, closely associated with membranes, highly expressed in neurons, and most enzymatically active in acidic compartments of the secretory pathway (221, 229). Initially, cathepsin D, a major endosomal/lysosomal aspartyl protease upregulated in AD and located in senile plaques, was thought to be responsible for β- or γ-site cleavage of APP (230-232). However, follow up studies in cathepsin D knockout mice indicated this protease had no role in amyloidogenic or non-amyloidogenic APP processing and was responsible only for lysosomal degradation of Aβ (233, 234). In 1999, a novel, 501 amino acid transmembrane aspartyl protease, BACE1, was identified by five independent groups as the major β-secretase enzyme (235-239). BACE1 was found to be expressed in most cell types and tissues throughout the body with the highest levels in the brain and pancreas. Within the CNS, BACE1 protein and mRNA are expressed at high levels in neurons and at much lower levels in astrocytes. Importantly, BACE1 protein and activity are
elevated in transgenic mouse models of AD and in brains of familial and sporadic AD patients compared to age-matched controls (240-242). Furthermore, immunohistochemical analysis by Vassar and colleagues showed BACE1 elevation occurs in swollen dystrophic neurites near amyloid plaques and co-localizes with APP, implicating BACE1 as a causal agent in AD pathogenesis (243).

Further characterization of BACE1 revealed that it is synthesized in the ER as a 60 kDa zymogen that undergoes rapid maturation to the active 70 kDa form following N-terminal cleavage by furin and N-linked glycosylation at four asparagine residues in the ER and Golgi. Mature BACE1 cycles between compartments of the secretory pathway, similar to APP, and displays peak enzymatic activity in endosomes at pH 4.5, from where it can be ubiquitinated and sent to the proteasome or transported to the lysosome for degradation. In addition to APP, BACE1 has been shown to cleave a variety of other targets, including neuregulin 1 (NRG1) type III, which plays an important role in myelination of the peripheral nervous system (PNS). Although BACE1 knockout mice were initially thought to be physiologically normal, it is now evident that these animals display hypomyelination in the PNS, similar to that observed in NRG1 type III knockouts, indicating that a key physiological role of BACE1 may be regulation and/or maintenance of myelination via cleavage of NRG1. Within the CNS, BACE1 is concentrated within pre-synaptic terminals and is believed to play a role in maintenance of synaptic function. Consistent with this idea, BACE1 /-/- mice display a variety of complex neurological and behavioral abnormalities including memory deficits, axon guidance defects, hyperactivity, reduced serotonin and dopamine levels in the hippocampus, and spontaneous seizures. These phenotypes are not recapitulated in APP or APP-like protein null mice, suggesting they are related to other established BACE1 substrates, such as NRG1 and Na,1 β2, or novel functions of BACE1 within the CNS.

BACE1 expression peaks in early postnatal brain indicating it may also play a role in growth and differentiation (244). Given the multitude of processes mediated by BACE1, it is thus
unsurprising that BACE1 expression is tightly controlled at the transcriptional and translational levels. The human BACE1 gene spans 30 kb and contains a complex promoter with binding sites for a host of transcription factors including Sp1, HIF-1α, and nuclear factor-κB (NF-κB), which suppresses BACE1 under normal cellular conditions but increases the transcription of BACE1 in the presence of stressors, such as Aβ peptides, indicating BACE1 may play a role in stress response or homeostasis signaling (245, 246). The BACE1 promoter is also differentially regulated by oxidative stress and inflammatory mediators, such as interferon gamma (INFγ) which activates Janus Kinase 2 (JAK2) and extracellular signal related MAP kinase 1/2 (ERK 1/2) leading to STAT1 phosphorylation and subsequent upregulation of BACE1 expression (247).

Yet, despite the presence of inflammation and oxidative stress in AD, upregulation of BACE1 appears to be mediated primarily at the post-transcriptional level given that protein levels are consistently elevated in the CNS without corresponding changes in mRNA (241, 248-252). BACE1 translation is known to be strictly regulated on multiple levels, consistent with the presence of a long, highly conserved leader sequence (246). In particular, the BACE1 5’ UTR is 446 nucleotides long, with 3 uORFs, two of which contain uAUGs that reside in optimal contexts for translational initiation, and has a GC content of 77%, which contributes to the formation of stable secondary structures (253). Translation of BACE1 occurs through a cap-dependent mechanism with no detectable IRES activity, indicating that uORFs and secondary structure are the primary mechanisms of BACE1 translational regulation (253, 254). Although there is disagreement in the literature, several mutagenesis analyses revealed that uAUG #2 has the strongest inhibitory effect on translation initiation while the other two uAUGs are largely bypassed via leaky ribosome scanning or ribosome shunting and do not contribute significantly to translational block (253-256). In contrast, a report by Lammich et. al. concluded that the uORFs in BACE1 contribute minimally to translational repression and that tightly folded secondary structures in the UTR with a predicted free energy of -215.3 kcal/mol were sufficient to inhibit translation (253). Additionally, there is evidence that the BACE1 5’ leader sequence undergoes alternative splicing that can reduce the number of uAUGs and improve translational efficiency.
In short, BACE1 is subject to complex translational controls that result in very low protein expression under normal conditions. However, recent evidence indicates that translational block of BACE1 is overcome during various stress conditions that activate mediators of the unfolded protein response. A report by O'Connor et. al. in 2008 was the first to describe that energy deprivation in neurons results in chronic activation of PERK, phosphorylation of eIF2α, and translational de-repression of BACE1 leading to enhanced amyloidogenesis in vivo (191). Others have shown that oxidative stress leads to translational upregulation of BACE1 through PKR-mediated phosphorylation of eIF2α (176, 178). Altogether, these observations strongly reinforce an emerging hypothesis that BACE1 may act as a stress response protein in the CNS. Potential mechanisms by which BACE1 activity could be protective to neurons remains an area of active investigation. A recent report demonstrated that BACE1 is upregulated in zebrafish in response to low oxygen levels via transcriptional upregulation by HIF-1, similar to what is seen in mammals, suggesting that activation of BACE1 is an evolutionarily conserved mechanism (258). Furthermore, BACE1 processing of APP to generate Aβ appears to be an activity dependent process in hippocampal neurons whereby Aβ protects cells from hyperactivity and excitotoxicity (259). Therefore, it is reasonable to assume that BACE1 upregulation serves a neuroprotective function during acute stress conditions in the brain. However, chronic inflammation, hypoxia, oxidative stress, and ER stress, which are hallmarks of many neurodegenerative disorders, might lead to unchecked, maladaptive BACE1 activation and over-production of Aβ. This would explain, in part, why amyloid pathology is often found in neurodegenerative disorders other than AD. Thus, BACE1 has emerged as a key therapeutic target that lies at the crossroads between cellular stress, stress response signaling, and Aβ production in AD and a growing list of other chronic, neurodegenerative diseases.

1.6: RATIONALE AND HYPOTHESIS

HAND has evolved into a complex, neurodegenerative condition that has persisted despite the use of successful antiretroviral therapy. Initial studies of the underlying mechanisms contributing to dementia in patients with HIV suggest that inflammation and excitotoxicity are key
mediators of neuronal damage and death. However, adjunctive therapies with anti-inflammatory and anti-excitotoxic properties have proved to be unsuccessful in the treatment of HAND. Additionally, the use of ART regimens with high CNS penetrance has yielded mixed neuropsychological results in patients with HIV, suggesting that ART is either unable to mitigate neuronal damage in HAND or contributes to the neurodegenerative process. We hypothesized that the persistence and evolution of HAND is a direct result of antiretroviral neurotoxicity in the CNS, leading to chronic ER stress, translational upregulation of BACE1, and aberrant amyloidogenic processing of APP. First, we provide an extensive review the contributing factors to HAND pathogenesis, including inflammation, aging, drugs of abuse, and antiretroviral neurotoxicity (Chapter 2). Next, we used in vitro models to demonstrate for the first time that ART drugs, and PIs in particular, potently activate the neuronal unfolded protein response leading to PERK-dependent phosphorylation of eIF2α and translational upregulation of BACE1 (Chapter 3). Importantly, enhanced BACE1 expression was accompanied by increased Aβ42 production in ART-treated cells, which was dose-dependently blocked with a cell-permeable beta-secretase inhibitor. These findings were corroborated by our in vivo findings that SIV-infected, ART-treated macaques and ART-treated, HIV-positive patients display increased expression of UPR markers, BACE1, and Aβ oligomers compared to age-matched controls (humans only) (Chapter 3).

We also sought to determine the role of BACE1 in neuronal survival under conditions of oxidative and ER stress (Chapter 4). In contradiction to the idea that BACE1 plays a protective in acute cellular adaptation to stress, we found that inhibition of BACE1 protected neurons against damage and death in the presence of hydrogen peroxide or ritonavir in vitro. Furthermore, using a pharmacological approach, we found that PKR may be critical for ART-mediated phosphorylation of eIF2α and BACE1 upregulation.

Altogether, this body of work implicates HIV protease inhibitor class ART drugs in the persistence and evolution of HAND pathology via chronic induction of ER stress in neurons. We have characterized a specific mechanism by which PIs induce translational upregulation of...
BACE1 leading to enhanced amyloidogenic cleavage of APP and corroborated these findings with post-mortem analysis in human patients. We have also identified BACE1 as a key therapeutic target in HAND, that appears to be mediate some of the neurotoxic effect of antiretroviral drugs and, more broadly, oxidative stressors, such as hydrogen peroxide. Ultimately, this work contributes to our understanding of HAND pathogenesis and the basic biological functions of BACE1 in neurons, which will enhance our ability to treat and prevent neurodegenerative disease.
Figure 1. Phosphorylation of eIF2α by four kinases integrates cellular stress responses. A wide variety of stress conditions result in phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) which is mediated by four distinct kinases, PERK, PKR, GCN2, and HRI that display cell- and tissue-specific expression levels. Phosphorylated eIF2α results in decreased translation of most mRNAs, which reduces the influx of new proteins into the ER to alleviate ER stress. Paradoxically, a subset of mRNAs that contain upstream open reading frames (uORFs) in the 5′ UTR, which are normally repressed, are translationally upregulated leading to increased expression of transcription factors, such as ATF4, that active gene transcription of molecular chaperones involved in protein re-folding. Alternatively, chronic induction of phospho-eIF2α can lead to expression of CHOP which activates apoptotic cell death.
1.7: BIBLIOGRAPHY


“Current understanding of HIV-associated neurocognitive disorders pathogenesis”

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2.1: ABSTRACT

Purpose: This review discusses current concepts of HIV-associated neurocognitive disorders (HAND) in the era of anti-retroviral therapy (ART). As the HIV epidemic enters its fourth decade (the second decade of ART), research must address evolving factors in HAND pathogenesis. These include persistent systemic and central nervous system (CNS) inflammation, aging in the HIV infected brain, HIV subtype (clade) distribution, concomitant use of drugs of abuse, and potential neurotoxicity of ART drugs.

Recent findings: Although the severest form of HAND, HIV-associated dementia (HAD) is now rare due to ART, the persistence of milder, functionally important HAND forms persist in up to half of HIV-infected individuals. HAND prevalence may be higher in areas of Africa where different HIV subtypes predominate, and ART regimens that are more effective in suppressing CNS HIV replication can improve neurological outcomes. HAND is correlated with persistent systemic and CNS inflammation, and enhanced neuronal injury due to stimulant abuse (cocaine and methamphetamine), aging, and possibly ART drugs themselves.

Summary: Prevention and treatment of HAND requires strategies aimed at suppressing CNS HIV replication and effects of systemic and CNS inflammation in aging and substance-abusing HIV populations. Use of improved CNS-penetrating ART must be accompanied by evaluation of potential ART neurotoxicity.

Key words: HIV, cognitive dysfunction, inflammation, antiretroviral therapy, HIV- associated neurocognitive disorders
2.2: INTRODUCTION

The term HIV-associated Neurocognitive Disorders, or HAND, represents a group of syndromes of varying degrees of impairment of cognition and associated functioning in HIV infected individuals [1,2]. Its clinical severity ranges from asymptomatic neuropsychological impairment (ANI) through Minor Cognitive Motor Disorder (MCMD) and HIV-associated dementia (HAD), grouped collectively as HAND. The neuropathogenesis of HAND is generally considered to be initiated and driven by HIV invasion and replication within the brain parenchyma, largely through productive infection of brain perivascular macrophages and endogenous microglia, and perhaps to some degree by restricted infection of astrocytes [3,4]. Associated with this infection is neuroinflammation and immune activation of resident glia (macrophages, microglia, astrocytes), which is associated with neuronal injury (both reversible and irreversible). Although the widespread utilization of anti-retroviral therapy (ART) has dramatically decreased the prevalence of the severest form of HAND, HAD, the overall prevalence of HAND and associated morbidity remain high (~50%) [5-8**]. The persistence of this high risk for HAND in individuals experiencing effective control of systemic HIV viral load is incompletely explained, and suggested factors include effects of aging on brain vulnerability, persistence of HIV replication in brain macrophages, evolution of highly neurovirulent CNS HIV strains, and even long-term CNS toxicity of ART [8**,9**]. This review will discuss several of these key factors implicated in modulating HAND pathogenesis: inflammation, HIV-1 subtype (clade), drugs of abuse, aging, and antiretroviral drug effects. Other important factors, including co-morbidity effects of Hepatitis C, host genetic susceptibility, viral gene adaptations, and others are discussed elsewhere [4,10*,11*].

2.3: ROLE FOR INFLAMMATION IN NEUROPATHOGENESIS OF HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

50
Inflammation is associated with HIV replication, both in the periphery and within the CNS where macrophage activation has been correlated with HAND [12,13]. In the last few years the inflammatory response in the systemic circulation has been recognized as a key driver of HIV pathogenesis, both in the periphery and in the CNS [14**-26]. In the CNS there is considerable evidence that this inflammatory response drives the development of HAND or worsens it, possibly independently of viral replication [4,27*-29].

Evidence for persistent inflammation in the CNS in the ART-experienced patients. The era of ART is associated with changes in the neuropathology of HIV infection, which reflects the partial efficacy of ART drugs in suppressing, albeit incompletely, CNS virus replication and associated inflammation [30-32]. Before the introduction of ART, robust neuroinflammation was frequently observed in brain autopsies from HIV-infected patients and the severity of inflammation generally increased throughout clinical disease progression from the early asymptomatic stage to AIDS to severe HAND [33-36]. Although inflammation is less severe since ART inception, it nonetheless persists within the macrophage/microglial populations, which represent the primary reservoir for HIV in the brain [37-38]. Perivascular monocyte-derived macrophages (MDM) and microglia are the primary CD4+ cells in the CNS and the major sources of productive HIV infection in the brain [39-42] and clinical disease severity correlates more strongly with the amount of monocyte infiltration and MDM/microglia activation than with the quantity of infected cells or viral load [12,13]. This suggests that MDM/microglia play a predominant role in the neuroinflammation and neurodegeneration seen in HAND. Immune activation of MDM/microglia is demonstrated by expression of CD14 (LPS receptor), CD16, CD68, and MHC class II in vivo [34,43-45]. Furthermore, cerebrospinal fluid markers of immune activation and inflammation are commonly detected in individuals with HAND. These markers include CCL2 [46,47], 2 microglobulin [48-51], quinolinic acid [52-55], arachidonic acid metabolites [56,57], oxidative stress markers [58,59], and platelet activating factor [60].

Although ART has limited the severity of pathological changes characteristic of HAND, it has not
eliminated them. These persistent pathological findings in ART-experienced individuals include neuronal loss with apoptosis, astrocytosis, myelin pallor, and at least some activated microglia and perivascular macrophages, although the neuropathological hallmarks of HIVE, multinucleated giant cells and microglial nodules, are typically absent [37]. Persistent CNS immune activation has also been documented in pediatric AIDS patients, as evidenced by detection of sCD14 and an elevated CSF IgG index despite prolonged (>4 years) ART use and undetectable serum viral loads [61]. Thus, despite some ART effectiveness in limiting the infiltration of infected cells (monocytes/macrophages) into the CNS, neuroinflammation still persists. Nonetheless, the primary sites of neuroinflammation are different; the characteristic involvement of the basal ganglia in pre-ART specimens is less commonly seen in post-ART specimens, which display inflammation in the hippocampus and in adjacent parts of the entorhinal and temporal cortices [32,38,62]. Overall these studies confirm the notion that neuroinflammation continues to be associated with HIV CNS infection in ART-experienced individuals [63].

Chronic systemic inflammation and microbial translocation in the gut as a driving force for CNS inflammation and HAND. Chronic systemic inflammation has been tightly linked to morbidity and mortality in HIV infected patients receiving ART, which suggests that adjunctive anti-inflammatory drug therapy is needed to improve outcomes [14**-26]. Studies have correlated systemic inflammation (elevated plasma sCD14, LPS), CNS inflammation and HAND [64] and persistence of CSF immune activation (sCD14, elevated IgG index) despite ART use and undetectable serum viral loads [61]. A strong association between the early and persistent damage caused to gut-associated lymphoid tissue (GALT) by HIV infection (SIV infection in macaques), increased microbial translocation resulting in systemic immune/monocyte activation, and disease progression has been established [21,22*,24-26,65]. An association between this systemic immune activation and HAND has also been established, and a causal relationship between increased systemic monocyte activation, increased transendothelial migration of activated monocytes into the brain, and neurocognitive decline secondary to neurodegeneration has been proposed [64]. Furthermore, the persistence of HAND (~50% prevalence) despite prolonged
ART use is associated with not only neuropathologic but also neuroradiologic evidence of persistent CNS inflammation [7,61,66*-68]. Persistent systemic and CNS inflammation in ART-treated individuals are thus clear targets for adjunctive therapies against disease progression.

2.4: ASSOCIATION OF HIV-1 CLADES/SUBTYPES AND RISK OF HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

Until recently, HAND has been studied nearly exclusively in developed countries (United States and Europe), where a single HIV clade, or genotypically-defined subtype predominates (HIV clade B). The distribution of HIV-1 clades varies worldwide, and differences in phenotypic characteristics including induction of immune responses, viral fitness, drug resistance, co-receptor utilization, antibody neutralization sensitivity, and neurovirulence among HIV clades have been described [69-76]. Several recent publications have suggested that HAND prevalence varies among populations based upon clade predominance, thus representing an independent risk factor for HAND [77,78]. The majority of clinical studies have been performed in cohorts infected with clade B, and the neuropathogenesis of HAND has, until recently, been exclusively described in these populations. Furthermore, HIV clades can be further modified through genetic recombination events, which could alter their pathogenic potential. Early studies in Uganda (where clades A and D predominate), have shown that prevalence of some HAND features is comparable to that observed in the United States during the pre-ART era, and that advanced age and low CD4+ T cell count are major risk factors [81*]. Other investigators observed a greater prevalence of HAND in anti-retroviral-naïve HIV+ individuals in Uganda who are infected with clade D strains in comparison with individuals infected with clade A strains (89% vs. 24%) [79*]. Notably, the use of ART can significantly improve neurocognitive function in these individuals within a few months [80].

More studies have focused on clade C, as it is the most common HIV clade and it accounts for approximately 50% of HIV infections worldwide, Clade C is linked to growing epidemics in sub-
Saharan Africa and parts of Asia, including China and India [81*]. Some studies have associated infection with clade C with a low risk for HAND (in Ethiopia) while others (performed by Australia-Pacific NeuroAIDS consortium in many countries in the Pacific Rim) associate it with a higher risk. Studies in India, where clade C accounts for 95% of HIV infections, have produced conflicting results. In southern India, approximately 60.5% of ART-naïve HIV+ individuals in one study (n=119) were found to have neuropsychological test impairments without clinically identifiable neurological symptoms (consistent with asymptomatic HAND, ANI), while another study indicated a higher than expected prevalence of clinically symptomatic HAND. A study of HIV+ (clade C) individuals in China, showed that the prevalence, pattern and severity of some HAND deficits were comparable to those reported for (clade B) in western countries. Finally, a recent study of clade C-infected ART-experienced (average 2 years on ART) individuals in Botswana, demonstrated a prevalence of neurocognitive impairment detected by neuropsychological testing and a modified International HIV Dementia Scale (IHDS) of greater than 33%, which exceeds the expected prevalence of HAD, even in the pre-ART era [82*]. Thus, several studies in distinct clade C cohorts worldwide suggest a potentially high risk for moderate to severe HAND complications with clade C infection. Notably, despite possible different risks for HAND among these different HIV clades, beneficial effects of ART have been demonstrated worldwide (reviewed in [83*]).

2.5: ASSOCIATION OF DRUGS OF ABUSSE AND RISK FOR HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

Although the strict definition of HAND requires the exclusion of other co-morbid conditions (besides HIV infection) as the cause of neurocognitive dysfunction, the contribution of drugs of abuse as a major co-morbidity risk for neurocognitive dysfunction in HIV+ individuals is a major concern worldwide [84-87*]. Among the major drugs of abuse contributing to HIV pathogenesis are opiates (morphine), and stimulants (cocaine, methamphetamine/METH). In developed countries, approximately 30% of HIV+ individuals are intravenous drug abusers, and the risk for
HAND is clearly greater among these individuals [87*]. The neuroinflammation associated with HAND appears to be exacerbated by drugs of abuse, as demonstrated by brain autopsy studies revealing a higher prevalence of HIV encephalitis (microglia activation, presence of multinucleated giant cells, and blood brain barrier disruption) in drug-abusing HIV+ individuals in comparison with non-abusing HIV+ controls [88-90]. These findings suggest that drug abuse, exerts an additive (if not synergistic) effect with HIV within the CNS. However, the inherently heterogeneous nature of drug abusing patient populations confounds the specific effects of drugs of abuse on neuronal function and survival \textit{in vivo}.

Both \textit{in vitro} and \textit{in vivo} studies, however, clearly implicate drugs of abuse in exacerbating neuronal injury induced by HIV (or the primate homolog, SIV/simian immunodeficiency virus), although conflicting evidence for certain drugs of abuse has been presented [91-97]. Enhanced HIV replication in MDM and T lymphocytes through opioid exposure has been demonstrated [98-100] as has enhancement of MDM-associated inflammation and oxidative stress [101]. Opiates (methadone) also activate HIV replication in latently infected macrophages \textit{in vitro} [102]. In nonhuman primate models of simian immunodeficiency (SIV) infection (the primate homologue of HIV infection), carefully controlled studies show that chronic morphine administration markedly increases viral loads in the plasma and cerebrospinal fluid (CSF) [103]. Interestingly, activation of mu opioid receptors, which are expressed in neurons, MDM, and T lymphocytes, can increase the expression of some of the chemokine receptors (CCR3, CCR5, and CXCR4) that serve as HIV co-receptors for HIV in susceptible cells (MDM, T lymphocytes) [104,105]. Furthermore, activation of Kappa opioid receptors (MDM, T lymphocytes) can decrease CCR5 expression, and thus decrease cell susceptibility to HIV infection [106,107]. Nonetheless, a role for opiates in exacerbating neurodegeneration in HAND remains controversial [85*,108].

A role for stimulants such as cocaine and methamphetamine in exacerbating the risk for HAND is more strongly established by \textit{in vivo} and \textit{in vitro} studies [109]. Enhancement of HIV replication in
MDM by stimulants (cocaine, methamphetamine) has been consistently demonstrated in vitro, and the expected consequence of enhanced HIV replication in MDM is enhanced neurodegeneration through enhanced production of neurotoxic factors from infected and activated macrophages within the CNS [3,84]. Cocaine can also increase HIV replication in monocytes, and even astrocytes in vitro [110,111]. The later observation could be significant, as restricted infection of astrocytes in vivo has been demonstrated in several studies, suggested that this could be a second HIV reservoir (in addition to the primary HIV reservoir, macrophages/microglia) [112-114]. In addition, cocaine can facilitate HIV infection by upregulating DC-SIGN, another HIV co-receptor, in dendritic cells, through dysregulation of mitogen-activated protein kinases [115]. Methamphetamine can increase macrophage HIV infection in association with increased expression of CXCR4 and CCR5, and perhaps by downregulation of extracellular-regulated kinase (ERK) and the upregulation of p38 mitogen-activated protein kinase [116]. Methamphetamine can also enhance HIV replication in monocyte-derived dendritic cells [116].

Alterations in blood-brain-barrier (BBB) integrity by cocaine and methamphetamine are another proposed mechanism for enhancing neurodegeneration through enhanced monocyte entry and disruption of cellular homeostasis. In vitro, cocaine can enhance monocyte transendothelial migration, induce the expression of adhesion molecules on endothelial cells, and disrupt intercellular junctions [117-119]. Methamphetamine and the HIV envelope protein, gp120, can modulate tight junction expression in brain endothelial cells, leading to decreased transendothelial resistance across the blood–brain barrier and enhanced transendothelial migration of monocytes [120]. Morphine alone, on the other hand, does not appear to alter the integrity of the blood–brain barrier, although in combination with the HIV transactivator protein, Tat, it can alter tight junction expression in brain endothelial cells in vitro. Interestingly, although morphine by itself is not toxic to striatal neurons in culture, it can significantly potentiate Tat toxicity in striatal neurons [121]. Thus, these studies of effects HIV-derived proteins and cocaine
and methamphetamine that demonstrate alterations in endothelial cell function and/or disruption of the blood–brain barrier suggest potential additive effects of HIV infection, cocaine and methamphetamine in vivo.

Evidence for in vivo neuropathologic effects of cocaine and methamphetamine in HIV+ individuals is also accumulating [85*]. Disruption of the blood–brain barrier in such individuals has been demonstrated in neuropathologic studies, and this disruption correlates with early inflammatory changes in the CSF, particularly with increased monocyte chemoattractant protein-1 (MCP-1/CCL2) levels [122]. However, whether BBB integrity is even further compromised in individuals abusing cocaine or methamphetamine is unknown. Autopsy studies of adult methamphetamine abusers have demonstrated neuronal loss within the substantia nigra and structural and metabolic changes within the brain have been detected in children after prenatal exposure [123,124]. Injury to dopaminergic pathways and the basal ganglia also occurs in HIV+ individuals in the presence and absence of abuse of cocaine, which can result in profound clinical symptoms of basal ganglia dysfunction [125]. Thus, drugs of abuse, particularly cocaine and methamphetamine are strongly associated with enhanced brain injury in HIV+ individuals, which is expressed as enhanced risk for HAND and other neurologic complications.

2.6: AGING AND HIV-ASSOCIATED NEUROCOGNITIVE DISODERS
The long-term prognosis for ART-treated HIV+ individuals continues to improve as the incidence of many AIDS-related complications declines, and by 2015 more than 50% of the HIV+ population in the United States will be over 50 years of age [14**]. Nonetheless, life expectancy for treated HIV+ individuals remains 10 to 30 years less than uninfected individuals [14**]. ART-treated patients are at increased risk for systemic and CNS diseases associated with aging: renal failure, osteoporosis, cancer, cardiovascular disease, and cognitive decline, which can be associated with Alzheimer’s disease (AD) and Parkinson’s disease (PD)-like pathology [14*,126*]. This suggests that the aging brain might be more vulnerable to neuronal injury associated with HIV
infection, although co-morbidity factors in aging patients complicate establishing a causal relationship between age and HAND risk.

Several published neuroimaging and neurobehavioral studies have suggested an increased risk for cognitive impairment with increased age in HIV+ individuals \[66^*,127,128\], although an additional study has suggested that the effects of HIV infection and aging in the brain might act independently \[129\]. Ernst and Chang \[127\] used brain proton magnetic resonance spectroscopy (MRS) to demonstrate that the combined effects of HIV+ serostatus resulted in a greater than five-fold acceleration of aging effects (rather than additive effects) in the basal ganglia, in a cohort of 46 HIV+ subjects in comparison with HIV negative controls. Cherner et al \[128\] showed that an HIV+ subject cohort with an age greater than 50 years and detectable CSF viral loads had a two-fold higher prevalence of neuropsychological impairment in comparison with a younger cohort (less than 35 years of age) showing undetectable viral loads. Notably, this relationship was not found in those individuals younger than 50 years of age. These studies suggest that older adults are at higher risk for neurocognitive dysfunction because of age-related brain vulnerability; however, whether this dysfunction reflects accelerated neuropathological processes associated more specifically with HAND or processes more specifically associated with other familiar neurodegenerative diseases, or neither, remains to be determined.

Some recent studies have begun to address the underlying neuropathology of age-related neurocognitive dysfunction in HIV+ subjects. AD and PD-like pathological changes observed in ART-treated patients \[130^*,131\] include elevated levels of hyperphosphorylated Tau (p-Tau) in the hippocampus and beta-amyloid (Aβ) deposition, both intra and extracellular, in the frontal cortex and hippocampus \[132-135\]. Recent evidence has also shown increased levels of alpha-synuclein in the substantia nigra and increased risk for PD in aging HIV+ patients on ART \[131\]. Although accumulation of neurodegenerative-related proteins might be accounted for by the increased lifespan associated with ART, possible toxic effects of ART in the CNS are now being
considered as a contributing factor in HAND [11*,136*]. One study demonstrated that pre-ART subjects who lived up 15 years with HIV infection did not express excessive levels of hyperphosphorylated Tau or beta-amyloid, nor were they associated with HAND [38]. Other studies have demonstrated increased levels of amyloid precursor protein in damaged axons in brain specimens from ART-naive patients without evidence for elevated p-Tau expression or neuritic plaque formation [137,138]. Thus, studies utilizing neuropsychological performance testing, neuroimaging, and neuropathological analyses strongly support a strong correlation between accelerated neurocognitive decline driven by HIV infection in aging individuals, even those with what is considered 'effective' suppression of systemic HIV replication. These studies further emphasize the need for developing new strategies, involving current ART and possibly adjunctive therapies, for protecting the brain against injury in the aging HIV+ population.

2.7: POSSIBLE ROLE FOR ANTIRETROVIRAL THERAPY DRUGS IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

The persistent high prevalence of less severe forms of HAND, including ANI and MCMD, after widespread implementation of ART was not anticipated, and several causes have been suggested, including effects of aging and associated co-morbidity factors on the brain [9*,39,66*]. Antiretroviral drugs, particularly nucleoside reverse transcriptase inhibitors (NRTIs) are highly neurotoxic, and ART drug-induced neuropathy is a major complication of HIV treatment [10*]. In addition, some clinical studies have also suggested a role for direct and/or indirect neurotoxic effects of ART drugs in the CNS [139-141]. In addition to direct neurotoxicity, ART has been linked to multiple risk factors for neurodegenerative disease, such as insulin resistance, lipodystrophy, atherosclerosis, coronary artery disease, and immune reconstitution syndrome [142-149]. These studies suggest possible direct and indirect effects of ART drugs in the CNS that could be linked to impaired neurocognitive performance.
However, other studies have demonstrated beneficial effects on neurocognitive functioning by ART regimens ranked according to their predicted effectiveness (termed CNS penetration-effectiveness ranking, CPE) in suppressing HIV replication within the CNS [8*, 150*-152]. Better neurocognitive performance was observed over a 15 week period in adult individuals beginning ART with regimens of higher CPE [151] and improved survival rates over 6+ years of follow up of pediatric HIV encephalopathy patients receiving higher CNS-penetrating regimens were also observed [153**,154**]. A cross sectional study of 2636 adults (ALLRT cohort) on effective ART (less than 50 HIV RNA copies/ml) also demonstrated better neurocognitive performance in those receiving higher CPE ART [150*]. Another recent study utilized MRS brain imaging and neurocognitive testing to demonstrate partial reversal of neuronal injury in patients and greater improvements in neurocognitive functioning in other patients receiving different ART regimens over a 48 week period, which might relate to CNS drug penetrance [155*]. These studies suggest a neuroprotective effect of ART based upon use of higher CPE regimens, and ongoing prospective clinical studies are further addressing this critical issue [8**,9**,154**].

2.8: CONCLUSION

HAND pathogenesis is driven by HIV replication and the factors associated with amplifying the inflammatory milieu within the CNS. Systemic immune activation, migration of activated monocytes, drugs of abuse, and secondary effects of aging all contribute to neuronal injury associated with HAND, which persists despite effective systemic control of HIV replication by current ART. Accordingly, drugs that suppress systemic immune activation and associated inflammation, both systemically and within the CNS compartment, could represent effective adjunctive neuroprotectants [4, 27*-29]. Investigating drugs in current clinical use that target these cellular pathways could rapidly facilitate testing and implementation of feasible adjunctive neuroprotective strategies against HAND.
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2.10: REFERENCES

Papers of particular interest, published recently, are highlighted as follows:

* Of importance

** Of major importance


*This cross-sectional observational study of 1,555 patients demonstrates a 50+% prevalence of HAND in ART-treated patients, with lowest impairment among those with suppressed viral loads and CD4 counts > 200 cells/mm3 and discusses criteria for initiating ART.*

This study confirms the consistent and robust association between HAND risk and nadir CD4 count in the pre-ART and post-ART eras and suggests that early ART treatment before immunosuppression may prevent neurological complications.


This update from the 2010 Conference on Retroviruses and Opportunistic Infections suggests additional HAND risk factors including vascular disease and apolipoprotein E and discusses conflicting evidence about potential ART toxicity in the CNS.


This review highlights recent studies addressing changes in the clinical, pathological and neuroradiological findings in ART treated patients and risk factors associated with HAND.


This perspective convincingly discusses the role for ongoing inflammation during ART as a treatable risk factor for disease progression, the need to aggressively manage inflammatory and vascular risk factors, and to consider early ART to prevent potentially reversible immune dysfunction.


This study confirms the persistent activation of HIV-associated inflammatory pathways in virally suppressed ART experienced patients among more than 5,000 participants in the Strategies for Management of Anti-Retroviral Therapy (SMART) trial in comparison with age-matched participants of two large population-based studies of atherosclerosis risk in non-HIV-selected individuals. This will strongly prompt further investigations into interventions to decrease these risk factors and their effect on HIV disease progression.


Plasma indicators of systemic inflammation (fibrinogen and CRP) in 922 HIV-infected individuals followed over 5 years were found to be strong and independent predictors of mortality even when CD4 counts exceeded 500 cells/mm3. Investigations are needed to determine if reducing these inflammatory factors affects mortality and other outcomes, including HAND.


_Microbial translocation associated with elevated LPS and soluble CD14 levels persist in HIV-1 infected infants even with ART suppression of virus replication, T cell reconstitution, and resolution of lymphocyte activation. Thus monocyte, macrophage persists in treated infants with as yet unknown consequences._


_This timely and thorough review links literature evidence for inflammation with neurocognitive dysfunction and discusses similarities with Alzheimer’s Disease. Important evidence from primate studies of SIV infection of natural and non-natural hosts is clearly discussed._


*This is a comprehensive yet succint review of evidence for enhanced progression of neurodegenerative diseases in HIV infected individuals.*


This study suggests that HIV subtype D (prevalent in Uganda) expresses greater neuropathogenic potential than subtype A, and it represents the first direct comparison of neurocognitive outcomes in populations infected with different HIV subtypes. Although small (n=60), the study shows highly significant and marked differences (89% vs. 24%) that require further studies to identify possible viral and host genetic determinants for enhanced neurovirulence of HIV subtype D.


This comprehensive overview of published and unpublished studies of neurological complications of HIV Africa presented at the 5th IAS Conference on HIV Pathogenesis, Treatment and Prevention (Capetown, South Africa) addresses prevalence, patterns of CNS manifestations, HIV subtypes, and treatment considerations in Africa.


This is the first study (n=120 patients) of neurocognitive impairment prevalence in Botswana, and it demonstrates a higher than expected prevalence of HIV-associated dementia (38%), based upon the International HIV Dementia scoring scale. It argues for initiation of ART before severe immunosuppression occurs, as a possible prevention strategy.

This review discusses altering ART treatment guidelines for neuroprotection against HIV and reviews the evidence for ART CNS neurotoxicity.


This is a balanced review of the possible effects of cocaine, methamphetamine, and opiates on several mechanisms of HIV-induced neurodegeneration: HIV replication, blood-brain barrier integrity, and glial cell functions relevant to HAND. Important therapeutic considerations are presented.


This review summarizes the international risks for HIV spread due to the spread of intravenous drug abuse, with implications for worldwide spread of different HIV subtypes.


This study demonstrates, although in a small number of patients, long term (47 months) neuroprotective effects of AZT and NNRTIs despite persistence of neurological impairment and HAD in ART-mediated HIV+ participants.


This review summarizes the potential contribution of HIV and ART to Alzheimer disease-like pathology in HAND patients and proposes several mechanisms for HIV-mediated intraneuronal beta-amyloid deposition.


*This book chapter details HAND neuropathology in the pre- and post-ART eras and proposes mechanisms for neurodegeneration, specifically relating to tau dysregulation, via HIV and ART.*


*The authors present a thoughtful discussion about potential neurotoxicity vs. neuroprotection by ART, and they recognize the need for a prospective randomized study to investigate CNS ART toxicity.*


>This prospective study demonstrates, in a small HIV+ cohort, a significant association between highly CNS-penetrant ART regimens and impaired long-term neuropsychological and motor performance, despite decreased CSF HIV RNA.


153. **Patel K, Ming X, Williams PL, et al. Impact of HAART and CNS-penetrating antiretroviral regimens on HIV encephalopathy among perinatally infected children and adolescents. AIDS. 2009 Sep 10;23(14):1893-901. This study demonstrates the effectiveness of CNS-penetrant ART in prolonging survival in perinatally infected children and adolescents, over a 6+ year follow up.

154. **Joska JA, Gouse H, Paul RH, et al. Does highly active antiretroviral therapy improve neurocognitive function? A systematic review. J Neurovirol. 2010 Apr;16(2):101-14. The first systemic review examining the effect of ART on neurocognitive functioning in HIV-positive patients across 15 different studies that details the use of neuropsychological examinations, ART regimens and duration of use, as well as the quality of each of the studies examined.
3.1: ABSTRACT

The clinical and pathologic presentation of HIV-associated neurocognitive disorders (HAND) has changed in the post-antiretroviral (ARV) era, evolving from a subacute, subcortical dementia to a cortical, neurodegenerative disease. Mounting evidence implicates ARVs as potential contributing factors to the persistence and evolution of HAND. Based on their ability to induce ER stress in a wide variety of cell types, we hypothesized that ARVs also cause ER stress in the CNS, resulting in chronic dysregulation of the unfolded protein response (UPR) and altered amyloid precursor protein (APP) processing. Here, we utilized in vitro and in vivo models to show that HIV protease inhibitor (PIs) class ARVs induce neuronal ER stress leading to PKR-like ER kinase (PERK)-dependent phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), and enhanced translation of β-site APP cleaving enzyme-1 (BACE1). Additionally, we demonstrate PI-mediated Aβ production, indicative of amyloidogenic APP processing, in primary rodent neuroglial cultures and Chinese Hamster Ovary (CHO) cells expressing human APP. These effects were abrogated by genetic excision of PERK or overexpression of a kinase-dead PERK mutant. Finally, ARVs administered to SIV-infected macaques resulted in elevated levels of APP in the CNS. Altogether, these findings implicate PIs as potential mediators of neurodegeneration in HAND.
3.2: INTRODUCTION

The advent of antiretroviral (ARV) therapy as the mainstay for HIV treatment has significantly reduced the prevalence of HIV-associated dementia (HAD), a severe motor/cognitive disorder afflicting AIDS patients (1-3), from ~20-30% to 1-2% (1). However, the prevalence of HIV-associated neurocognitive disorders (HAND), a spectrum of HIV-related CNS dysfunctions ranging from mild cognitive deficits to HAD, has remained high despite this dramatic reduction in HAD prevalence (1, 4-7). In addition, HIV-associated neuropathology has evolved from a subcortical encephalitis to a prolonged, cortical, neurodegenerative disease (8). Recently, several groups have reported beta-amyloid (Aβ) immunoreactivity, both intra and extracellular in the frontal cortex and hippocampus of ARV-mediated subjects, while others have observed only accumulation of hyperphosphorylated Tau (8-14). While increased longevity of HIV-infected populations is suggested to contribute to these changes in HAND neuropathology, other risk factors, such as peripheral toxicities of ARVs and potential central effects linked to enhanced amyloidogenesis and aging, remain largely underexplored.

Among the six classes of ARVs currently prescribed, nucleoside reserve transcriptase inhibitors (NRTIs) and HIV protease inhibitors (PIs) are the oldest and continue to provide the backbone for treatment regimens. NRTIs inhibit the viral reverse transcriptase enzyme, preventing the generation of proviral DNA from viral RNA (15). In addition, PIs are peptidomimetics that bind to the active site of the HIV aspartyl protease and prevent cleavage of gag-pol precursor polyproteins, blocking viral maturation (16). As a result of combination ARV therapy, HIV-infected individuals have experienced a 50% reduction in death rates and incidence of HAD (16). Despite their numerous benefits, ARVs have been linked to wide-ranging, peripheral metabolic and neural disturbances that could themselves influence the progression of HAND. Specifically, NRTIs have been linked to mitochondrial dysfunction, manifested clinically as peripheral neuropathy and myopathy. (17-19). On the other hand, PIs have been associated with dyslipidemia, hypercholesterolemia, and metabolic syndrome, characterized by lipodystrophy, atherosclerosis, and insulin resistance (20-27).
We have shown that ARVs administered to healthy adult rats and SIV-infected macaques lead to extensive synaptodendritic damage in the CNS. Furthermore, treatment of rat cortical neurons with ARVs led to accumulation of ROS and eventual synaptic damage and neuronal death (28). These data support a growing body of evidence that ARVs have significant neurotoxic effects in the CNS, as they do in the peripheral nervous system (PNS), and may influence the long-term neurocognitive outcome of patients. Importantly, NRTIs and PIs have each been shown to induce numerous cellular stressors, including ER and oxidative stress, both of which are commonly found in HAND and other neurodegenerative diseases, such as Alzheimer disease (AD) (29, 30). Recently, it has become clear that diverse cellular stressors converge on a ubiquitous intracellular response mechanism termed the unfolded protein response (UPR) or ER stress response (31, 32). In eukaryotes, the UPR entails three sensor proteins, pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), located in the ER membrane, which are kept inactive through complex formation with the ER-resident chaperone, Binding Protein (BiP)/GRP78 (33-35). Accumulation of misfolded proteins in the ER disrupts interaction between BiP and the luminal domain of one or more of the three UPR sensors, permitting their consequent activation (33). Downstream effects of UPR induction include global protein synthesis attenuation via eIF2α phosphorylation, upregulation of genes containing the ER Stress Response Element (ERSE), and ER-mediated protein degradation (35).

While transient UPR activation serves a protective function, the chronic nature of cellular stress and stress response activation in the CNS during HIV infection may ultimately overwhelm the capacity of the ER resulting in aberrant UPR activation and eventual cell death (30, 36). We and others have demonstrated a potential role for ER stress and the UPR in the pathogenesis of HAND as well as other neurodegenerative diseases (30, 37-40). The examination of post-mortem brain tissue from ARV-treated, HIV-positive patients and AD patients shows increased expression of BiP, p-eIF2α, ATF6, and other markers of the UPR primarily in neuronal cells (30, 35, 41). Interestingly, in vivo studies utilizing glucose deprivation or tunicamycin to induce UPR
activation in CNS neurons have demonstrated consequent increases in APP and Aβ deposition (42-44). Vassar and colleagues subsequently showed that impaired brain energy metabolism, induced by glycolysis inhibition, leads to chronic activation of the PERK arm of the UPR, allowing for sustained phosphorylation of eIF2α and upregulation of β-secretase (BACE1) (44). Extensive studies have shown that phosphorylation of eIF2α globally inhibits protein translation while upregulating the translation of a subset of mRNA species that contain multiple upstream open reading frames (uORF) in the 5'-UTR, one of which is BACE1 (44). BACE1 is an aspartyl protease critical for generation of Aβ from APP and is upregulated in brains of patients suffering from AD (45, 46). Increased BACE1 expression and activity in the brain have been correlated with plaque number and cognition status, and has been implicated as a biomarker of mild cognitive impairment (MCI) and AD (46). Altogether, these studies suggest that UPR-mediated upregulation of BACE1 may function as an early event in AD pathogenesis.

In the present study, we sought to determine the mechanisms by which ARVs induce damage and death in primary CNS neurons and whether BACE1 expression and function could contribute. Here, we report that PIs induce ER stress in primary neurons leading to PERK-dependent translational upregulation of the APP-cleaving enzyme, BACE1. Additionally, we found that PIs enhance production of Aβ in neurons and CHO cells stably expressing human APP. Moreover, these findings were corroborated with in vivo findings that chronic ARV drug treatment results in CNS damage and increased expression of APP. These findings suggest that PIs may be altering APP processing, and thus contributing to the changing neuropathology and the persistence of cognitive decline among ARV-treated, HIV-infected individuals.

3.3: EXPERIMENTAL METHODS

Chemicals and Reagents. The following antibodies used in this study were purchased from the indicated vendors: BACE1 (5606S); phospho-eIF2α (9721S); Total eIF2α (9722); Lamin A/C (2032S); Presinilin-1 (5643) [Cell Signaling Technology], BiP/Grp78 (610978) [BD Transduction Laboratories], APP (6E10) [Covance], Synaptophysin (ab8049); ADAM10 (ab1997); MAP2 (5392)
The following reagents used in the study were purchased from the indicated vendors: 4',6-diamidino-2-phenylindole (DAPI); Citifluor, Ltd. (London, UK): citifluor AF1; Dulbecco's Modified Eagle's Medium (DMEM); Neurobasal media and B27 supplement [Invitrogen], Protein assay dye (500-0005), Polyvinylidene fluoride (PVDF) membrane; Prestained broad range molecular weight ladder [BioRad], Neuromag Transfection Reagent (NAN14000) [Nanotherics], Fast Green FCF; Protease inhibitor cocktail, 4-hydroxytamoxifen (H7904); Anisomycin (A9789); Actinomycin D (A1410) [Sigma]. Antiretroviral reagents (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). Poly-L-Lysine (Peptide International). Tyramide Amplification System (New England Biolabs). Normal Antibody Diluent (Scytek Labs). All horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Thermo Scientific and all dye-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Labs.

Immunofluorescence. Paraffin-embedded tissue sections from the hippocampus of control and HIV(+) human autopsy cases obtained from the NNTC and paraffin-embedded tissue sections from hippocampus of pigtailed macaques (Macaca nemestrina) were prepared for immunofluorescent staining with minor modifications of previously described protocols (30, 47). The age, neurocognitive status, sex, and postmortem interval of each human specimen was provided by the NNTC (Table 1). Glass slides containing paraffin-embedded tissue sections (10 μM) were heated overnight to 55°C, deparaffinized in histoclear and rehydrated in 100%, 95%, 90%, and 70% ethanol washes. Tissue was then incubated in 3% H₂O₂ in methanol to inactive
endogenous peroxidase activity. Antigen unmasking was performed with target retrieval solution at 95°C for 1 h. Sections were then blocked with 10% normal goat serum and incubated with various primary antibodies overnight at 4°C. Tyramide amplification was used to detect BACE1 and DNA was visualized with DAPI staining. Slides were washed with PBS plus 0.1% Tween-20 (PBS-T) and mounted in Citifluor AF1 and analyzed by laser confocal microscopy at 600x on a BioRad Radiance 2100 equipped with Argon, Green He/Ne, Red Diode, and Blue Diode lasers (BioRad, Hercules, CA). Post-acquisition analysis was performed using Metamorph 6.0 (Universal Imaging, Downingtown, PA). Total intensity for MAP2 and APP were determined by the measurement of integrated pixel intensity per z-stack image, where integrated pixel intensity is defined as total pixel intensity per image times the area of pixels with positive MAP2 or APP signal. Averages are expressed as mean ± SEM. All data was analyzed by Prism 5.0 software (Graphpad Software, San Diego, CA).

SIV/pigtail macaque model of HIV CNS disease. Pigtailed macaques (Macaca nemestrina) were inoculated with SIV/DeltaB670 and SIV/17E-Fr, as described previously (48). 12 days after infection, animals were divided into placebo and ARV-treated groups until necropsy (range, day 161-175). ARV treatment was composed of the NRTI tenofovir (Gilead) at 30 mg/kg subcutaneously given once daily, saquinavir (Roche) and atazanavir (Bristol-Myers Squibb) at 205 and 270 mg/kg, respectively, given twice daily via oral administration, and an integrase inhibitor, L-870812 (Merck) (49) at 10 mg/kg orally twice daily (50). Tenofovir dosing was based on previous studies (51) while saquinavir and atazanavir dosing was based on pharmacokinetic analysis carried out in pigtail macaques to reflect similar area under the curve parameters as those detected in human treated with atazanavir and saquinavir (50). L-870812 integrase inhibitor dosing was based on previous work conducted in rhesus macaques (49).

Preparation of Primary Cortical Neuroglial Cultures. Primary rat or mouse cortical neuroglial cultures were isolated from brains of embryonic day 16 Sprague Dawley rat pups or embryonic day 16.5-17.5 floxed PERK mice, with modifications of previously described protocols (52). Briefly, to generate neuroglial cultures, cortical cell suspensions were isolated from rat or mouse
pups and plated in poly-L-lysine coated tissue culture dishes and maintained in neurobasal media supplemented with B27 at 37°C with 5% CO₂, as described previously (53, 54). Unless otherwise indicated, all experiments were performed at 21 days in vitro (DIV) on cultures containing approximately 90% neurons and 10% astrocytes/glia. Pure neuronal cultures were prepared by treating neuroglial cultures with 10μM Ara-C 48 hours after plating and were maintained in neurobasal media with B27 at 37°C with 5% CO₂.

Human primary cortical neurons were obtained from the Comprehensive NeuroAIDS Center (CNAC), Temple University, Philadelphia, PA. Cultures were prepared from fetal brain tissue (gestational age, 16-18 weeks) performed in full compliance with National Institutes of Health and Temple University ethical guidelines as described previously (55). Neurobasal medium supplemented with B27 was half-changed every 4 days and cultures were maintained at 37°C with 5% CO₂ for 21 DIV.

Previously characterized Floxed PERK (PerkloxP/loxP) mice (56) were crossed with tamoxifen-inducible cre (Cre-ERT2) heterozygous mice and the resulting PERKloxP/loxP; Cre-ERT2 mice were kindly provided by Dr. Alan Diehl. PERK deletion was achieved by treating 10 DIV mouse neuroglial cultures with various concentrations of 4-hydroxytamoxifen (4-OHT). Antiretroviral drug treatments were performed 4 days following 4-OHT treatment to allow for maximal PERK excision. 16 h following antiretroviral drug treatment, PERK-/- neuroglial cultures were harvested for Immunoblotting or genotyping using routine methods.

Drug Treatments. 21 DIV rat cortical neurons or human fetal neurons were treated with various log-fold concentrations of either AZT, prepared in sterile water at 25 mM stock, saquinavir, or ritonavir, both prepared in DMSO at 25 mM stock concentrations. All antiretroviral drugs were added to neuroglial cultures 16 h prior to harvest. Anisomycin and Actinomycin D were prepared in DMSO at 50 mM and 5 mM stock concentrations, respectively. 21 DIV rat cortical neurons were pre-treated with either 10 μM or 50 μM Anisomycin or 5 μM Actinomycin D for 12 hours then treated with 10 μM ritonavir 16 h prior to harvest.
**Immunoblotting.** Flash-frozen whole-brain tissue samples from HIV (-) control (n=4), HIV (+) neurocognitively normal (n=8), and HIV (+) HAND (n=6) human autopsy cases were obtained from the tissue banks of the National NeuroAIDS Tissue Consortium (NNTC). Tissue was prepared for western blotting as described previously (30). 30 μg protein for each sample was loaded into each lane of 10% Bis-Tris gels and transferred to PVDF membranes followed by blocking with TBS-T with 5% bovine serum albumin (BSA) for 30 min at room temperature. Membranes were probed with various primary antibodies overnight at 4°C.

Whole-cell extracts of mouse brain tissue samples were prepared by homogenization in ice-cold tissue extraction buffer (50 mM Tris pH 7.5, 0.5M NaCl, 1% NP-40, 1% SDS, 2 mM EDTA, 2 mM EGTA, 5 mM NaF, 0.4 mM Na₃VO₄, 1 mM dithiothreitol (DTT) and 1:100 protease inhibitor cocktail). Extracts were then centrifuged at 12,000 g at 4°C for 20 min.

Whole cell extracts of primary rat cortical cultures were prepared with ice-cold whole-cell lysis buffer containing 50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 0.4 mM Na₃VO₄, 100 mM, and 1:100 protease inhibitor cocktail. Lysates were centrifuged at 14,000 g at 4°C for 10 min. Protein concentrations were determined using the Bradford method and equal amounts of protein (15-20 μg) were loaded into each well of 4-12% Bis-Tris Gradient gels or 3-8% Tris-Acetate gradient gels (for confirmation of PERK overexpression) followed by transfer at 25 V at room temperature for 1 h to PVDF membranes. Membranes were subsequently blocked in TBS-T and 5% BSA for at least 30 min at room temperature. Membranes were then incubated in primary antibodies overnight at 4°C, washed with TBS-T, and incubated with appropriate HRP-conjugated secondary antibodies for 30 min. at room temperature. Membranes were developed using SuperSignal West Dura extended duration substrate and densitometric analysis was performed using the NIH ImageJ software program (Image J v1.44, NIH). Equal loading and transfer was confirmed by fast green staining of membranes. Band densities of interest were normalized to indicated loading controls or fast green stains of membranes. Immunoblots shown are representative of at least three independent biological replicates.
RNA Isolation and Quantitative RT-PCR. Total RNA was isolated from primary rat neuroglial cultures using the RNeasy Mini Kit (Qiagen, Valencia, CA). To assess the expression of spliced XBP-1 and BACE1 genes, 1 μg total RNA from each sample was subjected to the SuperScript II First-Strand Synthesis system (Invitrogen). Approximately 100 ng cDNA was amplified via quantitative RT-PCR using Custom TaqMan® Gene Expression Assays purchased from Applied Biosystems. Relative expression of mRNA levels from each sample were quantified using the comparative CT method and data was normalized to unspliced XBP-1 and TBP for spliced XBP-1 and BACE1 genes, respectively. All samples were run in triplicate and experiments were repeated in at least three biological replicates.

Human/Rat Aβ ELISAs. Cell media from 21 DIV primary rat neuroglial cultures and CHO cells stably expressing Human wild-type APP were diluted 25-fold and subjected to a human/rat Aβ<sub>42</sub>- or Aβ<sub>40</sub>-specific sandwich ELISA (Wako Chemicals, Richmond, VA) according to manufacturer’s protocols. For CHO cell experiments, cultures were pre-treated with 10 nM, 100 nM, or 1 μM Beta-Secretase IV (Calbiochem, Billerica, MA) for 12 h followed by treatment with 10 μM ritonavir or DMSO vehicle for 16 h at which time media was collected. For rat neuroglial cultures, cells were treated with combinations of antiretroviral drugs followed by media collection at 4 and 16 h.

Human sAPP ELISAs. Cell culture media from CHO cells stably expressing human wild-type or APP were pre-treated with 10 nM, 100 nM, or 1 μM Beta-Secretase IV (Calbiochem, Billerica, MA) for 12 h followed by treatment with 10 μM ritonavir or DMSO vehicle for 16 h at which time media was collected. Conditioned media was then diluted 25-fold and subjected to a human sAPPα or sAPPβ ELISA (IBL International, Toronto, ON) according to manufacturer’s recommendations.

Pulse-Chase and Immunoprecipitation. Primary rat neuroglial cultures were grown in 60 mM plates for 21 DIV in neurobasal media supplemented with B27. Cultures were pulse-labeled with 2 mL methionine/cysteine-free DMEM containing 0.4 mCi <sup>35</sup>S-labeled methionine and cysteine for 30 minutes. Cultures were washed twice with sterile DPBS and chased with neurobasal media supplemented plus B27 with or without 10 μM ritonavir. At 0, 6, 12, 18, and 24 hr after labeling,
cultures were harvested with ice-cold lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 0.4 mM Na$_3$VO$_4$, 100 mM, and 1:100 protease inhibitor cocktail). Lysates were immunoprecipitated with Dynabeads Protein A [Novex] and 2 μg anti-BACE1 [Cell Signaling]. Immunoprecipitates were run on a 4-12% Bis-Tris gradient gel for separation and transferred onto PVDF membranes for approximately 7 hr. Membranes were dried and exposed via autoradiography.

**Subcellular Fractionation.** To evaluate ATF4 nuclear translocation in the context of antiretroviral drug toxicity, 21 DIV rat neuroglial cultures were treated with 10 μM ritonavir, or DMSO vehicle, for 16 h and fractionated. To prepare nuclear extracts, cultures were washed twice in ice-cold PBS and lysed for 10 min in 10 mM HEPES (pH 7.9), 10 mM KCl, 10 mM EDTA, 1 mM DTT, and 0.4% Nonidet P-40 supplemented with protease inhibitor cocktail. Extracts were centrifuged at 4°C for 3 min at 15,000 x g and the supernatant (cytoplasmic fraction) stored at -80°C. The resulting nuclear pellet was resuspended in 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM DTT with protease inhibitor cocktail then incubated at 4°C, with shaking, for 2 h. The nuclear fraction (supernatant) was then generated by centrifuging the extracts at 15,000 x g for 5 min at 4°C. Protein concentrations were then determined using the Bradford method and lysates run on 4-12% Bis-Tris gradient gels. Membranes containing nuclear fractions were probed with rabbit anti-ATF4 (generous gift from Dr. Ron Wek, Indiana University).

**Nanomagnetic Transfection.** For transfection of primary rat neurons, pure neuronal cultures were generated by treating DIV 3 neuronal cultures with 10 μM AraC. DIV 10 pure neuronal cultures were transfected with plasmid DNA via magnetotransfection using the Magnefect-Nano II system (Nanotherics Ltd., Stoke-On-Trent, UK). Briefly, 0.5 μg, 1 μg, or 2 μg of plasmid DNA: pcDNA3.1Zeo(+) + BACE1 5' UTR and -5' BACE1 UTR (a generous gift from Robert Vassar), pLenti CMV/TO Puro destination vector (backbone), wild-type mouse PERK, or kinase-dead, K618A PERK was complexed with 2.4 μl of Neuromag transfection reagent (Nanotherics Ltd.) for 15-20 min in serum-free neurobasal media. Complexes were added drop-wise to 24-well plates of pure neuronal cultures containing 500,000 cells per well. Plates were incubated for 30 min at
37°C with 5% CO₂ over a magnet array oscillating at 3 Hz with 0.2 mm amplitude of displacement. Following transfection, plates were immediately removed from the magnet array and placed on a shelf in the incubator at the same temperature and CO₂ conditions. Plates were harvested after 48-72 h for Immunoblotting or cell staining following routine methods.

Statistical Analysis. All data were analyzed by Prism 5.0 software (GraphPad Software, San Diego, CA). All quantifications are expressed as mean ± SEM. Paired results were analyzed by Mann-Whitney U test or unpaired student t test while data with multiple categories were analyzed by one-way analysis of variance (ANOVA) plus Newman-Keuls or Dunnett post hoc test, as indicated. Values of p < 0.05 were considered significant.

Study Approval. All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and Johns Hopkins University.

3.4: RESULTS

BACE1 and Aβ oligomer immunostaining are elevated in brains of HIV(+) individuals. Previous studies have shown accumulation of neurodegenerative proteins in the brains of HAND patients. To investigate the mechanisms underlying age-related pathologies that are increasingly observed in HAND, we first assessed levels of Aβ oligomers (Aβ-oligo), the Aβ species suggested to be responsible for CNS neurotoxicity in AD, in the hippocampus of HIV(+) HAND patients (see Table 1 for a summary of cases). We observed elevated levels of intraneuronal Aβ oligomers in HAND patients compared to healthy controls (Figure 1A, Table 1). Importantly, we found no evidence of senile or diffuse Aβ plaque deposition in the hippocampus using the BC05 antibody, an Aβ₄₂-specific antibody generated by Dr. Virginia Lee (not shown). As oligomeric Aβ is derived from cleavage of APP by the β secretase enzyme 1, we determined whether BACE1 expression was altered in the same cohort of samples. When we stained for BACE1 in the CA1, CA3, and dentate gyrus regions of the hippocampus, we observed significantly elevated BACE1 immunoreactivity in HAND patients compared to healthy controls,
consistent with our finding of elevated Aβ oligomers, despite decreased microtubule-associated protein 2 (MAP2) expression (Figure 1B). Interestingly, no significant changes in BACE1 were observed in the dentate gyrus. To confirm our immunostaining findings, we assessed protein levels of BACE1 and APP in whole brain lysates from the mid-frontal cortex of uninfected (HIV(-)), HIV(+) neurocognitively normal (HIV(+) Normal) and HIV(+) HAND patients by immunoblotting.

As shown in Figure 1D, and quantified in Figure 1E, BACE1 was elevated both in HIV(+) Normal and HAND patients compared to uninfected controls; however, no differences were observed between HIV(+) Normal and HAND samples. As increased Aβ production may also result from changes in total APP expression, we also immunoblotted these lysates for full-length APP. As seen in Figure 1F, we found no significant differences in APP protein levels when groups were compared according to infection or cognitive status. Of note, levels of full-length APP showed more variation among different samples from the HIV(+) HAND group. The significance of this variation remains unclear. Importantly, we found that BiP levels were elevated in HIV(+) Normal brain samples, but not in HAND (Figure 1D), in agreement with our previous findings. However, p-eIF2α was unchanged with HIV infection and regardless of neurocognitive status, compared to healthy controls (Figure 1G).

**Antiretroviral therapy induces axonal damage and ER stress in vivo.** Several clinical studies have reported an inverse relationship between ARV regimens with high CNS penetration-effectiveness (CPE) scores and neurocognitive outcome (57-59). However, it has been difficult to separate out CNS toxicity induced by HIV versus that of ARV drugs themselves. Previously, we have shown that ARV drugs administered to SIV-infected pigtail macaques lead to decreased markers of synaptodendritic integrity, including synaptophysin in the hippocampus and calmodulin kinase II in the frontal cortex, compared to SIV-infected, placebo-treated or uninfected controls (28). In this model of lentiviral-induced neurodegeneration, adult pigtail macaques are inoculated with two strains of SIV, SIV/DeltaB670 and SIV/17E-Fr, as described previously (48). 12 days post-inoculation animals receive either a placebo or ARV therapy (ART) that includes tenofovir (NRTI), atazanavir (PI), saquinavir (PI), and L-870812a (integrase inhibitor). 90% of placebo-treated
animals develop neurologic disease within 12 weeks post-inoculation (p.i.), as described previously (48). However, animals that receive ART do not develop SIV encephalitis, nor do they demonstrate neurological dysfunction (50). Further, ART-treated animals display a rapid reduction in plasma and CSF viral load and suppression of viral replication with conservation of CD4+ T cell counts until elective euthanasia at day 160 p.i. To expand upon our previous findings, we utilized immunofluorescence and immunoblotting to retrospectively assess formalin-fixed, paraffin-embedded hippocampal tissue from SIV-infected animals for APP expression, which serves as a sensitive marker of neuronal damage and impaired axonal transport in the SIV/macaque model of HIV CNS disease (60). Consistent with our previous report, we found significantly elevated APP expression in the SIV(+)/ART group compared to both SIV(+)/placebo and uninfected controls (Figure 2A and 2B). Interestingly, placebo-treated animals showed a reduction in hippocampal APP expression compared to uninfected controls. These changes were corroborated by immunoblotting of total APP (Figure 2C). To validate our SIV/pigtail macaque model of HIV CNS disease we also immunoblotted for BiP, BACE1, and p-eIF2α (Figure 2C). Similar to our observations in human tissue, we observed increased BiP expression with infection (SIV(+)/placebo animals) compared to uninfected controls but no significant change in the SIV(+)/ART group (Figure 2D). Importantly, SIV(+) macaques treated with ART displayed a significant increase in BACE1 compared to both SIV(+)/placebo and uninfected controls (Figure 2E). However, no significant changes in p-eIF2α were observed (Figure 2G).

Antiretroviral drugs activate the unfolded protein response and upregulate BACE1 expression. We and others have shown that ARVs are toxic to primary neurons (28, 61, 62). Although it is suggested that such toxicity is mediated by mitochondrial DNA damage and/or pro-oxidant properties of these drugs, additional mechanisms may also contribute to ARV-mediated neuronal damage and death. Given our previous observations of elevated UPR markers in the post-mortem mid-frontal cortex of patients with HAND and the established role of PIs inducing ER stress in a variety of cell types, we hypothesized that antiretroviral drugs may be activating the UPR in neurons. Therefore, we examined canonical UPR markers in primary rat neuroglial
cultures in response to increasing concentrations of zidovudine (AZT), saquinavir (Saq), or ritonavir (Rit) (Figure 3A). Drug dose ranges used in our in vitro studies were based on reported plasma and CSF levels of ARVs (63, 64). At 16 h post-treatment with ritonavir or saquinavir, neurons displayed a five-fold increase in BiP expression and more than four-fold increase in phospho-eIF2α (p-eIF2α) (Figure 3B and 3C). 25 μM AZT treatment resulted in a three-fold increase in BiP expression, but had no significant effect on p-eIF2α. As an added parameter to ensure the presence of ARV-mediated UPR activation, we treated primary neurons with 10 μM Rit, 1 μM Saq, 25 μM AZT, or 1 μM thapsigargin (positive control) for 16 h and assessed levels of spliced XBP-1 (sXBP1) by TaqMan quantitative real-time PCR relative to unspliced XBP-1 (uXBP1) (Figure 3H). We observed nearly a two-fold increase in sXBP1 mRNA in Rit and Saq-treated cultures, but no change in AZT-treated neurons, compared to vehicle controls. To rule out the possibility of species-specific effects, we also treated 21 days in vitro (DIV) human fetal neurons with increasing doses of AZT, Saq, and Rit for 16 h and found similar trends in both BiP and p-eIF2α expression in Saq and Rit-treated cultures compared to rat cortical neuroglial cultures (Figure 3I).

Recent studies have shown that induction of ER stress in neurons can increase protein expression of the APP-cleaving enzyme, BACE1 (44, 65). Therefore, we sought to determine whether ARV-treated neuroglial cultures, which show robust activation of ER stress, display changes in BACE1 expression. Immunoblotting analysis revealed that 16 h drug treatment with AZT, Saq, or Rit resulted in 4 to 5-fold increases in BACE1 expression (Figure 3E). Similar results were observed in human fetal neurons treated with ARVs (Figure 3I).

In addition to BACE1, APP may be cleaved by two additional secretases, γ-secretase and α-secretase, the latter of which comprises the non-amyloidogenic cascade. To examine whether ARV-mediated BACE1 upregulation is specific to BACE1, or a shared mechanism among all APP secretases, we also immunoblotted for presenilin-1 (PS-1) (γ-secretase) and ADAM metalloproteinase domain 10 (ADAM10) (α-secretase) (Figure 3A). We found no significant changes in the expression of either of these enzymes regardless of drug treatment or
dosage (Figure 3F and 3G). Notably, SIV(+)/ART macaques displayed increased hippocampal expression of PS-1 and ADAM10 compared to uninfected controls (Figure 2H and 2I). Previous work has shown that ER stressors are capable of altering APP expression as well (38); therefore we also immunoblotted for APP and observed a small, but significant increase in APP levels in 25 μM AZT and Rit-treated neuroglial cultures (Figure 3D). Taken together, these data indicate that antiretroviral drugs, and PIs in particular, induce ER stress in neurons leading to enhanced APP and BACE1 expression.

*Ritonavir increases amyloidogenic APP processing in primary neurons.* Based on our observation that ARVs induce BACE1 expression in neurons, we sought to determine whether this translated into functional changes in enzymatic APP processing. To that end, we treated Chinese Hamster Ovary (CHO) cells stably expressing human APP with 10 μM Rit, as a representative PI, for 16 h and measured the relative amount of Aβ species in the culture media using a commercially available human/rat Aβ40- or Aβ42-specific sandwich ELISA. While ritonavir had no effect on secreted Aβ40 levels, Aβ42 peptide was increased by nearly two-fold (Figure 4A). Addition of a cell-permeable BACE1 inhibitor (BSI) blocked ritonavir-mediated elevations in Aβ42 in a dose-dependent manner. These findings contradict previous reports that changes in BACE1 activity affect the generation of all Aβ isoforms equally (66). To further understand how ritonavir affects human APP processing, CHO cell conditioned media was subjected to sAPPβ and sAPPα ELISAs (Figures 4B and 4C). In contrast to the observed elevations in Aβ42 peptide, no significant changes in sAPPβ or sAPPα were detected in the culture media from ritonavir-treated CHO cells. We also utilized the human/rat Aβ40- or Aβ42-specific sandwich ELISA to assess Aβ40 and Aβ42 release into the media of rat cortical neuroglial cultures treated with combinations of ARV drugs (Figures 4D and 4E). At 4 h post-treatment, no changes were observed in secreted Aβ40 or Aβ42 peptides. However, at 16 h post-treatment, all combinations of ARV drugs resulted in 1.5 and 3 fold increases in Aβ40 and Aβ42 levels, respectively. In conjunction with our in vivo findings of elevated Aβ oligomers in HAND patients and increased APP in SIV-infected, ARV-
treated macaques, these findings suggest that ARVs promote amyloidogenic APP processing in CNS neurons.

Ritonavir upregulates BACE1 expression in a translation-dependent manner. Vassar and colleagues first reported that chronic activation of ER stress results in translational upregulation of BACE1. To determine if antiretroviral drugs increase BACE1 expression by altering mRNA levels, we first treated 21 DIV rat neuroglial cultures with Rit, Saq, Azt, or thapsigargin for 16 h and harvested mRNA for quantitative real-time PCR (qRT-PCR). We observed no changes in BACE1 mRNA in any of the ARV-treated cultures compared to controls; however, there was a significant decrease in BACE1 mRNA in thapsigargin-treated neurons (Figure 5A). To confirm that BACE1 regulation is occurring via a post-transcription mechanism, we pre-treated neurons with a translation inhibitor, anisomycin, or a transcription inhibitor, actinomycin D for 12 hours followed by 16 h treatment with ritonavir and harvested protein lysates for analysis by immunoblotting for BACE1. Inhibition of transcription with 5 μM actinomycin D did not affect upregulation of BACE1 protein levels by ritonavir; however translation inhibition by 10 or 50 μM anisomycin abrogated Rit-mediated BACE1 protein upregulation (Figures 5B and 5C) indicating that PIs are exerting their effects on neuronal BACE1 expression through a translational or post-translational mechanism.

Activating transcription factor 4 (ATF4) is a canonical target of translational control by phospho-εIF2α. Similar to BACE1, ATF4 mRNA contains multiple uORFs in the 5’ UTR, which act to suppress translation under basal conditions, but undergo scan-through following phosphorylation of εIF2α, leading to selective translational upregulation during conditions of ER stress (44, 65, 67, 68). ATF4 subsequently translocates to the nucleus to upregulate expression of genes involved in the ER stress response. Thus, we determined whether ATF4 was increased in the nucleus of ritonavir-treated neurons following 16 h treatment. We observed nearly a five-fold increase in nuclear ATF4 in Rit- and thapsigargin-treated neuroglial cultures while cytoplasmic levels remained unchanged (Figures 5D and 5E). Similarly, BACE1 expression was increased greater than two-fold in the cytoplasm of Rit-treated cultures (Figures 5D and 5E).
To confirm that translational control of BACE1 is occurring through its 5' UTR, we transfected rat cortical neurons with vectors containing full-length human BACE1 with (+5' UTR) or without (-5' UTR) the 5' UTR then treated cultures with DMSO or ritonavir for 16 h. Similar to our wild-type rat cortical cultures, the neurons transfected with BACE1 +5' UTR displayed nearly a three-fold increase in BACE1 expression following treatment with ritonavir compared to vehicle controls (Figure 5F). Deletion of the 5' UTR resulted in a significant increase in overall BACE1 expression in transfected cells as described previously (44, 69), however overexpression of the -5' UTR BACE1 construct prevented ritonavir-mediated upregulation of BACE1 in neurons (Figure 5F and 5G).

To rule out the involvement of a post-translational mechanism in BACE1 upregulation by ritonavir, we performed a pulse-chase experiment using 35S-metabolic radiolabeling to determine the half-life of BACE1 in Rit-treated neurons compared to vehicle controls. After confirming immunoprecipitation of BACE1 from neurons (Figure 6A), we pulse-labeled 21 DIV neuroglial cultures for 30 min and then chased with neurobasal media with or without 10 μM Rit for up to 24 h (Figure 6B). Compared to controls, we observed no apparent difference in BACE1 half-life in Rit-treated neuroglial cultures over the course of 24 h (Figure 6C). In both treatment conditions, we observed that BACE1 half-life was approximately 12 h, similar to what has been reported previously (44, 70). Collectively, these data strongly suggest that ritonavir-mediated upregulation of BACE1 in neurons occurs through a 5' UTR-dependent translational mechanism.

**PERK is necessary for ARV-mediated BACE1 upregulation in neurons.** Several reports have proposed a role for p-eIF2α in post-transcriptional regulation of BACE1 under conditions of oxidative and ER stress (44, 65, 71). However, eIF2α can be phosphorylated by one of four kinases (PERK, PKR, HRI, and GCN2) depending on the type of stress impinging on cells. Given that PERK is the central kinase responsible for eIF2α phosphorylation under conditions of ER stress, we assessed whether PERK was involved in the stress response signaling in neurons treated with antiretroviral drugs. First, we determined the effect of blocking PERK signaling in neurons through overexpression of a kinase-dead mutant of PERK (dnPERK). We
overexpressed wild-type (PERK) or kinase-dead (dnPERK) PERK in 14 DIV pure neuronal cultures using magnetofection. 72 h after transfection, lysates were harvested for immunoblotting and probed for markers of ER stress and BACE1 (Figure 7A). In addition to successful overexpression of both wild-type and kinase-dead PERK, we observed a significant decrease in both p-eIF2α and BACE1 in neurons overexpressing dnPERK compared to cultures overexpressing wild-type PERK (Figures 7B-7D). Consistent with our hypothesis, BACE1 and p-eIF2α levels in dnPERK-expressing cultures were not significantly different from neurons transfected with the empty vector control (pLENTI).

Next, we sought to determine whether PERK signaling was necessary for ARV-mediated BACE1 upregulation. To that end, we isolated PERK-excised neuroglial cultures from brains of tamoxifen-inducible cre recombinase conditional PERK knockout embryonic mice. At DIV 10, neuroglial cultures were treated with 100 nM or 1 μM 4-hydroxtamoxifen (4-OHT) to excise the PERK gene (56). The estimated efficiency of PERK excision in neurons was approximately 50 to 60% after 96 h 4-OHT treatment (Figure 8A). Wild-type and PERK-excised neurons were treated with 10 μM ritonavir (Rit) or 1 μM saquinavir (Saq) for 16 h. As shown in Figures 8B and 8C, PERK-excised neuroglial cultures treated with Rit or Saq displayed reduced p-eIF2α, BACE1, and APP compared to wild-type cultures treated with the same drugs. These findings indicate that ARV-mediated BACE1 upregulation in neurons is dependent on ER stress response signaling.

3.5: DISCUSSION
The unanticipated persistence of neurocognitive deficits in HIV-positive individuals has become a growing public health concern as the average age of HIV-infected patients in the U.S. is expected to be over the age of 50 by the year 2015 (72). Increasing clinical and experimental evidence suggests that ARV drugs are toxic to CNS neurons, yet the potential mechanisms by which ARVs may be contributing to HAND neuropathology remain elusive. Previously, we have reported that ARVs induce ROS production in primary neurons leading to activation of the endogenous
antioxidant response (28). Additionally, there is extensive evidence that ARVs induce ER stress in a wide variety of peripheral cell types. Our findings provide a mechanistic link between ARV-mediated ER stress in cortical neurons and our observations of increased BACE1 and Aβ oligomers in post-mortem brain tissue of ARV-treated, HIV-positive individuals.

In the present study, we demonstrate for the first time the ability of ARV drugs, and PIs in particular, to robustly activate the UPR in primary rat neuroglial cultures and human fetal neurons as demonstrated by increased expression of BiP, p-eIF2α, and spliced XBP1. Additionally, we observed increased levels of nuclear ATF4, indicating that ARV-mediated eIF2α phosphorylation leads to functional changes in the translational profile of neurons. The specific mechanism by which PIs and NRTIs activate ER stress in neurons remains unclear, but is likely to be multifactorial. In other cell types, PIs have been shown to induce ER stress via proteasome inhibition and depletion of ER calcium stores (21, 22, 73-75). Interestingly, Zhang, et. al., reported that pharmacological inhibition of proteasome activity can activate PERK, the primary kinase responsible for phosphorylating eIF2α under ER stress conditions (76). Further studies will be necessary to determine if: 1) PIs can inhibit proteasome function in primary neuroglial cultures, 2) proteasome inhibition precedes phosphorylation of eIF2α, and 3) rescue of proteasome function abrogates phosphorylation of eIF2α and all purported downstream effects, including translational upregulation of ATF4 and BACE1.

In agreement with our in vitro studies, BiP was elevated in HIV(+) Normal cases, however, no significant difference was observed between HIV(+) HAND cases and uninfected controls, indicating that severity of CNS impairment may be less important than early events associated with HIV pathogenesis. Unexpectedly, phospho-eIF2α was unchanged with HIV infection in our cohort of patient samples, where it was previously reported to be elevated in HIV-positive cases (77). One potential reason for the conflicting results may be the variability of tissue used to generate the whole-cell protein extracts from patient samples, and the relative percentage of specific cell types, such as astrocytes and microglia, in the specimen used (77). As the inflammation in ARV-medicated, HIV-infected brains is typically focused in the
hippocampus and entorhinal cortex, there may be region-specific elevations in ER stress markers correlating with these focal points of inflammation. Many clinical factors are also likely contribute to the level of neuronal ER stress in HAND patients, including duration of HIV infection, co-morbidities, latency between infection and onset of ARV medication, adherence to ARV regimens, and the specific treatment regimen(s) prescribed during the course of infection. Despite these complications, our data are consistent with UPR activation, BACE1 upregulation, and Aβ oligomer generation as underlying contributors to pathology in HAND.

Our work also confirms a report by Vassar and colleagues that chronic activation of ER stress in neurons is linked to translational BACE1 upregulation. AZT, saquinavir, and ritonavir all increased expression levels of BACE1 in neuroglial cultures and human fetal neurons at 16 h post-treatment, parallel to an increase in nuclear ATF4. This increase appears to be at least partially mediated by PERK, although we cannot rule out the potential contribution of other eIF2α kinases. Interestingly, PERK excision decreased expression of p-eIF2α and BACE1, but had no effect on BiP or APP, indicating that the mechanism by which these markers are increased in ARV-treated cultures is likely distinct from that of BACE1. While extensive evidence has shown that BiP is increased transcriptionally following ER stress, the mechanism of APP upregulation remains unclear, and may be unrelated to ER stress induction. Additionally, we report elevated BACE1 expression in CA1 and CA3 hippocampal regions of HAND patients by IFA and confirmed by immunoblotting of whole brain lysates. Notably, BACE1 was elevated in HIV-positive cases, regardless of neurocognitive status, suggesting that BACE1 elevation may be an early event in the pathogenesis of HAND; similar to what has been reported in AD (46). Importantly, neither γ-secretase nor α-secretase, both of which cleave APP, showed any changes in expression following ARV treatment. This distinction is likely a result of the different mRNA structure of these enzymes. Unlike, γ-secretase and α-secretase, BACE1 mRNA contains three uORFs in its long, GC-rich 5’-UTR, which act to suppress translation under normal cellular conditions (44, 68). However, phosphorylation of eIF2α induces bypass, or scan-through, of the inhibitory uORFs leading to more efficient translation of BACE1 mRNA. Indeed, placement of multiple uORFs in
the 5’-leader sequence of mRNAs is an evolutionarily conserved mechanism among many species to control translation of stress-related genes (67). However, it remains unclear what protective role BACE1 might have, if any, in neurons undergoing ER stress.

One potentially detrimental consequence of elevated BACE1 expression is increased amyloidogenic processing of APP to generate Aβ peptides. Giunta, et. al., first demonstrated the ability of ARVs to increase Aβ production in N2a cells transfected with Swedish mutant APP (78). Similarly, our ELISA results demonstrate that ritonavir, as a representative PI, induces increased production of Aβ$_{42}$ in rat neurons and CHO cells expressing human APP. Unexpectedly, no changes in Aβ$_{40}$ production were observed in CHO cells treated with ritonavir, although Aβ$_{40}$ was increased in media of rat neuroglial cultures treated with all combinations of ARVs. This inconsistency may be a result of potential differences in APP processing in CHO cells versus neurons, or an artifact resulting from overexpression of human APP, which itself may be activating ER stress and the UPR. Ultimately, these data suggest that ARVs alter APP processing in CNS neurons, likely through upregulation of BACE1.

Contrary to these findings is a previous report by Ikezu and colleagues that PIs, including ritonavir and saquinavir, modestly inhibit Aβ degradation in human monocyte-derived macrophages, but decrease Aβ$_{40}$ production and BACE1 activity in human cortical neurons (79). Furthermore, they showed that oral administration of nelfinavir or lopinavir/ritonavir had no effect on Aβ accumulation in an APP SCID mouse model. While these results are striking, several caveats must be considered: 1) previous work indicates CRND8 transgenic animals begin to show plaque deposition at approximately 9 weeks of age, yet the APP SCID mice generated by Ikezu (BALB/cBy-Prkdc$^{scid}$ mice crossed with CRND8 mice) displayed no overt amyloid pathology at 12 weeks of age. Therefore, the inclusion of age-matched, CRND8 transgenic controls is necessary to ensure pathology in the APP SCID mice occurs in a similar, time-dependent manner, 2) concentrations of PIs tested in human fetal neurons were 2.5 – 5 fold greater than the highest concentrations of PIs tested in our experiments, 3) ARV concentrations tested on purified BACE1 protein in vitro were up to 30-fold greater than concentrations employed in our studies.
and 4) the effect of PIs on Aβ$_{42}$ was not tested by Ikezu and colleagues, while we found that PIs had the greatest effect on Aβ$_{42}$ production. Notably, we found that concentrations of ARVs similar to those employed by Ikezu were highly toxic to primary neurons even at time points less than 16 h. Thus, it is difficult to directly compare the results of our studies with those of Ikezu and colleagues due to variations in drug concentrations and in vitro/in vivo model systems. Clearly, further investigation is needed in this area.

Previous studies investigating amyloid pathology in HIV-positive, ARV-medicated brain tissue have yielded conflicting results. Green et al., were the first to show increased APP accumulation, primarily intraneuronal, and amyloid-associated pathology in ARV-treated, HIV-positive individuals compared to ARV-naïve controls (9). These findings were confirmed by Xu and Ikezu who observed intraneuronal Aβ immunoreactivity and Aβ plaques in ARV-treated, HIVE brains but only intraneuronal Aβ in ARV-untreated, HAD brains, with no evidence of extracellular deposition (8). In contrast, Anthony et. al., found no evidence of increased Aβ deposition or plaque accumulation in HIV-infected cases, but did observe increased hippocampal hyperphosphorylated Tau, especially in ARV-medicated patients, compared to unmedicated controls (10, 80, 81). To reconcile these differences and shed light on the clinical significance of our in vitro findings, we stained aged-matched HIV-negative and HIV-positive post-mortem brain tissue with Aβ$_{42}$ C-terminal-specific (not shown) and Aβ oligomer-specific antibodies. We found no evidence for Aβ plaque deposition in HIV-positive cases, but strikingly, we did observe intraneuronal Aβ immunoreactivity, consistent with previous reports. More importantly, we show for the first time, evidence for abundant intraneuronal Aβ oligomer formation in hippocampus of ARV-treated patients. As Aβ oligomers are believed to be highly cytotoxic, and thus responsible for neuronal damage and death in AD, this may be a contributing factor to early aging in HIV-positive individuals as well. However, future studies investigating ARV therapy as a specific contributor to HIV pathogenesis are warranted.

One major caveat associated with human studies in HAND is the inability to separate out pathological effects associated with HIV from those associated with confounding variables such
as co-morbidities, drug abuse and antiretroviral toxicity. To address these concerns, we utilized two animal models of chronic ARV drug treatment. We have previously shown that SIV-infected, pigtailed macaques treated with an ARV cocktail show increased hippocampal synaptodendritic damage compared to placebo-treated and uninfected controls. Here, we expand upon those findings to report elevated neuronal APP in hippocampus of SIV(+)/ART macaques compared to controls. Taken together, these observations strongly suggest that antiretrovirals in the presence of virus may contribute to the neurodegenerative process associated with HAND. Although it remains unclear why placebo-treated animals displayed decreased APP compared to uninfected controls, several caveats must be considered when interpreting these results. First, the time to euthanasia after the initial SIV infection is different between SIV(+)/placebo and SIV(+)/ART groups, as these samples were obtained from animals used to address a distinct question regarding use of ART. Second, there was persistent viral DNA in the CNS of SIV(+)/ART macaques, though it is uncertain if this DNA is replication competent (50). Lastly, there was no SIV(-)/ART control group to account for the effects of the drugs by themselves in the absence of virus. Future studies that include the use of SIV(-)/ART controls will be invaluable for separating out the contribution of virus to CNS damage from that of ARVs. Importantly, concentrations of ARV drugs used in the SIV/macaque studies have been demonstrated by Clements and colleagues to closely reflect dose ranges measured in human plasma and CSF.

Although CNS effects of ARVs are still poorly understood, clinical studies have begun to address the effect of drug regimens with high CNS penetration on populations of HIV-positive individuals. While many studies have shown that ARVs are beneficial for improving cognition in treated patients, others have found a significant association between ARV regimens with high CPE scores and poorer neurocognitive performance (5, 57-59, 82-90). Confounding the issue of ARV toxicity in the CNS is variation in blood brain barrier (BBB) permeability among different drug classes, persistence of viral load in the brain, and the resulting difficulty to separate out neuronal toxicity induced by HIV from that of antiretroviral drugs. While many ARV drugs are thought to suffer from various physicochemical properties that impair their ability to penetrate an intact BBB,
there is extensive evidence demonstrating increased entry of small molecules and serum proteins into the CNS following HIV-mediated disruption of BBB endothelial cells and tight junctions (91-98). Therefore, it is reasonable to consider that higher-than-anticipated concentrations of ARV drugs may penetrate the compromised BBB of HIV-infected individuals. Notably, pharmacokinetic analysis of ARVs in guinea pigs revealed significantly higher uptake of [3H]ritonavir into brain parenchyma compared to CSF, suggesting that clinical estimates of CNS penetration for PIs may be lower than actual drug concentrations in the brain (63). In addition to uncertainties in ARV CNS penetration with currently prescribed regimens, current efforts to enhance delivery of ARV drugs to the CNS via nanotherapeutics necessitate a careful examination of the potential neurotoxic effects these drugs may exert in the brain.

While concentrations of ARVs utilized in this study are significantly higher than peak CNS levels obtained in patients, the effects of ARV drugs on neurons we demonstrate here are likely to occur in vivo over long-term exposures. In the present study, we sought to model these chronic, low-dose effects of ARVs in a more acute time frame with higher doses of drug. Our results indicate that nanomolar concentrations of HIV protease inhibitors robustly activate the UPR in primary cortical neurons leading to PERK-dependent phosphorylation of eIF2α and translational upregulation of BACE1. ARVs alone and in combination were also shown to increase production of Aβ peptides in multiple cells types, which could be dose-dependently blocked by addition of a cell-permeable BACE1 inhibitor. Lending further support to these findings were our observations that ARV-medicated, HIV(+) patients display elevated levels of Aβ oligomers, BACE1, and BiP. Taken together with our previous reports of elevated p-eIF2α and ATF6 in HAND tissue, these data suggest a role for ER stress in CNS HIV pathogenesis. These results bear striking similarities to observations made in AD, PD, ALS and prion disease conditions in which markers of ER stress, including phospho-PERK are elevated in multiple brain regions. While transient eIF2α phosphorylation is protective to cells coping with ER stress, sustained suppression of global protein synthesis in the CNS may ultimately lead to impaired LTP and synaptic failure. However, promising therapeutics targeted at the UPR have already been reported. Several
studies have indicated that genetic and pharmacological suppression of PERK activity can decrease brain p-eIF2α expression, improve synaptic plasticity and rescue memory deficits in an AD mouse model and alleviate prion-mediated pathology and cognitive deficits in mice inoculated with prion proteins (99, 100). Additionally, BACE1 inhibitors, which have already been tested extensively in clinical trials, may have therapeutic benefits in patients suffering from HAND. Future investigations will be necessary to determine the role of BACE1 in the ER stress response. Furthermore, we propose to investigate the potential effect of ARVs on BACE1 redistribution to the trans-golgi network and early endosomal compartments as a mechanism of increased amyloidogenic APP processing, similar to what has been reported by Tan et. al (71). These studies will enhance our understanding of BACE1’s role in cellular maintenance under normal and stressed conditions in the CNS and provide insights into potential therapeutic targets for neurodegenerative disease.

3.6: ACKNOWLEDGEMENTS

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**Table 1**

Summary of human cases used for immunofluorescence staining in Figure 1

- **NNTC ID**: National NeuroAIDS Tissue Consortium identification.
- **HIV**: HIV infection status.
- **Neurocog**: Neurocognitive diagnosis.
- **PMI**: Postmortem interval (hours).
- **Aβ Oligo**: Extent of hippocampal Aβ oligomer burden as assessed by Nab61 staining.
- **N/A**: Not available.
- **F**: Female; **M**: Male.
Figure 1. BACE1 and Aβ oligomer immunostaining are elevated in the brain of HIV(+) cases. Paraffin-embedded tissue sections from hippocampus of HIV(-) control, HIV(+) Normal and HIV(+) HAND cases were prepared for immunofluorescent analysis and visualized by laser confocal microscopy. A) Representative images are shown from hippocampal sections triple-
labeled for Aβ oligomers (red), MAP2 (green), and nuclei (blue). Red and green colocalization appears yellow. Aβ oligomers (red) are increased in MAP2-positive neurons in hippocampus of HIV(+) cases compared to controls. B) CA1, CA3, and dentate gyrus hippocampal sections were double-labeled for BACE1 (green) and MAP2 (red). Representative images are shown. Despite a loss of MAP2 (red) in HIV(+) HAND cases compared to controls, BACE1 (green) immunostaining was increased in CA1 and CA3 regions in HAND cases. C) Whole brain lysates from the mid-frontal cortex of HIV(-) controls (n=4), HIV(+) neurocognitively normal (HIV(+) Normal) (n=7), and HIV(+) HAND (n=6) cases were prepared for immunoblot and probed for BiP, BACE1, APP, phospho- and total eIF2α. D-G) Densitometric analysis revealed no significant changes in expression levels of phospho-eIF2α or APP in HIV(+) cases, however, BiP was elevated in HIV(+) Normal cases and BACE1 was elevated in HIV(+) Normal and HIV(+) HAND cases compared to controls (*p<0.05, ns: not significant, one-way ANOVA plus Newman-Keuls post hoc testing).
Figure 2. Antiretroviral drugs administered to SIV(+) macaques induce CNS damage and elevated BACE1. Using double-label confocal microscopy, formalin-fixed, paraffin-embedded hippocampal brain sections from pigtailed macaques were labeled by immunofluorescence for: MAP2 (red) and APP (green). Pigtail macaques were either uninfected (n=6), SIV-infected and treated with placebo (n=7), or SIV-infected and treated with combination antiretroviral therapy (ART) (tenofovir, atazanavir, saquinavir, and L-870812a) (n=4). A) Representative images of cases stained with APP and MAP2 are shown. B) Quantification indicates a significant increase in intraneuronal APP in SIV(+) ART-treated macaques compared to uninfected and SIV(+)/placebo-treated controls (**p<0.01, ***p<0.001, one-way ANOVA plus Newman-Keuls post hoc testing). C) Fresh-frozen tissue sections from the hippocampus of pigtailed macaques were
subjected to standard protein extraction and immunoblotting for expression of BiP, BACE1, APP, p-eIF2α, total eIF2α, PS-1 CTF, and ADAM10. Fast green was used as a loading control. D-I) Quantification of band densities from immunoblots shown in Figure 2C (*p<0.05, **p<0.01, ***p<0.001, ns: not significant, one-way ANOVA plus Newman-Keuls post hoc testing).
Figure 3. Antiretroviral drugs activate the unfolded protein response and upregulate BACE1 expression in primary neurons. A) 21 DIV rat neuroglial cultures were treated with indicated concentrations of AZT, saquinavir, or ritonavir for 16 h, lysed, and immunoblotted for markers of ER stress (BiP, phospho- and total eIF2α), APP, BACE1, ADAM10, and PS-1). Actin was used as an internal control.
as a loading control for all targets except phospho-eIF2α which was normalized to total eIF2α. Representative blots shown. B-G) Quantification of immunosignals were normalized to actin and expressed as fold change relative to vehicle controls. BiP, p-eIF2α, APP, and BACE1 protein levels were significantly elevated compared to controls. ADAM10, and PS-1 did not show any significant changes (n=5, vehicle: 0.04% DMSO, *p<0.05, **p<0.01, one-way ANOVA with Dunnett post hoc testing). H) Rat neurons were treated with 0.04% DMSO (Vehicle), 10 μM Ritonavir, 1 μM Saquinavir, 25 μM AZT, or 1 μM thapsigargin for 16 h. Total mRNA was isolated and expression levels of spliced XBP1 mRNA measured via TaqMan using the comparative Ct method relative to unspliced XBP1 (uXBP1). qRT-PCR assays were performed in triplicate. Ritonavir and saquinavir induced significant increases in spliced XBP1 (n=4, *p<0.05, ***p<0.001, one-way ANOVA with Dunnett post hoc testing). I) 21 DIV human fetal neurons were treated as in (A) and lysates subjected to immunoblotting. Representative blot shown. Similar to (A), ARVs appeared to induce increased protein expression of BiP, phospho-eIF2α, and BACE1, relative to vehicle controls (n=3).
Figure 4. ARV drugs increase amyloidogenic APP processing in primary neurons. CHO cells stably expressing human APP were treated with 10 μM ritonavir for 16 h or pre-treated with various doses of a cell-permeable beta secretase inhibitor (BSI) for 12 h then treated with ritonavir for 16 h. After 16 h, culture media was collected and subjected to a human/rat Aβ42- or Aβ40-specific sandwich ELISA (A) or a human sAPPα or sAPPβ ELISA (B, C). A) Ritonavir treatment resulted in a two-fold increase in Aβ42 production that was dose-dependently blocked...
by addition of a beta secretase inhibitor, while Aβ_{40} levels were unaffected by addition of ritonavir (n=6, ***p<0.001, ns: not significant, one-way ANOVA plus Newman-Keuls post hoc testing). B) No statistically significant changes in sAPPβ or sAPPα were observed in ritonavir-treated cultures, though sAPPβ was dose-dependently decreased by the presence of a beta secretase inhibitor. D, E) Primary rat neuroglial cultures were treated with various combinations of ARV drugs (10 μM ritonavir, 1 μM saquinavir, and 25 μM AZT) for 4 or 16 h. Culture media was then subjected to a human/rat Aβ_{42}- or Aβ_{40}-specific sandwich ELISA as in (A). At 16 h, both Aβ_{42} and Aβ_{40} levels were increased in most treatment conditions. AZT appeared to blunt PI-mediated enhancement of amyloidogenic APP processing in combination treatment (n=3, *p<0.05, **p<0.01, one-way ANOVA plus Dunnett post hoc testing).
Figure 5. Ritonavir upregulates neuronal BACE1 expression via translation-dependent control.

A) Rat neurons were exposed to the indicated treatments for 16 h. mRNA was isolated for RT-PCR and cDNA subjected to real-time PCR. Levels of BACE1 mRNA were determined using the ΔΔCt method relative to TBP. qRT-PCR assays were performed in triplicate. No significant changes in BACE1 mRNA were observed in ARV drug-treated cultures (n=4, **p<0.01, one-way ANOVA plus Dunnett post hoc).

B) Rat neurons were treated with ritonavir alone or pre-treated with anisomycin or actinomycin D for 12 h then treated with ritonavir. Fast green was used as a loading control.

C) Compared to ritonavir-only treated neurons, anisomycin pre-treatment resulted in decreased BACE1 expression. Actinomycin D had no significant effect (n=4, **p<0.01, ns: not significant, one-way ANOVA plus Newman-Keuls post hoc).

D) Rat neurons were treated with the indicated drugs for 16 h, separated into cytoplasmic and nuclear fractions, and analyzed by immunoblotting.

E) Densitometric analysis revealed a significant increase in ATF4 and BACE1 (n=4, **p<0.01, one-way ANOVA plus Dunnett post hoc).

F) 14 DIV rat cortical cultures were transfected via magnetotransfection with pcDNA3.1Zeo(+) vector containing the entire human BACE1 coding region including the BACE1 5' UTR but missing the 3' UTR (+5' UTR) or pcDNA3.1Zeo(+) vector containing the human BACE1 coding region including the 3' UTR but missing the 5' UTR (-5' UTR). 48 hours following transfection, cultures were treated with 0.04% DMSO or 10 µM ritonavir for 16 h, lysed, and immunoblotted for BACE1. Representative blots shown.

G) Quantification of BACE1 immunosignal was normalized to actin (n=3, *p<0.05, ns: not significant, one-way ANOVA plus Newman-Keuls post hoc testing).
Figure 6. Ritonavir does not affect BACE1 protein stability in primary neurons. A) Primary rat neurons were pulse-labeled for 30 min with $^{35}$S-methionine/cysteine-containing DMEM and chased with neurobasal media + B27. Radiolabeled BACE1 was immunoprecipitated with Dynabeads Protein A and 2 μg anti-BACE1 12h post-labeling to confirm enrichment. B) Neurons were pulse-labeled as in (A) and chased with neurobasal media +/- 10 μM ritonavir for 24 h. BACE1 was immunoprecipitated as in (A), and visualized via autoradiography. Note that immature BACE1 runs at ~60 kDa while mature, fully glycosylated BACE1 runs at ~70 kDa. Representative blots shown (n=3). C) The levels of $^{35}$S-methionine/cysteine-labeled BACE1 were quantified and the percentage changes, relative to 0h time point, are plotted. No significant differences in BACE1 half-life were observed between control and ritonavir-treated neurons (n=3, linear regression analysis).
Figure 7. PERK regulates basal BACE1 expression in neurons. A) DIV 14 pure neuronal cultures were transfected via magnetotransfection with one of three plasmids: pLENTI CMV/TO Puro destination vector (backbone), wild-type mouse PERK, or kinase-dead, K618A mutant
PERK (dnPERK) and complexed with Neuromag transfection reagent. 72 h after transfection, cultures were harvested and lysates prepared for immunoblotting. Representative blots shown. B) Densitometric analysis of PERK immunosignals indicate significant overexpression of wild-type and kinase-dead PERK in pure neuronal cultures compared to empty vector controls (n=3, *p<0.05, ***p<0.001, one-way ANOVA plus Newman-Keuls post hoc testing). C, D) Quantification of p-eIF2α and BACE1 blots show a significant decrease in expression of these respective proteins in neuronal cultures overexpressing dnPERK compared to neurons overexpressing wild-type PERK when transfected with 2 μg of plasmid DNA (n=3, Student’s t test).
Figure 8. HIV protease inhibitor-mediated BACE1 upregulation is PERK-dependent. A-C) Floxed PERK mouse cortical neuroglial cultures were generated by isolating neurons from brains of tamoxifen-inducible cre recombinase conditional PERK knockout embryonic mice at E16.5-17.5. At DIV 10, neuroglial cultures were treated with 0.1 μM or 1 μM 4-hydroxtamoxifen (4-
OHT), for 96 h to excise the PERK gene (note that estimated PERK half-life is 13 h). Efficiency of PERK excision was estimated to be approximately 50 to 60% via densitometric analysis (A) (n=4, *p<0.05, **p<0.01, one-way ANOVA plus Newman-Keuls post hoc testing). Wild-type and PERK-excised neuroglial cultures were treated with 10 μM ritonavir (B) or 1 μM saquinavir (C) for 16 h and lysates prepared for immunoblotting. Representative images shown (n=4). Compared to wild-type cultures treated with ritonavir or saquinavir, PERK-excised neuroglial cultures treated with ARVs showed reduced expression of p-eIF2α and BACE1.
3.7: REFERENCES


CHAPTER 4

“Neuronal BACE1 inhibition is protective against antiretroviral-mediated neurotoxicity”

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4.1: ABSTRACT

Despite advances in antiretroviral therapy (ART), HIV remains the leading cause of dementia among young adults in the United States. Approximately 50% of HIV+ individuals develop some form of cognitive/motor dysfunction, which can range from asymptomatic neurocognitive impairment (ANI) to frank dementia. Collectively, the spectrum of neurological dysfunctions caused by HIV infection is known as HIV-associated neurocognitive disorders (HAND). A growing body of evidence suggests that HAND in the post-ART era has evolved from a subacute dementia to a more insidious, neurodegenerative disease with pathological hallmarks similar to Alzheimer disease (AD). Common features among HAND and AD include the presence of chronic oxidative stress and inflammation, as well as misfolded proteins in the CNS. Additionally, we recently demonstrated that beta-site APP cleaving enzyme 1 (BACE1), which is the rate-limiting enzyme in the production of beta amyloid (Aβ) from the amyloid precursor protein (APP), is upregulated in brains of ART-medicated, HIV+ individuals and that ART drugs increase BACE1 expression and Aβ peptide production in primary neurons via chronic activation of the unfolded protein response (UPR). Therefore, we hypothesized that ART-mediated BACE1 upregulation in neurons may be contributing to neurological dysfunction in patients with HIV via aberrant production of Aβ peptides. In this report, we demonstrate that pharmacological inhibition of BACE1 is neuroprotective against ART-mediated toxicity and that inhibition of the unfolded protein response can mitigate ART-mediated upregulation of BACE1 in neurons. Together, these results suggest that inhibiting neuronal BACE1 function, either directly or upstream, may be a viable adjunctive neuroprotective therapy in the treatment of HAND.
4.2: INTRODUCTION

Beta-site APP cleaving enzyme 1 (BACE1) is a transmembrane aspartyl protease that was initially identified as the primary beta-secretase enzyme responsible for cleaving the amyloid precursor protein (APP) to generate beta amyloid (Aβ), the primary component of senile plaques in AD (1, 2). Post-mortem analyses have revealed that BACE1 expression and enzymatic activity are significantly upregulated in AD brains compared to age-matched controls and BACE1 co-localizes with amyloid plaques (3-6). BACE1 expression is controlled at the transcriptional and translational level by a host of stress factors including inflammation, ischemia, oxidative stress, and energy deprivation (7-9). Oxidative stress, which is a common feature among many neurodegenerative diseases, is particularly important in the pathogenesis of AD as it has been shown to increase expression of β- and γ-secretase leading to overproduction of Aβ, which itself induces oxidative stress, yielding a positive feedback loop (10).

Recent studies have shown that oxidative stress and BACE1 upregulation are salient features of many CNS inflammatory and neurodegenerative diseases. In particular, HIV infection is associated with a spectrum of neurocognitive impairments, termed HIV-associated neurocognitive disorders (HAND), characterized by chronic neuroinflammation, oxidative stress, and neurodegeneration (11-13). Despite the successes of antiretroviral therapy (ART) in suppressing HIV viral loads to undetectable levels and decreasing HIV-related morbidity and mortality, the prevalence of HAND continues to escalate. Furthermore, increasing evidence suggests that HAND pathology has evolved in the post-ART era from a subcortical encephalitis to a more cortical and hippocampal, neurodegenerative condition with Alzheimer disease (AD)-like pathological hallmarks, including neurofibrillary tangles and intracellular Aβ accumulation (14-19). We recently showed that ART-medicated, HIV+ individuals also display increased expression of intraneuronal Aβ oligomers and beta-site APP cleaving enzyme (BACE1) compared to ART-naïve and uninfected controls, suggesting that antiretrovirals may be contributing to enhanced Aβ production in the CNS of HAND patients through a BACE1-dependent mechanism (20).
Although it remains unclear what specific role ART has on the persistence and progression of HAND, many classes of ART drugs induce a wide range of systemic metabolic and neural disturbances that could themselves influence the neurocognitive status of HIV+ individuals. Nucleoside reverse transcriptase inhibitors (NRTIs) have been linked to oxidative stress and mitochondrial dysfunction, manifested clinically as peripheral neuropathy and myopathy, while HIV protease inhibitors (PIs) are associated with atherosclerosis, lipodystrophy, and diabetes (21-31). Recently, several groups have shown that PIs can also induce oxidative stress via production of reactive oxygen species (ROS) leading to neuronal damage and death in vitro (12, 32). Through these and numerous other cellular mechanisms, including 26S proteasome inhibition, ART drugs, and PIs in particular, induce ER stress and activate the unfolded protein response (UPR) in a wide variety of cell types (25, 26, 28, 33-36). In eukaryotes, the UPR is tightly regulated by the ER-resident chaperone, Binding Protein (BiP)/GRP78, which binds to and keeps inactive, three sensor proteins, pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), under basal cellular conditions (37-39). Following induction of ER stress, BiP is recruited to aid in protein refolding allowing for the subsequent activation of PERK, ATF6, and IRE1α. Transient activation of the UPR initially results in PERK-mediated phosphorylation of the translation initiation factor, eIF2α, and global suppression of protein translation. Paradoxically, this event is accompanied by selective translational upregulation of a subset of mRNAs, characterized by the presence of multiple uORFs in the 5’ UTR, that typically encode for molecular chaperones and transcription factors involved in cellular adaptation to stress, such as ATF4 (40). Despite having no known chaperone function, BACE1 mRNA, which contains three uORFs and 4 uAUGs in the 5’ leader sequence, is also translationally upregulated in response to ER stress and has been shown to increase via a translational mechanism in neurons treated with ART drugs (7, 41, 42).

Altogether, these results indicate that ART drugs activate the unfolded protein response in neurons, likely through a pro-oxidative mechanism, leading to translational upregulation of BACE1, aberrant production of Aβ, and cellular toxicity. Therefore, we hypothesized that
pharmacological inhibition of UPR signaling and/or BACE1 activity in ART-treated neurons would mitigate cellular damage and death. Using an acute, in vitro model of ART exposure, we show that inhibition of two eIF2α kinases, PERK and PKR, suppresses ART-mediated BACE1 upregulation in neurons while direct BACE1 inhibition is protective against ART-mediated neuronal damage, suggesting that BACE1 may be a potential therapeutic target in the treatment of HAND.

4.3: EXPERIMENTAL METHODS

Preparation of Primary Neuronal Cultures: All primary rat cortical neuroglial cultures were isolated from frontal cortices of embryonic day 16.5-17.5 Sprague-Dawley rat pups, with modifications of previously described protocols (43). Briefly, cortical cell suspensions were isolated from rat pups and plated in poly-L-lysine (Peptide International) coated tissue culture dishes and maintained in neurobasal media containing B27 supplement at 37°C with 5% CO₂, as described previously (44, 45). Unless otherwise indicated, all experiments were performed at 14-21 days in vitro (DIV) on neuronal cultures containing approximately 90% neurons and 10% astrocytes/glia. 50% of neurobasal media volume was replaced with fresh media 10 days after plating cells.

Drug Treatments: To model mild oxidative stress or ER stress in neurons, 10 DIV rat cortical cultures were treated for 12 hours with 10 and 20 µM hydrogen peroxide diluted in water from a 30% w/w stock solution (Sigma, St. Louis, MO) or 10 µM ritonavir, respectively. All antiretroviral drugs were obtained from the AIDS research and Reference and Reagent Program (Division of AIDS, NIAID, NIH) and prepared in DMSO at 25 mM stock concentrations. To assess the neuroprotective properties of BACE1 inhibition, neuronal cultures were pre-treated with 1 µM Beta-Secretase Inhibitor IV (Calbiochem, Billerica, MA) for 30 minutes followed by treatment with hydrogen peroxide or ritonavir, as indicated. eIF2α kinase inhibition assays were performed using the 7DG PKR inhibitor (Sigma; IC₅₀ = 5 µM) and GSK2606414 PERK inhibitor (EMD
Millipore; IC$_{50}$ = 0.4 nM) and were prepared in DMSO stocks and added to cultures by dilution at the indicated final concentrations.

**Immunofluorescence Staining of Neuronal Cultures:** To assess neuronal damage and death, 15 µM propidium iodide was added to neuronal cultures grown on coverslips 15 minutes prior to the end of drug treatments. Cultures were washed twice with PBS and fixed for 30 minutes in 4% paraformaldehyde/4% sucrose in PBS, followed by permeabilization in 0.2% BSA + 0.1% Triton X-100/PBS for 1 hour at room temperature. Coverslips were then washed twice with PBS and incubated with anti-MAP2 antibody (1:1000) in normal antibody diluent overnight at 4°C. After washing in PBS-T, coverslips were incubated in a fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (1:200 for 30 min at room temperature, Jackson ImmunoResearch Lab, West Grove, PA). Coverslips were mounted on slides and the number of MAP2+ cells determined through blind counting of all fields (15 at 200x) in three adjacent vertical columns through the center of each coverslip. Images were captured using a Nikon Eclipse E400 fluorescent microscope (Nikon Corp, Tokyo, Japan) equipped with an Olympus DP70 digital camera (Olympus Corp, Tokyo, Japan). Propidium iodide puncta were counted by hand using the NIH ImageJ software program (Image J v1.44, NIH) and plotted as fold change compared to untreated controls. Averages are expressed as mean ± SEM.

To determine the effect of oxidative and ER stress on synaptic integrity, neuronal cultures were treated with H2O2 or ritonavir as described above, fixed, and incubated in anti-synapsin antibody (1:300) overnight at 4°C. Coverslips were then incubated in a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody (1:200) for 30 minutes at room temperature. For each treatment group, 15 images were captured on three coverslips from three independent experiments using fluorescence microscopy. Post-acquisition analysis was performed using Metamorph 6.0 (Universal Imaging, Downingtown, PA). Briefly, intensity of synapsin staining was determined by the measurement of integrated pixel intensity (defined as total pixel intensity times the area of pixels with positive synapsin signal). This value was divided by the total MAP2 area.
(also quantified using Metamorph) to account for global changes in neuronal density. For all imaging quantification, three coverslips were counted from three independent experiments. Averages are expressed as mean ± SEM.

**Immunoblotting:** Whole cell extracts of primary rat cortical cultures were prepared using ice-cold whole-cell lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 0.4 mM Na₃VO₄, 100 mM DTT, and 1:100 protease inhibitor cocktail. Lysates were centrifuged at 14,000 g at 4°C for 10 minutes and protein concentrations determined using the Bradford method and equal amounts of protein (20 µg) loaded into each well of a 4-12% Bis-Tris Gradient gels followed by transfer to PVDF membranes at 25 volts at room temperature for 1 hour. Membranes were then blocked in tris buffered saline (TBS) with 0.1% tween (TBS-T) plus 5% bovine serum albumin (BSA) for 30-60 minutes at room temperature. Blocked membranes were incubated in BACE1 primary antibody (Cell Signaling) overnight at 4°C, washed with TBS-T, and incubated with HRP-conjugated goat-anti rabbit secondary antibody for 30 minutes at room temperature. Membranes were developed using SuperSignal West Dura extended duration substrate and pixel intensities of ROIs quantified via densitometric analysis using the NIH ImageJ software program. BACE1 band intensity was normalized to actin loading controls. Immunoblots shown are representative of three independent biological replicates.

**Statistical Analysis:** All data were analyzed by Prism 5.0 software (Graphpad Software, San Diego, CA). All quantifications are expressed as mean ± SEM. Data with multiple categories were analyzed by one-way analysis of variance (ANOVA) plus Newman-Keuls post hoc test, as indicated. Values of p <0.05 were considered significant.

### 4.4: RESULTS

**BACE1 inhibition mitigates MAP2 and synapsin loss in primary neurons treated with hydrogen peroxide.** Numerous studies have shown that neurons exposed to mild oxidative stress display increased expression of BACE1 at the transcriptional and translational levels leading to enhanced...
production of Aβ species (8, 46, 47). To investigate the role of BACE1 in neurons undergoing oxidative stress we first treated primary rat neuroglial cultures with 10 and 20 µM hydrogen peroxide (H2O2) for 24 hours in the presence or absence of a cell-permeable beta secretase inhibitor (BSI) and assessed cellular damage and death via MAP2 (blue), synapsin (green), and propidium iodide staining. We observed that BACE1 inhibition alone had no apparent effect on cellular or synaptic density, nor did it have any effect on propidium iodide uptake compared to vehicle controls (Figure 1). Neuronal cultures treated with 10 µM H2O2 showed decreased MAP2 expression and increased uptake of propidium iodide, but no evident changes in synapsin, while cultures pre-treated with 1 µM BSI showed reduced uptake of propidium iodide but no apparent rescue of MAP2 expression (Figure 1). At 20 µM H2O2, neuronal MAP2 and synapsin levels were reduced, while propidium iodide uptake was increased compared to vehicle controls but decreased compared to 10 µM H2O2-treated neurons. Pre-treatment with 1 µM BSI partially rescued MAP2 loss and completely rescued loss of synapsin. The number of propidium iodide puncta was increased in BSI pre-treated 20 µM H2O2 neuronal cells, suggesting BACE1 inhibition may result in a delayed death response (Figure 1).

**BACE1 inhibition prevents MAP2 loss in ritonavir-treated neurons.** Our previous studies have shown that ritonavir, and other PI class drugs, induce toxic oxidative stress in primary neurons followed by translational upregulation of BACE1 (12, 20). Therefore, we sought to determine whether BACE1 has a protective or deleterious effect on neuronal survival following chronic exposure to ritonavir. Primary rat cortical cultures were pre-treated with DMSO vehicle or 1 µM BSI for 30 minutes then 10 µM ritonavir for 48 hours. Cultures were stained with propidium iodide to assess cellular viability and immunostained for MAP2 and synapsin, as described above. Similar to our previous observations, BACE1 inhibition had no effect on MAP2+ cell count, synapsin intensity, or propidium iodide uptake compared to untreated and vehicle controls (Figure 2A). 48 hour ritonavir treatment resulted in a three-fold decrease in MAP2+ cell count and nearly a three-fold increase in propidium iodide uptake, but no change in synaptic integrity relative to MAP2 area in 10 DIV neuronal cultures (Figure 2A-D). Pre-treatment with BSI rescued MAP2+
cell loss in ritonavir-treated neurons and decreased propidium iodide uptake (Figure 2F). Together, these results suggest BACE1 may contribute to neuronal damage and death in the context of oxidative stressors, such as hydrogen peroxide and PI-class antiretroviral drugs.

Small molecule inhibitors of PKR and PERK block HIV protease inhibitor-mediated phosphorylation of eIF2α in neurons. ART drugs have been shown to increase expression of neuronal BACE1 through an eIF2α-dependent translational mechanism in vitro (20). Thus, we hypothesized that pharmacological inhibition of eIF2α phosphorylation would block ritonavir-mediated BACE1 upregulation in neurons. Among the four known eIF2α kinases, we first tested an inhibitor (7DG) of double-stranded RNA-dependent protein kinase (PKR), which has been shown to respond to various stressors including viral infection, calcium dysregulation, and oxidative/ER stress and has been implicated in translational BACE1 regulation (47-49). We observed that neurons pre-treated with 7DG and exposed to 16 hour ritonavir or saquinavir treatment showed significantly reduced levels of phospho-eIF2α compared to ART-only treated cultures (Figure 3A and 3B). Notably, 7DG also reduced levels of phospho-eIF2α to half the level of untreated and vehicle controls, indicating PKR may play a role in maintaining basal levels of phospho-eIF2α in neurons. We also observed that PKR inhibition in the presence of ritonavir or saquinavir reduced BACE1 expression levels compared to ART-only controls, however, this change was not statistically significant (Figure 3C).

Based on our previous observations that PERK is involved in ART-mediated BACE1 upregulation in neurons, we tested the effects of a PERK inhibitor (GSK2606414, abbreviated GSK) on phospho-eIF2α and BACE1 levels in ritonavir and saquinavir-treated neurons. We found that 0.5 µM GSK significantly reduced phospho-eIF2α levels in ritonavir and saquinavir-treated neurons compared to ART-only controls (Figures 3D-E and G-H). Similar to 7DG, administration of GSK reduced BACE1 levels in the presence of ART, but the change was not statistically significant (Figures 3F and 3I). These results suggest that PKR and PERK both contribute to BACE1 upregulation in ART-treated neurons through phosphorylation of eIF2α.
However, inhibition of only one eIF2α kinase may not be sufficient to fully suppress BACE1 upregulation mediated by ART.  

*PERK inhibitor compound, GSK2606414, does not alleviate ritonavir-mediated neurotoxicity in primary neuroglial cultures.* Genetic and pharmacologic knockdown of PERK activity has been shown to have protective effects in the CNS of animals infected with prions as well as in Alzheimer disease mouse models (7, 50-52). Therefore, we tested whether PERK inhibition abrogates ritonavir-mediated neurotoxicity. Unexpectedly, we found that use of the GSK PERK inhibitor augmented ritonavir-mediated losses in MAP2 and increased propidium iodide uptake (Figure 4). Notably, administration of GSK alone appeared to have toxic effects on neuronal cultures, as indicated by reduced MAP2 expression and a robust increase in propidium iodide uptake compared to untreated and vehicle controls, suggesting that this compound may have toxic side-effects unrelated to PERK inhibition or that PERK itself may play an important role in cellular maintenance and homeostasis in neurons, thus limiting its therapeutic potential.

**4.5: DISCUSSION**

The persistence of less severe manifestations of HAND, including ANI and mild neurocognitive disorder (MND), despite the widespread implementation of combination ART in 1996 highlights the need for new therapeutic targets and adjunctive therapies in the management of HAND. Previous attempts to mitigate neurocognitive decline in HIV+ individuals focused on the use of ART regimens with high CNS penetrance, as estimated by the CNS-penetrance effectiveness (CPE) scoring system. While many clinical studies found that higher CPE scores were initially successful at improving patients’ neuropsychological performance, others found an inverse relationship between CPE scores and neurocognitive/motor performance despite decreased CSF HIV RNA levels (53-57). To date, promising adjunctive therapies with anti-oxidant and anti-inflammatory properties such as selegiline, minocycline, and memantine, have been largely unsuccessful at reversing long-term cognitive decline in HAND patients. Here, we build on our previous observations that ART drugs increase BACE1 expression in vitro and in vivo to show
that BACE1 inhibition is protective against ART-mediated neurotoxicity, thus identifying BACE1 as a potential therapeutic target in HAND and other CNS inflammatory diseases in which oxidative and ER stress are prevalent.

We first sought to understand the role of BACE1 in neuronal survival under conditions of oxidative stress. Previous studies have demonstrated that oxidative stress can induce cellular redistribution of BACE1 to endosomal compartments co-localizing with APP and/or lead to transcriptional and translational upregulation of BACE1 expression in neurons (8, 47). Given that BACE1 is translationally repressed under normal conditions, but selectively upregulated during ER and oxidative stress, we initially hypothesized that BACE1 may play a role in cellular stress adaptation and/or survival. Consistent with this idea, Kamenetz et. al. reported that Aβ produced by BACE1 cleavage of APP was protective against neuronal hyperactivity and excitotoxicity in vitro (58). However, our observations in H202-treated neurons suggest that BACE1 contributes to neuronal damage and death in the presence of mild to severe oxidative stress over 24 hours. These findings were corroborated with our 48-hour ART treatment studies in which BACE1 inhibition partially rescued ritonavir-mediated losses in MAP2+ cell count and propidium iodide uptake. While informative, these observations raise questions regarding the basic physiological functions of BACE1 in neurons, which remain largely elusive. Among its few known roles, BACE1 appears to be involved in peripheral, and potentially CNS, myelination via cleavage of neuregulin-1, and is thought to be important for maintenance of synaptic functioning (2, 59). Additionally, BACE1 is upregulated in neurons in response to a host of inflammatory mediators, including interferon-γ, tumor necrosis factor α, interleukin-1β, and Aβ, and contains transcription factor binding sites for Sp1, CREB, NF-κB, STAT1, and PPARγ among others.

Based on our studies and previous reports, neuronal upregulation of BACE1 appears to be deleterious to cell survival during stress conditions. However, the cellular context of the specific stressor may be critical in determining whether the upregulated protein in question is pro-survival or pro-death. Recently, Baleriola et. al. demonstrated that Aβ_{42}, when applied to distal axons of rat hippocampal neurons, induces phosphorylation of eIF2α and local translation of a
subset of mRNAs, including ATF4 (60). ATF4, typically considered to be a neuroprotective gene involved in cellular adaptation to ER stress, was then shown to propagate a neurodegenerative signaling cascade in Aβ42 –treated neurons, leading to cell death. Therefore, it is likely that stress-induced genes, such as ATF4 and BACE1, that are translationally upregulated in response to phospho- eIF2α, which is elevated in brains of AD and HAND patients, play both protective as well as deleterious roles in neuronal survival depending on the chronicity and localization of the stressor. Careful time course experiments will be necessary to determine if BACE1 has an initial protective role in neurons undergoing oxidative and ER stress that becomes toxic over time, similar to what has been reported with ATF4.

The unfolded protein response has recently become an area of therapeutic interest in the treatment of neurodegenerative diseases and is of particular interest in HAND given our previous observations of elevated UPR markers in the CNS of HAND patients and ART drug-mediated BACE1 upregulation via phospho-eIF2α-dependent translational control in neurons (61, 62). Here, we used a pharmacological approach to assess the contribution of two eIF2α kinases, PERK and PKR, to ART-mediated BACE1 upregulation. In agreement with our previous work, we found that inhibition of PERK blocked ART-mediated phosphorylation of eIF2α; however, BACE1 was not significantly reduced compared to ART-only controls, emphasizing the redundant nature of the four eIF2α kinases. Furthermore, use of the GSK PERK inhibitor did not alleviate ritonavir-mediated loses in MAP2, indicating that this inhibitor, although protective in an AD mouse model, may be neurotoxic in vitro. Alternatively, PERK may be critical for neuronal health and survival during normal and/or acute stress conditions, limiting its therapeutic potential. Indeed, disruption of PERK signaling in CNS neurons has been shown to induce cognitive and behavioral deficits in mice (63). Unexpectedly, we found that PKR played a major role in maintaining basal levels of neuronal phospho-eIF2α and PKR inhibition completely abrogated ritonavir and saquinavir-mediated phosphorylation of eIF2α, but only partially reduced BACE1 levels. Future studies with 7DG will be utilized to determine if PKR inhibition is protective against ART-mediated neurotoxicity in vitro and in vivo.
In the present study, we did not test the effect of GCN2 and HRI inhibitors, however, work by Vassar and colleagues suggests that GCN2 does not contribute significantly to ER-stress mediated upregulation of BACE1 in neurons (7). Nonetheless, GCN2 remains an intriguing therapeutic target for HAND, as it has been shown to have inhibitory effects on HIV replication and is directly cleaved by the HIV protease to overcome this antiviral effect (64). Ultimately, successful adjunctive therapies for HAND will likely have to include pharmacologics that alleviate ER and oxidative stress, thereby slowing or preventing the toxic accumulation of neurodegenerative proteins, such as Aβ, that have become increasingly prevalent in the pathology associated with HIV infection in the CNS.
Figure 1. BACE1 inhibition mitigates MAP2 and synapsin loss in primary neurons treated with hydrogen peroxide. Rat cortical neuroglial cultures grown on coverslips for 10 DIV were treated with DMSO vehicle or pretreated with 1 µM cell-permeable beta secretase inhibitor (BSI) for 30 minutes then treated with 10 or 20 µM hydrogen peroxide (H2O2) for 24 hours. Cultures were stained with propidium iodide (red) and immunostained for MAP2 (blue) and synapsin (green). Neurons pre-treated with 1 µM BSI displayed decreased propidium iodide staining, increased expression of synapsin and increased MAP2+ cells compared to untreated cultures in the presence of 10 and 20 µM H2O2. Images were captured using epifluorescent microscopy at 40x magnification. Representative images shown.
Figure 2. BACE1 inhibition prevents MAP2 loss in ritonavir-treated neurons. A) Rat cortical cultures were grown on coverslips for 10 DIV and treated with DMSO vehicle or 1 µM cell-permeable beta-secretase inhibitor (BSI) for 30 minutes followed by 48 hour treatment with 10 µM ritonavir. To assess cellular damage and death, cultures were stained with propidium iodide (red) and immunostained for MAP2 (blue) and synapsin (green). B-D) In the presence of ritonavir, neurons pre-treated with 1 µM BSI showed a significant increase in the number of MAP2+ cells and a small decrease in propidium iodide staining. No changes in synapsin were observed between treatment groups (n=4, *p<0.05, **p<0.01, ns: not significant, one-way ANOVA plus
Newman-Keuls post hoc testing). Images were captured at 40x magnification using epifluorescent microscopy. Representative images shown.
Figure 3. Small molecule inhibitors of PKR and PERK block HIV protease inhibitor-mediated phosphorylation of eIF2α in neurons. Primary rat neuroglial cultures aged 21 DIV were pretreated with DMSO vehicle or an inhibitor of PKR (7DG) or PERK (GSK2606414) for 1 hour followed by treatment with 10 µM ritonavir or 1 µM saquinavir for 16 hours. Neuronal lysates were immunoblotted for phospho- and total eIF2α and BACE1. Actin was used a loading control for BACE1 while phospho-eIF2α was normalized to total eIF2α. A-C) PKR inhibition with 10 µM 7DG in neurons significantly reduced basal levels of phospho-eIF2α and prevented ritonavir and
saquinavir-mediated phosphorylation of eIF2α. 7DG also reduced neuronal BACE1 expression in ART-treated neurons, however the change was not significant (n=3 **p<0.01, ***p<0.001, one-way ANOVA plus Newman-Keuls post hoc testing). D-I) Inhibition of PERK with 0.5 µM GSK2606414 did not affect basal levels of phospho-eIF2α, but resulted in a significant decrease in phospho-eIF2α expression in ritonavir and saquinavir-treated neurons compared to vehicle pre-treated controls (n=3, *p<0.05, one-way ANOVA plus Newman-Keuls post hoc testing). BACE1 levels were not significantly altered in neurons treated with GSK2606414, although we observed a trend towards decreased BACE1 expression in PERK-inhibited, ART-treated neurons.
**Figure 4.** Pharmacological inhibition of PERK does not alleviate ritonavir-mediated neurotoxicity in primary neuroglial cultures. Rat cortical neuroglial cultures were grown on coverslips for 10 DIV and pre-treated with DMSO vehicle or 0.5 µM PERK inhibitor, GSK2606414 (GSK) for 30 minutes followed by treatment with 10 µM ritonavir for 48 hours. Cells were stained with propidium iodide (red) and immunostained for MAP2 (blue) and synapsin (green) to assess neuronal damage and death. Neuronal cultures pre-treated with GSK did not appear significantly different compared to controls in the presence of ritonavir. MAP2 and synapsin levels appeared similar while propidium iodide puncta were increased in GSK pre-treated neurons.
4.6: REFERENCES


157


5.1: OVERVIEW

The advent of combination antiretroviral therapy in 1996 represented a revolutionary breakthrough in the treatment of HIV and validation of rational drug design via structural biology as an essential methodology in the treatment of human disease. When on ART, patients experience suppressed viral replication, reconstitution of the immune system, and improved long-term survival. However, with aging of the HIV-infected population, the prevalence of HAND has continued to escalate. Furthermore, the life expectancy of ART-mediated, HIV-positive patients remains 10-30 years less than that of uninfected individuals, highlighting the need for advancements in the understanding of HIV comorbidities including HAND neuropathogenesis (1).

Since the implementation of ART, HAND has evolved from a rapidly progressing, subcortical encephalitic condition to a prolonged, cortical, neurodegenerative disease. While contributions of HIV replication, immune activation, and drugs of abuse are known to drive neuronal damage, it is less clear what effect antiretroviral drugs have in the onset and progression of HAND, however, a plethora of data suggests that ART toxicity is likely to play a key role in CNS toxicity, as it does in the peripheral nervous system. The aim of our work has been to determine the contributions of ART, on a cellular and molecular level, to the evolution and persistence of HAND pathology with the long-term goal of uncovering novel therapeutic strategies to combat cognitive deficits in aging, HIV-infected populations. To that end, we employed in vitro models of ART exposure to show that HIV protease inhibitors, and NRTIs to a lesser extent, induce UPR activation in primary rat and human fetal neurons (Chapter 3). Consistent with previous studies, activation of the UPR resulted in translational de-repression of BACE1 and enhanced production of Aβ peptides. These effects were mitigated by knockdown of neuronal PERK or overexpression of a kinase-dead PERK mutant. Importantly, we found evidence of UPR activation and upregulation of
BACE1 protein levels in ART-treated humans and macaques infected with HIV and SIV, respectively. In Chapter 4, we sought to elucidate the role of BACE1 in neuronal survival and stress response by challenging neurons with oxidative and ER stressors in the presence of a pharmacological inhibitor of BACE1. We found that BACE1 inhibition partially protected cells against hydrogen peroxide and ART-mediated neuronal damage and death as indicated by decreased uptake of propidium iodide and rescue of MAP2 and synapsin protein expression. Additionally, we observed that inhibition of PKR, one of the four eIF2α kinases, blocked ART-mediated phosphorylation of eIF2α and upregulation of BACE1. Ultimately, a detailed understanding of the molecular mechanisms by which antiretrovirals induce toxicity in neurons is vital to the development of safer drug regimens and the discovery of novel therapeutics sorely needed in HAND.

5.2: ANTIRETROVIRALS AND THE UNFOLDED PROTEIN RESPONSE

In Chapter 3, we built upon previous observations of ART toxicity in neurons to determine which specific molecular pathways were affected in cells upon exposure to commonly prescribed antiretroviral drugs. Consistent with findings in hepatocytes, adipocytes, and macrophages, PI and NRTI-class ART drugs were found to activate ER stress in rat and human fetal neurons in a dose-dependent manner, as indicated by elevated expression of BiP, p-eIF2α, and splicing of XBP1 mRNA by IRE1α. We did not test the ability of other ART drug classes to induce ER stress in neurons; however, we are currently conducting an extensive analysis on a panel of NRTIs, nNRTIs, and PIs to determine if our findings with ritonavir, saquinavir, and AZT are representative of their drug class. Current efforts are also underway to determine the precise mechanism by which PIs and NRTIs induce ER stress, although the answer is likely to be multifactorial (Figure 1). A 2005 study by Parker et. al. suggested that PIs activate ER stress in adipocytes via inhibition of 26S proteasome activity and blockade of glucose transporter 4 (GLUT4) (2). Intriguingly, Vassar and colleagues found that inhibition of glucose utilization resulted in PERK-dependent phosphorylation of eIF2α in vivo and in vitro (3). Furthermore, mutation of the Ser51
eIF2α phosphorylation site in mice results in fatal hypoglycemia and defective gluconeogenesis, indicating that the primary physiological function of p-eIF2α in mammals is nutrient-sensing and regulation of glucose homeostasis (4). Given that PIs induce insulin resistance in patients, it stands to reason that inhibition of glucose transport and utilization may be a key mechanism of cellular stress in the periphery as well as in the CNS, where GLUT4 is known to be expressed in the hippocampus. We will first assess glucose uptake in insulin-stimulated hippocampal neurons treated with increasing doses of ritonavir using a [3H]2-Deoxyglucose uptake assay. Based on previous work with PIs in other cell types, we predict that ritonavir will dose-dependently block glucose uptake in neurons. Using immunofluorescence analysis, we will then assess whether any of the major glucose transporters in neurons, including GLUT1, GLUT3, or GLUT4 fail to translocate to the plasma membrane after insulin-stimulation in the presence of ritonavir. A previous study in 3T3-L1 adipocytes demonstrated that GLUT3 and GLUT1 properly translocate to the plasma membrane in the presence of indinavir, but that indinavir inhibits the intrinsic transport activity of GLUT4 after its translocation (5). As glucose uptake by GLUT4 in muscle cells is dependent upon PI3-kinase activation (6), we will determine if ritonavir inhibits PI3-kinase in neurons as a mechanism of GLUT4-specific glucose transport inhibition. Ultimately, a comprehensive analysis of the effects of PIs on neuronal insulin sensitivity and signaling will be critical for advancing our understanding of the potential metabolic side-effects of ART in the CNS of drug-treated, HIV-positive individuals.

Inhibition of 26S proteasome activity by PIs has been confirmed in various cell types and cell-free systems and is likely to be important as an ER-activating mechanism in neurons as indicated by a recent report showing that pharmacological inhibition of proteasome activity with MG115 or MG132 directly activates PERK in neural cells (7). Future studies are necessary to determine if PIs can inhibit proteasome function in primary neuroglial cultures prior to phosphorylation of eIF2α. This can be assessed with the use of a fluorometric assay wherein a peptide substrate of the proteasome (LLVY) is linked to a fluorophore (7-amino-4-methylcoumarin) which fluoresces after cleavage of the LLVY-AMC bond. In this manner it will be
possible to determine if proteasome inhibition is an early event in PI-mediated cell toxicity and if rescue of proteasome activity abrogates PI-mediated activation of ER stress.

We have previously shown that antiretrovirals induce production of reactive oxygen species (ROS) in primary neurons as early as 6 hours after treatment, hypothesized to be the result of disrupted mitochondrial DNA synthesis and depletion of enzymes necessary for the electron transport chain (8-11). ROS, in turn, can activate ER stress via disruption of cellular calcium homeostasis or widespread oxidation of proteins leading to impaired protein degradation (12). To assess whether ART-induced oxidative stress is sufficient and/or necessary for downstream activation of the UPR, we would inhibit ROS production with the use of a fumaric acid ester, monomethyl fumarate (MMF). MMF, the hydrolyzed, active metabolite of dimethyl fumarate (DMF), is an anti-inflammatory that activates the Nrf2-dependent antioxidant response pathway leading to upregulation of detoxification enzymes, such as NQO-1 and heme oxygenase 1 (HO-1), and has been shown to protect neurons from ART-induced damage and death (8, 13, 14). DMF is currently used for the treatment of psoriasis in Europe and ongoing clinical trials indicate DMF shows promise as a disease-modifying agent in multiple sclerosis, highlighting its favorable in vivo efficacy and safety profile (13, 15-18). Thus, DMF represents an intriguing candidate for adjunctive therapy in the treatment of HAND, as reviewed by Gill and Kolson (19).

5.3: MECHANISMS OF BACE1 REGULATION IN NEURONS

Our work also confirms a 2008 report by O’Connor et. al. that UPR activation in neurons results in translational upregulation of BACE1. Importantly, we used a 5’UTR deletion construct to confirm that translational control of BACE1 during ER stress is dependent upon the presence of the 5’UTR. Whereas 16 hour ritonavir treatment in neurons overexpressing wild-type BACE1 resulted in increased BACE1 expression compared to vehicle controls, neurons overexpressing the -5’UTR construct did not show any changes in BACE1 protein when treated with ritonavir. Importantly, we observed a much larger increase in BACE1 protein due to deletion of the 5’ UTR as compared to induction of ER stress with ritonavir. These results suggest that ritonavir partially
de-represses BACE1, likely through the use of p-elf2α-mediated scan through of uORFs after re-initiation. However, secondary structures in the 5’UTR likely remain intact and therefore could contribute to partial repression of translation. Elevated BACE1 in neurons containing the 5’UTR deletion-construct thus reflects a coincident loss of all uORFs and secondary structures leading to translational initiation of all associated ribosomes at the true start codon, compared to neurons transfected with the BACE1 cDNA containing the full length 5’ UTR where initiation at the bonafide start codon remains sub-maximal due to secondary structures or incomplete scan-through of all three uORFs. Based on previous studies of BACE1 translational control, we predict that mutation or deletion of uAUG #2 and/or #4 would further enhance BACE1 expression during ER stress by limiting translation re-initiation at these start codons. A thorough analysis of BACE1 5’UTR uAUG mutants will be necessary to confirm if these uAUGs are critical to BACE1 translational control during ER stress or if secondary structures play a more prominent role in repression.

Although not supported by our data, there is also evidence that BACE1 transcript and protein stability can be influenced by microRNAs and non-coding antisense mRNAs (20). In particular, four microRNAs (miR-107, miR-9, miR-29a, miR-29b) are known to bind BACE1 mRNA and regulate translational efficiency while increasing the stability of the transcript (21, 22). Interestingly, several of these microRNAs were shown to be downregulated in AD brains and were inversely correlated with BACE1 mRNA levels in the CNS (21). Alternatively, BACE1 stability can be affected by proteasome activity, as shown in a 2004 report where inhibition of proteasome activity with lactacystin, but not other protease inhibitors, led to accumulation of ubiquitinated BACE1 and increased amyloidogenic processing of APP (23). We found that ritonavir had no effect on BACE1 protein stability in vitro, however, a parallel post-mortem analysis of BACE1 protein and mRNA levels in the CNS of HAND patients will be necessary to determine if BACE1 is increased only at the translational level as in AD.
In addition to increasing the expression of BACE1, we also observed that ritonavir and combinations of antiretrovirals increased production of Aβ peptides by two-fold in CHO cells stably expressing human APP and in primary rat neuroglial cultures. Unexpectedly, we found that CHO cells treated with ritonavir showed an increase in Aβ42 secretion but not Aβ40, conflicting with a report by Vassar et. al. which showed that overexpression of BACE1 in cells stably transfected with wild-type human APP increased production of Aβ40 and Aβ42 peptides equally (24). Our results in CHO cells appear to be a cell-specific artifact, as we observed comparable increases in Aβ40 and Aβ42 in primary rat neuronal cultures treated with combinations of antiretrovirals. Alternatively, our observations in rat neurons may be a result of combination ART treatment compared to the ritonavir-only treatments in CHO cells. Rigorous in vivo validation of ART effects on Aβ production are needed to determine the long-term effects of these drugs on amyloidogenesis in the brain. Although several groups have reported elevated Aβ accumulation and deposition in ART-treated, HIV-positive individuals compared to ART-naïve controls, there are several technical concerns with these studies regarding the antibody (4G8) used to detect amyloid. Specifically, 4G8 can detect full-length APP in addition to sAPPβ, and Aβ peptides, therefore, it is unclear which Aβ domain-containing species these groups were observing in HAND patients. Given the limitations associated with post-mortem human analyses, the use of transgenic mouse models of AD will be critical to ascertain a clear understanding of the interplay between antiretrovirals and amyloid pathology in the CNS. Previously, Ikezu and colleagues found that 30-day oral administration of nelfinavir (PI) or lopinavir/ritonavir had no effect on Aβ accumulation in two-month-old APP SCID mice (generated by crossing CRND8 transgenic mice with BALB/cBy-Prkdcscid mice) despite achieving CSF/IC₅₀ ratios of 4.3 and 3.3, respectively (25). However, previous studies using CRND8 transgenics indicated that Thioflavin-S-positive amyloid deposits were not prevalent until 3 months of age and dense-core neuritic plaques were not observed until 5 months of age (26). Importantly, none of the animals included in the Ikezu study displayed evidence of amyloid pathology; therefore, inclusion of age-matched, CRND8 transgenic controls will be necessary to ensure pathology in the APP SCID mouse.
develops in a similar, age-dependent manner. We propose using the APP/PS1 double knock-in mouse model of AD, in which FAD-causing mutations were targeted into their endogenous genes, resulting in age and region-dependent amyloid deposition beginning at 6 months of age (27). Treatment of these animals with antiretrovirals at 6 and 12 months of age will provide critical insights into the effects of ART on the initiation of amyloidogenesis, while avoiding the caveats of a transgenic AD model that utilizes exogenous overexpression of APP.

5.4: eIF2α KINASES AS THERAPEUTIC TARGETS IN HAND

PERK is the primary kinase responsible for eIF2α phosphorylation during conditions of oxidative and ER stress. Our results suggest that PERK is also involved in maintenance of basal p-eIF2α in neurons and ART-mediated increases in p-eIF2α and BACE1 (Chapters 3 and 4). Notably, while overexpression of kinase-dead PERK resulted in a significant decrease in p-eIF2α and BACE1 expression in neurons, overexpression of wild-type PERK increased p-eIF2α but did not change BACE1 levels, suggesting that in the absence of an oxidative or ER stressor, phosphorylation of eIF2α does not necessarily lead to translational upregulation of BACE1. To confirm this hypothesis, future experiments will be conducted in which wild-type and kinase-dead PERK constructs are overexpressed in neurons in the presence and absence of ritonavir. While overexpression of wild-type PERK is predicted to augment the effects of ritonavir through enhanced and/or sustained phosphorylation of eIF2α, overexpression of kinase-dead PERK should partially block ritonavir-mediated upregulation of BACE1, as observed in our PERK conditional knockout cells (Chapter 3) and PERK inhibitor studies with the novel compound GSK2606414 (Chapter 4).

Accumulating evidence suggests that aberrant UPR signaling through PERK is a key mechanism underlying amyloidogenesis and cognitive decline in neurodegenerative diseases, such as AD (3, 28-32). We and others have shown that ART contributes to enhanced amyloidogenic processing of APP in primary neurons via ER stress activation (33-35). Therefore, to determine if PERK is directly involved in antiretroviral-mediated Aβ production in vivo, we will
cross APP/PS1 double knock-in mice (described previously) with conditional PERK knockouts and treat the resulting APP PERK-/- mice with ART for up to 30 days and assess changes in Aβ production and amyloid pathology beginning at 6 months of age. As PERK haploinsufficiency is sufficient to prevent BACE1 elevation, reduce Aβ and plaque burden, and rescue memory deficits in a 5XFAD mouse model, we predict that conditional knockout of PERK in adult mice will abrogate ART-mediated phosphorylation of eIF2α and upregulation of BACE1, while preventing or slowing any accumulation of intraneuronal or extracellular Aβ that might occur. These experiments will also reveal whether PERK inhibition represents a promising therapeutic avenue in the treatment of HAND.

Phosphorylation of eIF2α is mediated by three kinases in addition to PERK, including HRI, GCN2, and PKR. While HRI is not expressed in neurons, GCN2 is expressed at high levels in the brain compared to other organs and responds to a variety of stress conditions, similar to PERK. Additionally GCN2 has been shown to play a role in learning and memory via regulation of early and late-phase long-term potentiation (LTP) (36). Paradoxically, genetic deletion of GCN2 in APP/PS1 mice was shown to rescue impairments in synaptic plasticity and spatial memory, suggesting this kinase becomes dysregulated or maladaptive in neurodegenerative disease (31). Additionally, GCN2 has been shown to inhibit HIV replication and is directly cleaved by the HIV-1 protease (37). Therefore, future experiments involving in vivo and in vitro knockdown of GCN2 will be informative in understanding the contribution of this kinase to CNS health in the presence of HIV and/or antiretroviral drugs.

PKR also appears to have a role in AD pathogenesis via stress-mediated upregulation of BACE1 (38-40). Our results in Chapter 4 indicate that PKR is partially responsible for ART-mediated increases in p-eIF2α and BACE1 and may play a role in maintaining basal levels of p-eIF2α in neurons. Although PKR knockout in APP/PS1 mice did not prevent amyloid pathology or rescue cognitive deficits (31), it is likely that PKR, GCN2, and PERK all contribute to eIF2α phosphorylation in chronic, neurodegenerative diseases given the widespread inflammation, energy deprivation, and accumulation of misfolded proteins that routinely accompany these
diseases. Additionally, inhibition of any one of the eIF2α kinases via targeted therapeutics in vivo is likely to be compensated by the overlapping functions of the other kinases, suggesting that direct targeting of eIF2α itself may be more efficient at ameliorating aberrant UPR signaling in chronic CNS disease. However, it remains unclear whether sustained eIF2α phosphorylation is an early, driving force behind the neurodegenerative process or a downstream cellular adaptation to the accumulation of misfolded proteins and gradual deterioration of protein folding and clearing mechanisms associated with normal aging. Whether stress-induced eIF2α phosphorylation is ultimately protective or deleterious to neuronal survival in the brain is incompletely understood and likely depends on the chronicity and severity of the stressor. Canonical activation and regulation of the UPR in general, and the PERK pathway in particular, is designed to be rapid and transient, exemplified by the fact that many of the translational targets of p-eIF2α are themselves transcription factors. A 2007 report by DeGracia and Hu concluded that neuronal survival during transient cerebral ischemia was dependent upon recovery from translational inhibition by p-eIF2α (41). However, long-term translational arrest via chronic UPR activation in neurons is associated with enhanced production of neurodegenerative proteins, impaired LTP, and in the case of ischemia/reperfusion, cell death. These conflicting roles for the UPR suggest that its activation is biphasic: adaptive and protective during transient stress but apoptotic during chronic/severe stress (42). Extensive animal studies with modulators of eIF2α, such as growth arrest and DNA damage inducible protein 34 (GADD34), a specific eIF2α phosphatase, will be critical to determine the safety of this therapeutic approach in the context of HIV infection and long-term exposure to antiretroviral drugs.

5.5: CONSIDERATIONS FOR IN VIVO ANALYSIS OF ART-ASSOCIATED CNS DAMAGE

We confirmed the biological significance of our in vitro findings with a well-characterized, SIV/pigtail macaque model of HIV CNS disease in which 90% of infected animals treated with placebo develop neurologic disease within 12 weeks post-inoculation, while animals treated with ART do not develop SIV encephalitis nor do they develop neurological dysfunction (43).
Strikingly, SIV-infected macaques treated with combination ART showed significantly elevated expression of BACE1 and APP, a sensitive marker of neuronal damage, in the hippocampus compared to SIV-infected, placebo-treated controls (Chapter 3). Together with our previous reports of ART-associated synaptic injury in this same macaque model (8), these results demonstrate that oral administration of successful ART to primates negatively impacts neuronal and synaptic viability in the CNS either through direct or peripheral toxicities. Furthermore, these data strongly suggest that antiretroviral agents themselves, not viral infection, are responsible for increased CNS expression of BACE1. Treatment of uninfected pigtailed macaques with ART is currently ongoing and will be necessary to confirm this hypothesis. Notably, we did not observe statistically significant changes in BiP or p-eIF2α in ART-treated macaques compared to uninfected or placebo-treated controls, however, changes in ER stress markers are likely to be region- and cell-specific, thus, immunofluorescent analysis will be necessary to properly assess the level of UPR activation in the macaque brain. Furthermore, successful detection of phosphoproteins in post-mortem tissue is highly dependent upon post-mortem interval (44), therefore, we will measure changes in alternative UPR markers, such as sXBP1, ATF6α/β, ATF4, and CCAAT-enhancer-binding protein homologous protein (CHOP) via immunofluorescent and western blotting analysis.

Our initial post-mortem analysis of brain tissue from a small cohort of 13 patient samples from the NNTC revealed two important findings: BACE1 levels are significantly upregulated in the hippocampus of HAND patients compared to HIV-negative controls and intraneuronal Aβ oligomers are elevated in the hippocampus of HAND patients despite the absence of overt plaque pathology or changes in total APP (Chapter 3). Interestingly, we did not find an association between the severity of HAND and Aβ oligomer burden in the hippocampus, indicating dysregulated APP processing may be an early event in pathogenesis. Notably, the patients tested in this cohort ranged from 31 to 53 years of age, therefore, advanced amyloid pathology would not be expected even in the presence of FAD-causing mutations. Similar to our findings in macaques, HAND patients did not display statistically significant changes in BiP or p-eIF2α
compared to uninfected or HIV-positive, neurocognitively normal controls by western blotting analysis. These results conflict with our previous reports in which immunofluorescent analysis revealed elevated markers of UPR activation, including BiP and p-eIF2α, in HAND patients (45, 46), emphasizing the importance of this approach to examine changes in protein levels that can vary across subcellular compartments, cell types, and brain regions. Our analysis of HAND patients is also limited by low statistical power owing to small sample size and lack of data on antiretroviral regimens. To address these concerns, we have begun extended analysis of a large cohort (n=60) of patients from the NNCTC grouped into three categories: 20 HIV(-), 20 HIV(+)/ART-naïve, 20 HIV(+)/ART-treated for at least 12 months (Table 5.1). Importantly, the ART regimens of our drug-treated patient samples were carefully documented which will allow us determine for the first time if long-term use of specific ART regimens/antiretroviral drug classes correlate with changes in UPR activation, BACE1 expression, and aberrant APP processing in the CNS. The importance of these studies are highlighted by the conflicting neuropsychological data obtained in cross-sectional and longitudinal clinical studies of HIV-positive patients beginning ART or switching to ART regimens with higher predicted CNS penetrance. While these studies have largely assessed the initial effects of ART on neurocognitive function in drug-naïve patients, our studies will provide critical information on the cumulative, long-term effects of ART on neuropathology in HIV-positive individuals.

5.6: BACE1 FUNCTIONS IN NEURONS: IMPLICATIONS FOR A NOVEL THERAPEUTIC TARGET IN HAND

A growing body of literature suggests that BACE1 is induced by a variety of stress conditions including oxidative stress, cerebral ischemia, traumatic brain injury, and impaired energy metabolism associated with neurodegeneration (3, 47-54). In Chapter 4, we investigated the role of BACE1 in cell survival and adaptation to oxidative and ER stress. Although the regulation and timing of BACE1 expression in neurons suggest it may be involved in an acute neuronal stress response, we found that inhibition of BACE1 activity protected cells from
hydrogen peroxide-induced damage and death. At acute doses of hydrogen peroxide, BACE1 inhibition mitigated loss of MAP2 and synapsin, but did not change the number of propidium iodide puncta, suggesting that loss of BACE1 activity either slowed the death response in neurons or restricted cell loss to glia. Recent preliminary work in our lab has demonstrated that toxic doses of hydrogen peroxide induce acute loss of astrocytes prior to the loss of neurons, suggesting that astrocytes buffer and protect neurons from changes in redox potential and that BACE1 may be involved in a delayed death response in neurons. In support of this conclusion, previous reports have shown that during apoptosis BACE1 protein degradation in lysosomes is inhibited by caspase-3-mediated cleavage of Golgi-localized γ-ear-containing ARF-binding protein 3 (GGA3), an adaptor protein required for BACE1 lysosomal trafficking and degradation, leading to enhanced Aβ production and cell death (55). Thus, BACE1 may be involved in programmed cell death as a means to prevent spread of cytotoxic ROS in the brain. However, what role BACE1 might play in sub-toxic oxidative stress conditions remains unclear. An additional consideration is the possibility that the beta-secretase inhibitor utilized in our study is protecting neurons via off-target effects on other proteases, such as caspases, however, this is unlikely given the high-specificity of the compound, which has an IC₅₀ of 15nM for BACE1, 230nM for BACE2, 7.6uM for cathepsin D, and greater than 50uM for the related aspartic protease renin. However, off-target effects of the BACE1 inhibitor on initiator and effector caspases will be verified by western blotting for active caspase-3 and caspase-9 in neurons following treatment with hydrogen peroxide, an important next step for our studies.

Alternatively, BACE1 might exert a specific neuroprotective role against excitotoxic insults based on its ability to cleave voltage-gated sodium channels (VGSCs). Excitotoxins, such as N-methyl-D-aspartate (NMDA), which bind to glutamate receptors, induce acute excitotoxicity in neurons via influx of calcium and sodium (56). We propose to examine the role of BACE1 during excitotoxicity by treating primary neurons with NMDA in the presence or absence of a BACE1 inhibitor. Neuronal viability will be assessed by changes in MAP2, synapsin, and propidium iodide inclusion. Additionally, we will correlate cell damage and death with levels of
calcium and sodium influx after NMDA treatment in BACE1 inhibited neurons with the use of fluorescent dye assays. Although BACE1 inhibition is not predicted to mitigate NMDA-mediated influx of calcium, this assay will allow us to determine if BACE1 mediates neuroprotective effects by regulating sodium influx via VGSC activity and function, which would have larger implications in diseases such as epilepsy where neuronal hyperactivity is prevalent. Hypothetically, BACE1 cleavage of VGSCs could also counteract the enhanced, region-specific expression of cortical VGSCs found in a rodent model of absence epilepsy, which was thought to correlate with the determined regions of seizure onset (57).

Finally, we found that BACE1 inhibition also partially protected neurons from ritonavir-mediated toxicity over 48 hours. Although we have data to suggest that ritonavir and other PI-class drugs exert their neurotoxic effects through production of ROS, there is an abundance of data indicating ART drugs have a plethora of other toxic effects mediated through calcium dysregulation, inhibition of proteasome function, and mitochondrial toxicity, suggesting BACE1 might play a role in balancing cell survival and death in response to a wide variety of insults. As such, BACE1 is an appealing target for therapeutic intervention in HAND, wherein patients are chronically exposed to toxic effects of antiretroviral drugs, low levels of insidious HIV replication, neuroinflammation, and accelerated aging. One major advantage of targeting BACE1 in HAND versus sporadic neurodegenerative diseases, such as AD, is the ability to identify HIV as a causative agent, and not merely a risk factor, for HAND, lending certainty to physicians for recognizing the at-risk patient population. Presumably, BACE1 inhibition in HAND would exert neuroprotective effects primarily through inhibition of Aβ production, however, the failure of previous amyloid-directed therapeutic agents in AD, where amyloid pathology is abundant, warrants significant skepticism with this approach in HAND. In particular, plaque disruptors, anti-Aβ immunotherapy, and γ-secretase inhibitors and modulators all failed to demonstrate substantial reductions in CSF Aβ in clinical trials and many of these agents displayed significant, dose-limiting side-effects, which in turn restricted their Aβ-lowering capabilities (58).
Early BACE1 inhibitors also failed to exhibit sufficient in vivo potency due to their poor pharmacokinetic profiles. Notably, first generation BACE1 inhibitors were structurally similar to the peptidomimetic HIV protease inhibitors, highlighting the structural homology between BACE1 and the HIV protease. Yet, despite their similarities to BACE1 inhibitors, HIV protease inhibitors have not displayed any apparent anti-BACE1 activity in vitro or in vivo. Current efforts in BACE1 inhibitor design have shifted towards the use of small molecule inhibitors, several of which have progressed into phase 3 clinical trials. The new generation inhibitors, including MK-8931 (Merck and Co.) and LY-2886721 display high affinity for BACE1, potently reduce CNS levels of Aβ in non-human primates and avoid the off-target effects on cathepsin D and renin that plagued earlier compounds. Additionally, these compounds avoid the insurmountable mechanism-based side effects that accounted for the failure of γ-secretase modulators and inhibitors. Although complete loss of BACE1 activity is known to cause a multitude of phenotypes, including peripheral hypomyelination, the use of potent pharmacological agents against BACE1 avoids these effects by only partially inhibiting BACE1 activity, which appears to be sufficient to achieve a desirable neuroprotective outcome. Genetic studies in AD mouse models showed that 50% knockdown of CNS BACE1 in PDAPP/BACE1⁺⁻: transgenic mice resulted in only a 12-20% reduction in Aβ levels, but which translated to 75% less amyloid plaque burden compared to PDAPP/BACE1⁺⁺ mice (58, 59). Importantly, BACE1 heterozygous knockout mice display none of the phenotypes of homozygous BACE1 knockouts, suggesting that complete loss of BACE1 function is necessary for phenotypic abnormalities (58). In patients with HAND, amyloid plaque burden has not been definitively established as a common pathological feature, however aberrant intraneuronal Aβ accumulation has been observed in addition to reduced CSF Aβ42 levels, which is a biomarker of AD and is thought to be the result of accumulation of insoluble Aβ within the brain parenchyma (60). Taken together with our studies, these observations suggest that patients suffering from HAND would stand to benefit from BACE1-directed therapeutics as a means to normalize Aβ levels within the CNS and improve cognitive functioning.
In summary, the body of work presented in this thesis advances our understanding of the cellular and molecular effects of antiretroviral drugs on CNS neurons in vitro and in vivo while further delineating the basic biological functions of BACE1 as a stress response protein. Additionally, we present novel features of HAND pathogenesis in the post-ART era, including intraneuronal accumulation of oligomeric Aβ, that have not previously been described pre-ART. Importantly, our work provides the first mechanistic link between the persistence and evolution of HAND as a chronic, neurodegenerative condition with the widespread use of antiretroviral drugs, taking into account the known toxicities of ART in the peripheral nervous system. Our data demonstrate that PI-class antiretroviral drugs potently activate the UPR in neurons leading to p-eIF2α-mediated translational upregulation of BACE1 and enhanced amyloidogenic processing of APP. Furthermore, toxic effects of chronic ART treatment in neurons were reversible with the use of a cell-permeable beta-secretase inhibitor, thus providing preliminary evidence for BACE1 inhibition as an adjunctive neuroprotective therapy in the treatment of HAND. These results highlight the role of antiretroviral drugs as mediators of neuronal damage and death in HAND and underscore the importance of novel therapeutic strategies in the treatment of HIV CNS disease.
Table 1

Summary of human samples from NNTC for post-mortem analysis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV -</th>
<th>HIV + No ART</th>
<th>HIV + w/ ART</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Subjects</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Age at death, mean ± SD</td>
<td>42.8 ± 5.6</td>
<td>39.7 ± 7.3</td>
<td>45.3 ± 6.7</td>
</tr>
<tr>
<td>Hours postmortem, mean ± SD</td>
<td>9.9 ± 5.7</td>
<td>10.0 ± 6.4</td>
<td>12.4 ± 7.9</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>80%</td>
<td>90%</td>
<td>80%</td>
</tr>
<tr>
<td>Female (%)</td>
<td>20%</td>
<td>10%</td>
<td>20%</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>11 (55%)</td>
<td>12 (60%)</td>
<td>15 (75%)</td>
</tr>
<tr>
<td>Black (%)</td>
<td>7 (35%)</td>
<td>8 (40%)</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>Other/Unknown (%)</td>
<td>2 (10%)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic (%)</td>
<td>5 (25%)</td>
<td>3 (15%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Not-Hispanic (%)</td>
<td>15 (75%)</td>
<td>17 (85%)</td>
<td>17 (85%)</td>
</tr>
<tr>
<td>Disease Parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV+ (%)</td>
<td></td>
<td>6 (30%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>Log plasma HIV c/mL, mean ± SD</td>
<td></td>
<td>4.1 ± 1.4</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>Log Brain HIV c/g, mean ± SD</td>
<td></td>
<td>3.4 ± 1.9</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Neurocognitive Impairment Status</td>
<td>HAND (%)</td>
<td>Subsyndromic (%)</td>
<td>MCMD (%)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------</td>
<td>------------------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6 (30%)</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td></td>
<td>14 (70%)</td>
<td>1 (5%)</td>
<td>9 (45%)</td>
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<table>
<thead>
<tr>
<th>ART Treatment Status</th>
<th>PI-experienced (%)</th>
<th>PI-naïve (%)</th>
<th>NRTI-experienced (%)</th>
<th>NRTI-naïve (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>17 (85%)</td>
<td>3 (15%)</td>
<td>20 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Log CSF HIV c/mL, mean ± SD</th>
<th>-</th>
<th>3.4 ± 1.8</th>
<th>2.8 ± 1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ lymphocytes/mm³, mean ± SD</td>
<td>-</td>
<td>108 ± 118</td>
<td>85 ± 118</td>
</tr>
</tbody>
</table>
Figure 1. Proposed mechanisms of antiretroviral drug-induced ER stress in neurons. ART drugs induce ER stress in a wide variety of cell types via multiple, distinct mechanisms including inhibition of GLUT4 transport activity, generation of reactive oxygen species, inhibition of 26S proteasome activity, and dysregulation of intracellular calcium stores. These insults can induce chronic activation of ER stress in neurons leading to suppressed global protein synthesis and impaired LTP, accompanied by translational de-repression of BACE1 and ATF4, both of which have been implicated in apoptosis and neurodegeneration.
5.7: REFERENCES


