Shruthi Nooka, Uncovering the Multifaceted HAND of Reactive astrocytes in **H**IV-**A**ssociated **N**eurocognitive **D**isorders Doctor of Philosophy (Biomedical Sciences), July 2017, 149 pp., 304 bibiliographies

Despite the advent of antiretroviral therapy (ART), central nervous system (CNS) complications associated with HIV-1 infection, collectively referred to as HIV-associated neurocognitive disorders (HAND), continue to increase. HIV infection promotes cognitive dysfunction and neurodegeneration through persistent inflammation, oxidative stress from infected and/or activated macrophages, astrocytes and neurons. Additionally, recent studies demonstrated that neurotoxic side effects of antiretroviral (ARV) drugs are among several contributing factors to this continued prevalence of HAND. In recent years, a new appreciation of the role of astrocytes in regulating HIV-1 CNS infection has emerged. Thus, investigating the elusive cellular and molecular mechanisms regulated by astrocytes during HIV-1/ART-induced neurotoxicity could provide insight into HAND pathogenesis. The work presented in this thesis contributes to the documentation of astrocyte dysfunction in HIV-1-infection. The accumulated data reveal the multifaceted mechanisms and roles of astrocytes in HIV-1 CNS infection.

A neuropathological feature of HIV-1 infection includes reactive astrogliosis, which is a hallmark of many neurodegenerative diseases. Our studies reveal the regulation of β-catenin signaling on major aspects of reactive astrogliosis *i.e.*, proliferation, wound healing and inflammation. HAND-relevant inflammatory stimuli activate β-catenin signaling in astrocytes. Our *in vitro* studies reveal knockdown of β-catenin impairs astrocyte responses to injury. Further, reduced levels of β-catenin also show less

proliferation and inflammatory responses in astrocytes. We also demonstrated that Wnt/β-catenin and NF-κB crosstalk links with inflammation during HIV-1 CNS infection. We next investigated endoplasmic reticulum (ER) stress associated with HAND-relevant neuroinflammation. Our studies show that ART (abacavir) and interleukin-1β increase cytosolic calcium in astrocytes, which in turn regulates ER stress and mitochondrial depolarization. We also identify astrocyte elevated gene (AEG)-1 as an ER stress inducible gene. In addition, AEG-1 interacts with calnexin, which emphasizes AEG-1 as a scaffolding protein regulating ER calcium signaling. Further, HIV-1-coupled inflammation and oxidative stress significantly increase regulator of ribosome synthesis (RRS1) expression, suggesting inhibition of rRNA transcription in astrocytes. Further, AEG-1 overexpression enhanced oxidative stress-induced RRS1 expression, *i.e*., nucleolar stress in astrocytes. Taken together, this study identified novel regulatory mechanisms in reactive astrocytes during HIV-1-induced neurodegeneration that might serve as innovative therapeutic targets for HAND.

Uncovering the Multifaceted HAND of Reactive astrocytes in **H**IV-**A**ssociated **N**eurocognitive **D**isorders

DISSERTATION

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LIST OF PUBLICATIONS

1. Neha Vartak-Sharma*****, **Shruthi Nooka*** and Anuja Ghorpade (*co-first authorship). Astrocyte Elevated Gene-1 (AEG-1) and the A(E)Ging HIV/AIDS-HAND. **Prog Neurobiol.** 2016, Apr 14. DOI: 10.1016/j.pneurobio.2016.03.006

2. **Shruthi Nooka** and Anuja Ghorpade. HIV-1-associated inflammation and antiretroviral therapy regulate astrocyte endoplasmic reticulum stress responses (*In press, Cell Death and Discovery.*

3. **Shruthi Nooka** and Anuja Ghorpade. The Endoplasmic Reticulum, Mitochondria and the Nucleolus: Intersecting Astrocyte Stress Responses in HIV-associated Neurocognitive Disorders. (*Invited for News & Commentary in Cell Death & Disease – In preparation*)

4. **Shruthi Nooka** and Anuja Ghorpade. Role of β-catenin pathway in reactive astrocytes during injury and HIV-1-associated inflammation (*In preparation for submission to PlosOne)*

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ABBREVIATIONS

ABC	Abacavir
AEG-1	Astrocyte elevated gene-1
AP-1	Activating protein-1
ART	Antiretroviral therapy
ARV	Antiretroviral
ATF	Activating transcription factor
AUC	Area under the curve
BAPTA-AM	Bis (2-aminophenoxy) ethane-N,N,N',N'-tetraaceticacid (acetoxymethyl ester)
BBB	Blood brain barrier
CCL/ CXCL	Chemokine (cystine) motif ligand
CHOP	CCAAT/enhancer-binding protein homologous protein
CNS	Central nervous system
CPE	CNS penetration effectiveness
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DFC	Dense fibrillary component
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ERSE	ER stress response element
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter-1
GS-1	Glutamine synthetase-1
GSK	Glycogen synthase kinase
HAART	Highly active antiretroviral therapy
HAD	(HIV)-associated dementia
HAND	HIV-1-associated neurocognitive disorders
HIVE	HIV-1-associated encephalitis
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
IRE	Inositol-requiring enzyme
LEF	Lymphoid enhancer-binding factor
LPS	Lipopolysaccharide
MRC	Mitochondrial respiratory chain
MTDH	Metadherin
MTR	Mitotracker red
NF-κB	Nuclear factor-κB
NLS	Nuclear localization signal
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NoLS	Nucleolar localization signal
NRTI	Nucleoside reverse transcriptase inhibitors
PERK	Protein kinase R-like endoplasmic reticulum kinase
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
RRS	Regulator of ribosome synthesis
TCF	T-cell factor
TMD	Transmembrane domain
TNF-α	Tumor necrosis factor-α
UPR	Unfolded protein response
WNT	Wingless type
XBP	X-Box binding protein

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CHAPTER 1

Introduction

(Adapted from Neha Vartak-Sharma*, **Shruthi Nooka***, Anuja Ghorpade.
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1.1 Introduction to HAND

1.1.1 Global HIV statistics

Globally, an estimated 36.7 million people were infected with Human immunodeficiency virus (HIV) at the end of 2015 (UNAIDS fact sheet, 2016). In the United States, more than 1.2 million people have HIV infection, and almost 1 in 7 (14%) are unaware of their infection (www.hiv.gov/hiv-basics/overview/data-and-trends/statistics). In 2015, estimated incidences of about 50,000 were diagnosed with HIV new infections in the United States (www.cdc.gov/hiv/statistics/overview/ataglance.html)

1.1.2 HIV-associated neurocognitive disorders (HAND)

The initiation of combined antiretroviral therapy (ART) has prolonged the life of HIV-1-infected individuals and reduced the progression to acquired immune deficiency syndrome (AIDS). HIV-1 rapidly enters the central nervous system (CNS) after infection and establishes as persistent viral reservoir. With the advent of ART, there is tremendous success in keeping HIV patients alive; yet, the commonly termed 'successful failure' is that ART has also increased the incidence of HAND (1), a spectrum of neurological complications occurring in HIV-1-infected individuals. Currently, HAND is prevalent in 18-50% of those diagnosed with HIV and is considered as a major factor decreasing their quality of life (1, 2). In the early 1990s, prior to highly effective ART, HIV-associated dementia (HAD) affected 15-20% of AIDS patients (3). In 2007, a more precise and sensitive "Frascati" classification system was introduced, which broadly classified HAND into asymptomatic neurocognitive impairment, mild neurocognitive impairment and HAD, the most severe form of neurocognitive deficit (4).

Successful therapeutic advancements in the post-ART era decreased HAD prevalence to 5% (1, 5); whereas the incidences of milder cognitive deficits remain high.

HAND is characterized by milder forms of brain parenchymal inflammation that can be detected by magnetic resonance spectroscopy and measurable markers of inflammation in cerebrospinal fluid (6). CD4+ T lymphocytes, dendritic cells and monocyte-derived macrophages are the major cells types that can support productive replication of HIV-1. Despite its immune privileged status, the CNS is now recognized as an important target of HIV-1 productive infection and an early viral reservoir following primary infection (6). While many theories have been postulated to explain HIV-1 neuroinvasion ranging from free viral entry to cell-mediated entry, the most widely accepted is the “Trojan horse” hypothesis, which suggests that HIV-1 crosses the blood brain barrier (BBB) *via* infected leukocyte infiltration from peripheral blood (7). CNS tissue penetration leads to HIV-1-infected monocyte maturation into perivascular macrophages that allow productive replication and release viral particles. These progeny virions infect resident brain microglia and initiate a neuroinflammatory cascade that promotes development and progression of HAND. Other neurological comorbidities manifest as well. These include acute aseptic meningitis, distal sensory polyneuropathy and vacuolar myelopathy, other opportunistic viral or non-viral neurological infections, cytomegalovirus encephalitis, toxoplasmosis encephalitis, cryptococcal meningitis, and progressive multifocal leukoencephalopathy, primary CNS lymphoma, cerebral vascular disease, and nucleoside neuropathy (8, 9). Amongst the multitude of neurological complications associated with HIV-1 infections, HAND is the most prevalent problem in the current ART era (8).

1.1.3 Adverse effects of ART

Classification of ARV drugs

Standard ART consists of the combination of antiretroviral (ARV) drugs to dramatically suppress the HIV infection and curtail the progression of HIV disease. Six main classes of antiretroviral agents, each targeting at different step of the viral life cycle currently exists, (**Fig 1.1**) as follows:

- Nucleoside reverse transcriptase inhibitors (NRTIs)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Protease inhibitors (PIs)
- Integrase inhibitors (INSTIs)
- Fusion inhibitors (FIs)
- Chemokine receptor antagonists (CCR5 antagonists)

The HIV-1 life cycle and mechanism of action of antiretroviral drug classes

Entry inhibitors interfere with viral entry into the host cell and are comprised of a complex group of drugs with multiple mechanisms of action. Virus spread can be mitigated by blocking key proteins that mediate virion attachment process, co-receptor binding and fusion to the host cells (10). **Fusion inhibitors** act extracellularly to prevent the fusion of HIV to the CD4 or other target cell. **Chemokine receptor antagonists** are a class of molecules that antagonize CCR5 receptor. **NRTI**s are structurally similar to endogenous deoxyribonucleotides and have a high affinity for the viral reverse transcriptase, thus facilitating incorporation into the viral DNA chain, resulting in termination of proviral DNA formation. NRTI incorporation leads to transcriptional termination as they all lack the 3'-OH group necessary for the formation of

phosphodiester bond in DNA strand elongation (11). **NNRTIs** are compounds bind to the allosteric pocket site of the HIV-1 reverse transcriptase and disrupt its enzymatic activity and blocks HIV-1 transcription (12). **Integrase inhibitors** bind to the viral integrase cofactors needed for host DNA interaction and block proviral DNA covalent link to the cellular DNA (13). **Protease inhibitors** function as competitive inhibitors that bind to the viral protease active site with high affinity and inhibit subsequent cleavage of viral polypeptides and viral budding from the host cell (14).

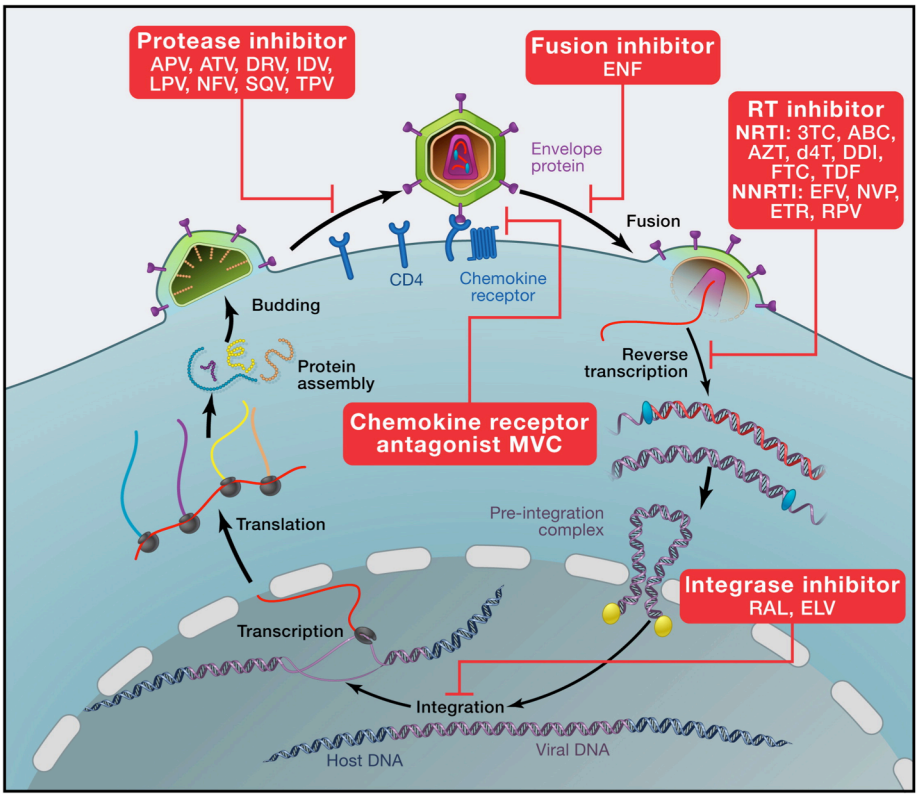

Figure 1.1. Schematic representation of various types of antiretroviral drugs intervention points in HIV-1 life cycle (15). HIV-1 entry into host cells can be blocked by the chemokine receptor antagonist (CRA) or by the fusion inhibitor. Nucleoside/non-nucleside reverse transcriptase inhibitors (NRTIs, NNRTIs) interrupt HIV-1 life cycle *via* inhibition of HIV-1 reverse transcriptase and terminating viral DNA synthesis. The integrase strand transfer inhibitors curb proviral DNA attachment to the host chromosome. Protease inhibitors bind to HIV-1 protease and inhibit the maturation and assembly of new virus. Abbreviations: lamivudine (3TC), abacavir (ABC), amprenavir (APV), atazanavir (ATV), zidovudine (AZT), stavudine (d4T), didanosine (DDI), darunavir (DRV), efavirenz (EFV), elvitegravir (ELV), enfuvirtide (ENF), etravirine (ETR), emtricitabine (FTC), indinavir (IDV), lopinavir (LPV), maraviroc (MVC), nelfinavir (NFV), nevirapine (NVP), raltegravir (RAL), rilpivirine (RPV), saquinavir (SQV), tenofovir disoproxil fumarate (TDF), tipranavir (TPV).

The ART toxicity is an increasingly important concern in the management of HIV-infected population. Current ART for the treatment of HIV infection is associated with long-term side effects. The need to monitor toxicity of ARV drugs has increased substantially. Typical ARV regimens include a "backbone" of two NRTI and a "base" of PI or NNRTI. Each drug class has its own side effects: NRTIs affect large spectrum of clinical and biological abnormalities that include peripheral and autonomic neuropathy, pancreatitis, cardiomyopathy, lactic acidosis, lipodystrophy, and hyperlipidemia; NNRTIs are associated with neuropsychiatric symptoms, rash, liver toxicity, and lipid abnormalities; and PIs are associated with osteoporosis, gastrointestinal intolerance and glucose and lipid abnormalities (16), (17), (18). In many of the HIV-1 treating patients, a serious complication, hepatotoxicity, was observed (19). All ARV drug classes, NRTIs, NNRTIs and PIs may cause hepatotoxicity but in different pathways (20). Numerous mechanisms have been proposed to explain the deleterious impact of highly active ART (HAART) drugs, including mitochondrial DNA depletion, impaired mitochondrial replication *via* inhibition of the mitochondrial DNA polymerase gamma (POLγ), and subsequent increase in reactive oxygen species and oxidative stress (21), (22).

NRTIs toxic mechanisms were majorly associated with mitochondrial dysfunction due to inhibition of mitochondrial (mt) DNA polymerase activity (23). Disturbed mitochondrial function summarized in (**Fig. 1.2**) due to polymerase-γ inhibition has been proposed as a major mechanism for NRTI-induced adverse events (21), (24). Due to high affinity of NRTIs for polymerase-γ, NRTIs are frequently incorporated into the new DNA strand which results in chain termination as they all lack the 3′ -OH group necessary for

phosphodiester bond formation in DNA strand elongation. This results into low mtDNA and possibly a reduction in mtDNA encoded proteins, essential components of the mitochondrial respiratory chain (MRC) complexes. In turn, this leads to disrupted electron transport through the MRC and a concomitant reduction in proton efflux, reducing the membrane potential and ATP production by the mitochondria. This disturbed mitochondrial function can result in augmented reactive oxygen species (ROS) production and morphological changes. Patients treated with NNRTIs experienced neurological disorders including distal symmetric polyneuropathy, peripheral and autonomic neuropathy (25), (26).

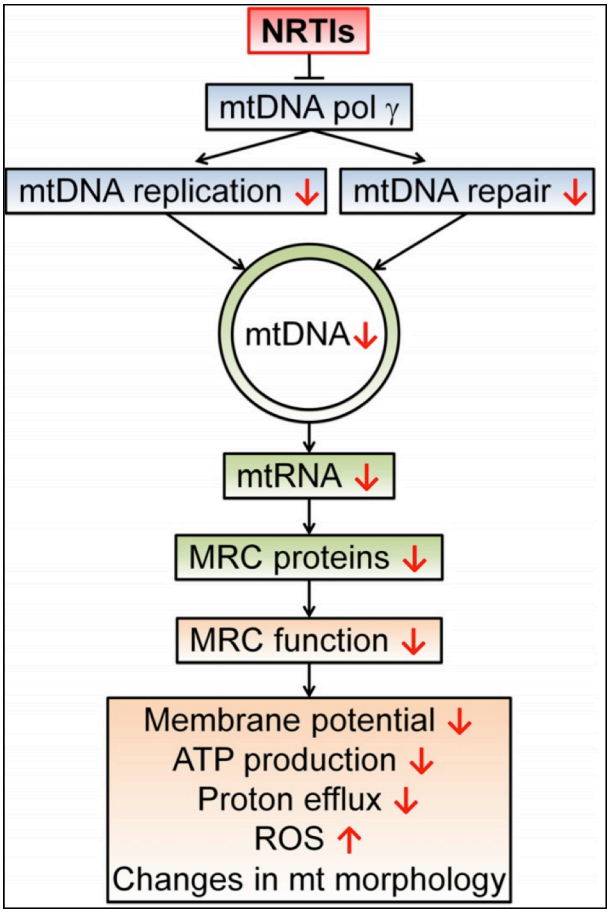

Figure 1.2. ARV drugs mitochondrial toxicity (27). High affinity of NRTIs for polymerase-γ causes integration of NRTIs into the new DNA strand, resulting into termination of mitochondria DNA synthesis, less mtDNA molecules and mtDNA encoded proteins, which are the main components of the mitochondrial respiratory chain (MRC) complexes. This, in turn, causes disrupted electron transport through the MRC and a concomitant reduction in proton efflux, reducing the membrane potential and ATP production by the mitochondria. This disturbed mitochondrial function can augment reactive oxygen species (ROS) and generate morphological changes.

Neurological impairment in HIV-infected patients is still prevalent, even in the post-HAART era. Therefore, the best option for treating HAND is development of ARV drugs with good CNS penetration (**Table 1.1**). Multiple studies have shown that higher CNS penetration effectiveness (CPE) is correlated with neurocognitive improvement, especially in impaired patients (28), (29), (30). Though, ARV drugs with higher CPE score were more effective in suppressing cerebrospinal fluid (CSF) HIV RNA, risk of neurotoxicity was still high (31). Other studies showed conflicting results *i.e.*, worsening of cognitive performance in patients treated with higher CPE value ARV drugs (32), (30). Fifteen ARV drugs were tested for their neurotoxic effects, including eight NRTIs, three non-NRTIs (NNRTIs), three PIs and maraviroc, MVC, a CCR5 receptor antagonist. Overall levels of neurotoxicity varied widely. Greater neurotoxicities were associated with NRTIs; while NNRTIs and PIs displayed a lower level of toxicity (33). Thus, further exploration of the mechanism of neurotoxicity of these compounds individually and in combinations is warranted.

Drug	CNS Penetration Score			
	4	3	2	1
NRTI	Zidovudine	Abacavir Emtricitabine	Lamivudine Stavudine Didanosine	Tenofovir Zalcitabine
NNRTI	Nevirapine	Efavirenz Delaverdine	Etravirine	
PI	Indinavir/r	Darunavir/r Fosamprenavir/r Lopinavir/r Indinavir	Atazanavir Atazanavir/r Fosamprenavir	Nelfinavir Ritonavir Saquinavir/r Saquinavir Tipranavir/r
CCR5 Inhibitor		Maraviroc		
Fusion Inhibitor				Enfuvirtide
Integrase inhibitor		Raltegravir		
CNS, central nervous system; NRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor.				

Table 1.1. CNS penetration-effectiveness of ARV drugs (adapted from(34)). Various ARV drugs CNS penetration scoring system (4, the highest)

1.2 Classical mechanisms of HIV-induced neurotoxicity

1.2.1 Inflammation

The pathogenesis of HAND is multifaceted, and several studies indicate that inflammation plays one of the major roles (35). Inflammation is a complex and tightly regulated process that recruits immune cells to and clears pathogen from the site of infection. Any sort of tissue damage generally induces acute inflammation, a beneficial process characterized by swelling, redness, pain and warmth. This process involves a complex array of soluble pro-inflammatory mediators including cytokines, chemokines and acute phase proteins (36), (37).

Persistent immune activation is recognized as a primary risk factor for neuropathological outcomes in chronic HIV infection. HIV-1 proteins, such as HIV

transactivator of transcription (Tat), negative regulatory factor and glycoprotein (gp)120, trigger inflammation by regulating cytokine signaling (38-40). HIV-1 infected or activated brain microglial cells cause neuronal damage and cognitive dysfunction by releasing neurotoxic agents (41), inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β (42), (43), (44), glutamate (45) and quinolinic acid (46). In addition to the above-mentioned inflammatory molecules, HIV+ demented patients showed higher levels of chemokines such as CCL2 and chemokine (CXCL)-10 in CSF (47). The specific contributions and regulation of these cytokines, chemokines and bioactive molecules in reactive astrocytes during HIV-1 infection is well understood. HIV-1 Tat-induced expression of pro-inflammatory chemokines, such as CXCL10, CXCL8, and CCL2 in astrocytes (48). HIV-1 gp120 enhanced the expression of intercellular adhesion molecule-1 (ICAM-1), critical mediator of CNS immune responsiveness in CNS glial cell types including astrocytes and microglia (49). ART causes metabolic disorders and plays an indirect role in inflammation. Though ART reduces systemic HIV-1-induced inflammation, the low-grade chronic inflammation through the disease span remains a significant contributing factor for HAND. For example, non-nucleoside reverse transcriptase inhibitors, such as efavirenz, could inhibit adipocyte differentiation, leading to lipid accumulation and atherosclerosis (50). Efavirenz also promotes IL-6 accumulation, a phenomenon also observed in elderly people (50). In addition, the *Strategies for Management of ART* study showed patients on abacavir have elevated proinflammatory and coagulation biomarkers (51). Identifying the mechanism(s) driving chronic inflammation of the CNS will likely provide important insights into the pathogenesis of HAND and may reveal potential therapeutic targets.

1.2.2. Oxidative stress

Oxidative stress reflects an imbalance between free radicals levels, or ROS, and the availability of detoxifying antioxidants. The resultant oxidative damage to biomolecules, including lipids, proteins and DNA, impacts chronic ailments such as cancer, atherosclerosis, stroke, septic shock, aging and other neurodegenerative diseases. Oxidative stress is often associated with HIV-1 infection. Tat-mediated neurotoxicity is associated with increased oxidative modifications of proteins (52). HIV-1 proteins induce oxidative stress; viral Tat protein released from HIV-infected cells increase mitochondrial reactive oxygen species (ROS) production (53). Moreover, lower intracellular glutathione levels are observed in Tat transgenic mouse liver and erythrocytes through modulation synthesis of glutathione, an important antioxidant pathway (54). HIV-1 viral protein R also dampens the astrocyte glycolytic pathway by impairing glyceraldehyde 3-phosphate dehydrogenase activity, leading to reduced intracellular ATP and glutathione concentrations, along with increased ROS and neurotoxicity (55). Likewise in Jurkat T cells, reactive oxygen intermediates, by activating nuclear factor (NF)-κB, may indirectly activate the HIV-1 long terminal repeat promoter NF-κB regulatory elements to enhance HIV-1 infection (56). In addition to the direct consequences of HIV-1 inducing a cytotoxic extracellular environment, ARV drugs may also exacerbate oxidative stress and cause neuronal damage (57). In HIV-1 patients undergoing therapy, serum oxidant levels increased while antioxidant status decreased when compared to untreated and control groups (58). Moreover in HIV-1-infected adults, higher plasma F2-isoprotane concentrations, a reliable index of oxidative stress, were observed. Further, plasma F2-isoprotane concentrations were

more likely to be elevated in patients on ART compared to untreated HIV+ subjects (59). NRTI and PI cause premature immunosenescence by accelerating oxidative stress (27). Overall, oxidative damage caused by ROS impairs physiological function and can cause neurological impairments in HIV+ patients.

1.2.3 Mitochondrial Dysfunction

Oxidative damage affects mitochondrial DNA transcription and disrupts mitochondrial function that in turn enhances ROS production (60). Higher rates of oxidative stress-induced DNA damage are seen in mitochondrial DNA as compared to nuclear DNA, suggesting that mitochondrial DNA is more susceptible to oxidative stress (61, 62). A positive correlation between plasma HIV RNA and plasma mtDNA was also observed in HIV-infected patients and ART-naïve subjects (63). HIV and HIV polypeptides (in the absence of ART) have been shown to contribute to mitochondrial dysfunction and apoptosis in CD4+ and CD8+ T cells (64). HIV-proteins may directly induce mitochondrial dysfunction and increase oxidative stress in neurons. Studies showed HIV-1 gp120-mediated neuronal dysfunction *via* mitochondrial dysfunction (65).

Mitochondrial DNA damage and replication defects are induced by NRTIs (66, 67). ART weakens mitochondrial integrity, and induces oxidative stress and inflammation. The principal theory for this observation is that NRTIs affect DNA polymerase γ function, the only known mitochondrial DNA polymerase. Both the HIV reverse transcriptase and the mitochondrial DNA polymerase γ are typically sensitive to NRTIs-guided abnormal DNA replication. A competition between the NRTI triphosphates with the physiological nucleoside triphosphates leads to irregular mitochondrial DNA replication, and loss of mitochondrial DNA, ultimately leading to

reduction in mitochondria. In addition, NRTIs also compromise mitochondrial membrane integrity resulting in electron leakage, thus increasing the load of ROS in the cell (66, 67). Overall, increased mitochondrial oxidative stress is an important contributing factor for HAND, particularly in the setting of ART.

1.2.4 ER Stress

Aging and age-related neurodegenerative diseases are commonly accompanied by accumulation of disease-specific misfolded proteins in the CNS, supporting a relationship between age and protein misfolding. Classical examples include amyloid beta (Aβ) peptides and microtubule-associated tau proteins in alzheimer's disease (AD) (68, 69), α-synuclein in Parkinson's disease (70), huntingtin in Huntington's disease (HD) (71) and superoxide dismutase in amyotrophic lateral sclerosis (72). The link between aging and protein misfolding is well accepted; for instance, quality control of protein synthesis wanes with age. Under stress, the ER initiates adaptive mechanisms termed the "unfolded protein response (UPR)" to limit accumulation of misfolded proteins. However, prolonged or sustained ER stress leads to apoptosis and inflammatory response.

Another promising therapeutic target in HAND is the endoplasmic reticulum (ER)–associated stress pathway, also known as the unfolded protein response (UPR). Under physiological conditions, the UPR is an adaptive response, activated by the accumulation of misfolded proteins in the ER, to maintain cell survival. Specifically, ER stress leads to the activation of 3 major UPR sensors: protein kinase R-like ER kinase- kinase (PERK), inositol-requiring enzyme1α (IRE1α), and activating transcription factor (ATF6) (**Fig 1.3**). PERK phosphorylates the eukaryotic translation

initiation factor-2α (eIF2α), which results in attenuation of general translation initiation and the selective translation of the transcription factor ATF4. In turn, ATF4 induces growth arrest and DNA damage-inducible protein *i.e.*, CCAAT-enhancer-binding protein homologous protein (CHOP). IRE1α- mediates splicing of x-box–binding protein (Xbp1), which increases transcription of ER-resident chaperones, folding enzymes, protein degradation component machinery. ATF6, after cleavage, contributes to the up-regulation of folding or degradation proteins.

ER stress response is activated in HIV-infected cortex and phenotypic analysis of immunofluorescence showed cell type-specific increases in binding immunoglobulin (BiP) levels in neurons and astrocytes (73). *In vitro* and *in vivo* studies showed HIV-1 infection and antiretroviral drugs also induce ER stress and UPR activation (74). During infection, increase in viral load and proteins activate UPR. HIV Tat, and HIV-1 gp120 induced ER stress and lead to increased glial fibrillary acidic protein (GFAP) activation, and cell death in astrocytes (75, 76). HIV-1 Tat upregulates X-box binding protein that could further activate UPR pathway (77). HIV protease inhibitors induce endoplasmic reticulum stress and disrupt barrier integrity in intestinal epithelial cells (78). However, certain downstream mechanisms are not beneficial for viral infection *e.g.*, ER-associated degradation. Quality control mechanism *via* the ER-associated degradation pathway induces proteosomal degradation of gp160, an essential precursor for productive viral replication (79). Above all suggest the role of ER stress response in HIV-induced neurodegeneration and the resulting neurocognitive impairment.

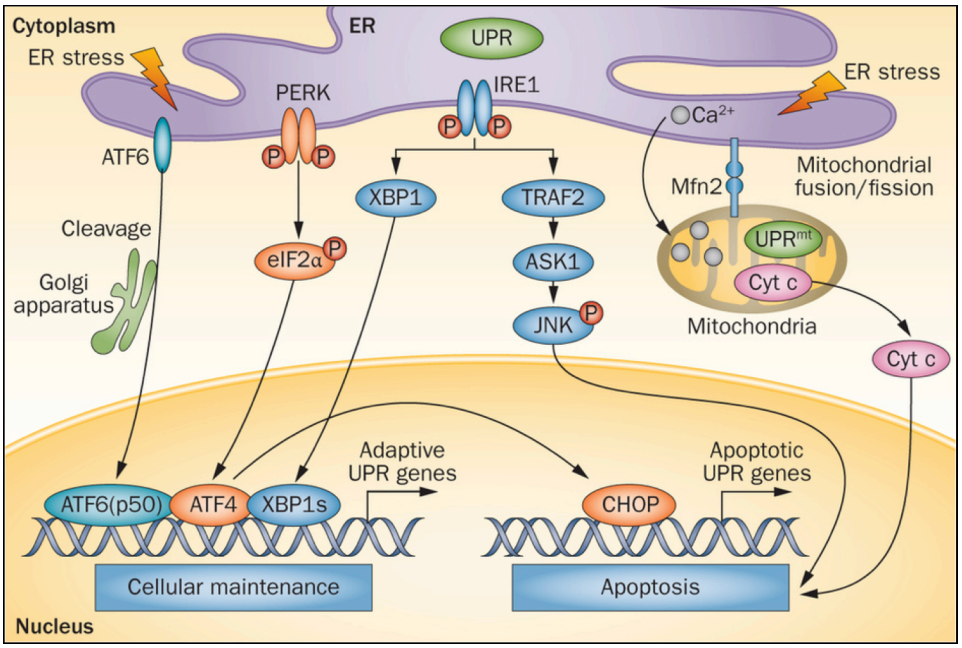

Figure 1.3 Unfolded protein responses during ER stress (80). The UPR involves three parallel signaling branches: PERK, IRE1α and ATF6. The outcome of UPR activation increases protein folding, transport and ER-associated protein degradation (ERAD), while attenuating protein synthesis. However, long-term ER stress activates inflammatory and apoptotic UPR pathway. Calcium, ca^{2+} plays a critical role in ER and mitochondrial cross talk. Ca^{2+} released from ER during stress is taken up by mitochondria, resulting into calcium overload and depolarization of permeability transition pore (PTP) that induces Ca^{2+}–Cyt c-dependent apoptosis signaling. Abbreviations: C/EBP-homologous protein (CHOP), cytochrome c (Cyt c), eukaryotic translation initiation factor 2α (eIF-2α), endoplasmic reticulum (ER), inositol-requiring protein 1 (IRE1), c-Jun N-terminal kinase (JNK), mitofusin (Mfn), phosphate (P), tumour necrosis factor receptor-associated factor 2 (TRAF2), unfolded protein response (UPR), X-box binding protein 1 (XBP1), spliced form of XBP1 (XBP1s).

1.3 Role of Astrocytes in HAND

Inflammatory responses orchestrated through activated glia have emerged as important mechanisms in neurodegeneration associated with multiple diseases including HAND pathogenesis. Initially considered as merely passive bystanders, astrocytes are now recognized as important regulators of neuronal function by integrating neuronal inputs and modulating synaptic activity at the neuroglial tripartite synapse (81). As astrocytes have both a 'neural' and an 'immune' repertoire, through which they are able to exert both neuroprotective and neurotoxic outcomes, an imbalance in any facet of astrocyte function likely has lasting repercussions, perpetuating neuropathogenesis.

In the case of HIV-1 CNS infection; however, the major targets for productive infection are not astrocytes, but rather, mononuclear phagocytes are primary sources of progeny virions in the CNS. Productive HIV-1 infection of perivascular macrophages and microglial cells can also activate surrounding microglia, which express higher levels of cell surface antigens, such as CD14, CD16, CD68 and major histocompatibility complex class II, leading to further leukocyte trafficking into the CNS, strongly implicated in HIV-1 neuropathogenesis (82). Current research established significant clinical correlations for cerebrospinal biomarkers of monocyte activation and chemotaxis, including soluble CD14, CCL2 and others, to magnetic resonance spectroscopy metabolites during chronic HIV disease (83). HIV-infected patients with ongoing drug abuse also exhibit CD14+CD16+ monocyte migration and accumulation in the brain induced *via* elevated extracellular dopamine in the CNS (84). Dendritic cells, macrophages or microglia, the primary targets of productive HIV-1 infection in the brain, along with astrocytes release several pro-inflammatory cytokines, such as IL-1β, TNF-α,

and interferon-γ, in addition to HIV-1 proteins Tat, gp120 and negative regulatory factor. These cells also release platelet activation factors and other neurotoxic compounds like nitric oxide, glutamate, and inducible nitric oxide synthase, along with chemokines, such as CCL2, IL-6 and CXCL8. Early studies indicated that the percentage of infected astrocytes may be as high as 19%, and taking into account the relative abundance in the brain, astrocytes presented as significant HIV-1 reservoirs in the CNS (85). Recent work suggests that pH-mediated-endocytosis and subsequent viral destruction in the endocytic vesicles leads to non-productive infection of astrocytes (86).

Interactions of infected and/or activated mononuclear phagocytes and astrocytes propagate disease processes. Resultant persistent CNS inflammation has been recognized as a hallmark of HIV-1 infection and is accompanied by cerebrospinal fluid pleocytosis and immune activation (35). HIV-1 proteins, particularly Tat, potentiate inducible nitric oxide synthase in astrocytes through the activation of NF-κB and CCAAT/enhancer binding protein β (87). In addition, IL-1β and TNF-α also trigger nitric oxide synthase production in astrocytes (88, 89). Astrocyte exposure to HIV-1 proteins, Tat and gp120 were reported to induce IL-6 and TNF-α, which in turn, induce the release of excitatory amino acids, for example glutamate and potassium, that further trigger toxic calcium accumulation in astrocytes and neurons (90). HIV-1 gp120 animal models documented that this protein disrupts the astrocyte glutamate-glutamine homeostasis by modulating the glutamate transporter expression on astrocytes, leading to memory impairment (91). Besides the direct influence of viral proteins, oxidative stress induced by pro-inflammatory cytokines is also implicated in CNS disease progression. Reactive astrocytes exhibit impaired glutamate clearance leading to

excitotoxicity, which is a source of ROS in addition to inflammatory stimuli (92). Astrocytes also extend foot processes surrounding the endothelial cell tight junctions and remain critical regulators of BBB integrity. Both brain microvascular endothelial cells and astrocytes express molecular drug pumps and transporters, such as permeability glycoprotein (P-gp) and organic ion transporters, that dictate drug elimination (93, 94). Taken together, inflammation, excitotoxicity and oxidative stress result in neurotoxicity, leading to dysfunction or, ultimately, neuronal death.

Although release of toxic levels of pro-inflammatory cytokines, inability to clear extracellular glutamate and blood brain barrier (BBB) damage endorse astrocytes as the central offenders in HAND pathogenesis, astrocytes are innately neuroprotective. Thus, it is imperative to take into consideration the neuroprotective repertoire of astrocytes and its dysregulation in disease. Astrocytes exposed to HIV-1 proteins secrete neuroprotective tissue inhibitor of metalloproteinases-1 (95) and also induce nuclear factor erythroid-2 related factor-2 (Nrf-2), a universal regulator of antioxidant cellular mechanisms, thereby triggering oxidative stress combating enzymes (96). Astrocytic sirtuin elicited neuroprotective effects by attenuating oxidative stress *via* increased production of super oxide dismutase 2 and catalase (97). Similarly, astrocytic tumor growth factor β-mediated neuroprotection was demonstrated in a mouse model for subacute neuroinflammation (98). Although deleterious effects of intracellular accumulation of Aβ is well known in AD, a recent study using an amyloid precursor protein/presenilin-1 transgenic mouse model revealed that HIV-1 Tat can contribute to HAND by modifying amyloid precursor protein processing to increase Aβ production (99).

Reactive astrogliosis, a pathological hallmark of HIV-1-associated encephalitis (HIVE) is evident in mouse and human HIV+ brain tissues. Histologically, astrogliosis is visualized by increased GFAP staining, near CNS areas of active HIV-1 replication in multinucleated giant cells and microglial nodules (100, 101). Several neurotoxic factors and cytokines implicated in HIVE pathogenesis, including TNF-α, IL-1β, NO and glutamate are reported to upregulate GFAP expression in astrocytes (102-104). However, molecular mechanisms mediating reactive astrogliosis are not well known. β-catenin signal cascade, a principal mediator of canonical wingless type (Wnt) signaling, plays a fundamental role in regulating various biologic processes such as organ development, tissue homeostasis, and pathogenesis of human diseases (105); (106). In the presence of Wnt, binding of Wnts to a frizzled receptor and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor triggers the recruitment of the cytoplasmic component, disheveled, which inhibits the phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β). This results in an increase in the stability of β-catenin and its translocation to the nucleus, where it can interact with members of the lymphoid enhancer factor/T-cell factor (TCF/LEF) transcription factors and subsequently regulate the expression of downstream target genes, such as c-myc and cyclin D1 (**Fig. 1.4**). Recent studies showed inhibited expression of β-catenin and alleviated reactive gliosis in the hydrocephalic rat brain tissue (107). It is interesting to note that Wnt and β-catenin signaling is activated in proliferating astrocytes and in NG2 glia after traumatic brain injury (108). Knockdown of β-catenin in the prefrontal cortex reduced glutamate transporter (GLT)-1 and glutamine synthetase (GS) expression confirm that β-catenin regulates key proteins responsible

for excitatory glutamate neurotransmission. Thus, further investigations discovering HIV-1-induced molecular mechanisms in astrocytes and how they influence astrocyte function during HAND are warranted.

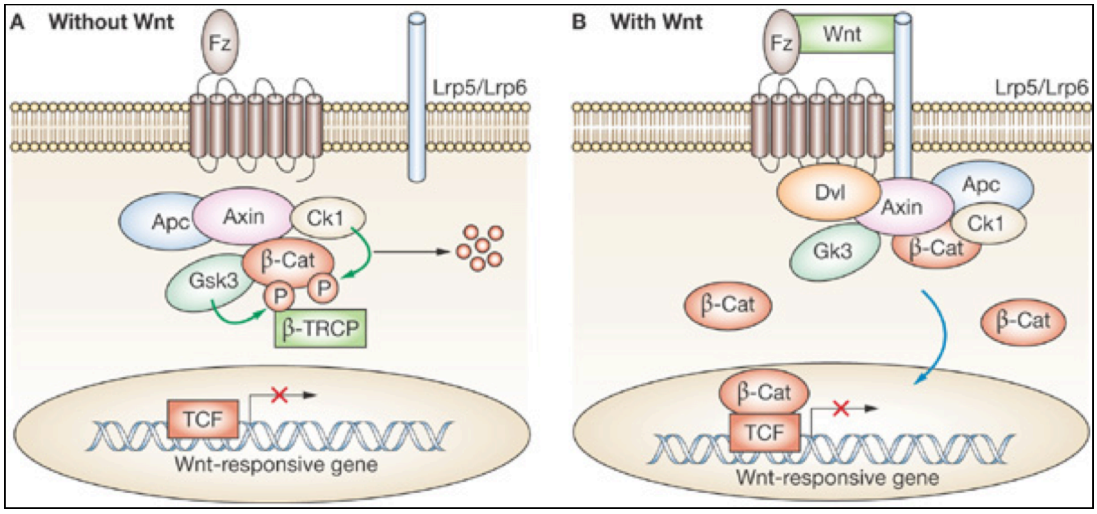

Fig 1.4. Canonical Wnt β-catenin signaling mechanism (109). (A) In the absence of Wnt ligands, β-catenin bound to multi-destruction complex is phosphorylated, ubiquitinated, and degraded by proteosome. (B) With Wnt, β-catenin is not phosphorylated or degraded, and is translocated to the nucleus. β-catenin functions as transcriptional co-activator in the nucleus, where it binds to TCF transcription factor and activates Wnt-responsive genes. Abbreviations: Antigen presenting cell (Apc), Casein kinase 1 (Ck1), Disheveled (Dvl), Glycogen synthase kinase 3 (GSK3), Frizzled protein (Fz), Lipoprotein receptor related protein (LRP), T-cell factor (TCF), Transducin repeat containing protein (TRCP)

1.4 Astrocyte elevated gene (AEG)-1

1.4.1 AEG-1: Historical Perspectives

AEG-1 was originally described in 2002 as an HIV-1 neuropathology-associated gene whose expression is significantly elevated upon HIV-1 or TNF-α exposure (110). Basic local alignment search tool analysis of AEG-1 gene indicated that AEG-1 had a unique gene structure, which did not resemble any other gene and is evolutionarily conserved among mammals and higher vertebrates. AEG-1 genomic environment is a known hot spot for genetic alterations in many cancers (111, 112) and in other CNS pathologies such as migraine (113, 114). AEG-1 is recognized as a pleiotropic protein localizing in various intracellular locations, including plasma membrane, cytoplasm, ER, nuclear envelope, nucleus and nucleolus. The physiological or cellular stimuli responsible for this differential localization remain unknown. At the same time, AEG-1 interaction with various protein/RNA partners and its wide-ranging intracellular localization are considered critical determinants of its function (115-117). Despite detection of AEG-1 in the nucleus of many cancers, no DNA binding sites have been described in its protein structure. However, a putative RNA binding site and many protein-protein interaction sites on AEG-1 have been mapped, which suggest its role as a scaffolding protein or as a transcriptional co-activator/repressor (**Fig 1.5**).

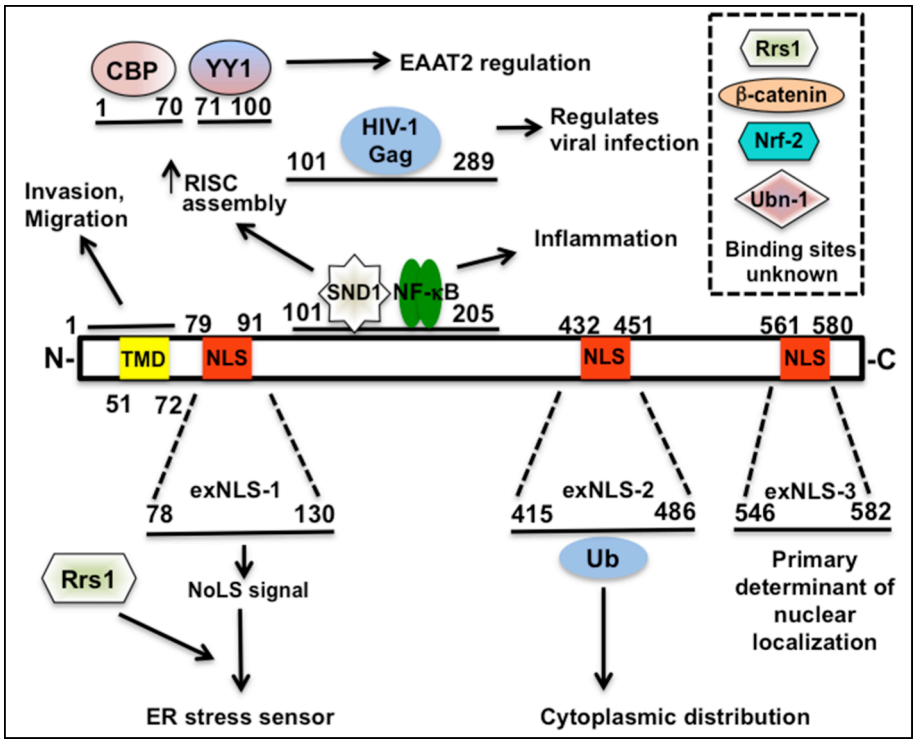

Figure 1.5 AEG-1 sub-cellular localization and protein interaction domains. AEG-1, together with other proteins, exerts diverse functional outcomes. The open reading frame for AEG-1 spans from 220 to 1968 base pairs, and encodes for a 582 amino acid protein with a predicted molecular weight of 64 kDa. This diagram highlights select AEG-1 binding domains and their respective functions. In addition to the known interactions, specific elements that are responsible for sub-cellular localization of AEG-1 are also shown. AEG-1 has one trans membrane domain (TMD), multiple nuclear localization sequences (NLS) with embedded extended segments (exNLS). However, AEG-1 regions that bind β-catenin, ubinuclein-1 (Ubn-1), and regulator of ribosome synthesis (Rrs)1 are not yet identified. Nuclear factor erythroid-2 related factor-2 (Nrf-2) binding site on AEG-1 is also unknown. Future investigations for mapping specific AEG-1 binding regions for Nrf-2, other proteins, and related functional changes will facilitate better understanding of its pleotropic role in health and disease.

1.4.2 AEG-1 Discovery

AEG-1 research has evolved since its discovery and first report. While astrocytes are not the primary targets of HIV-1 CNS infection, in the current ART era inflammatory responses orchestrated by astrocytes continue to play a vital role in HIV-1 neuropathogenesis and hence remain a target of extensive research. In an attempt to understand the astrocyte-driven etiology of HAND, alterations in gene expression patterns in HIV-1-infected cultured primary human astrocytes were analyzed, and 15 "astrocyte elevated genes", *i.e.* AEGs, were detected. Subsequently, mouse AEG-1, which was cloned using a phage screening approach, was found to mediate the metastasis of breast cancer cells to lung tissue (118). Here, the AEG-1 protein was named metadherin (MTDH) to specify its role as a metastatic adhesion protein. Further cloning approaches categorized AEG-1 as a tight junction protein and was suitably named lysine-rich carcinoembryonic antigen-related cell adhesion molecule 1 co-isolated (LYRIC)/3D3 (119). AEG-1 was often induced as a late onset gene 3-7 days post-HIV-1 exposure and/or TNF-α treatment (110). In cultured human astrocytes, HIV-1 and TNF-α induced AEG-1 expression earlier, within 8 h for RNA and 24 h for protein (120).

1.4.3. AEG-1 Biochemical interactions and Functional implications

The AEG-1 gene consists of 12 exons and 11 introns. The resultant mRNA encodes a single pass transmembrane protein consisting of 3611 nucleotides, excluding its poly A tail (121). AEG-1 promoter is regulated by the presence of negative transcriptional control elements located in the -2710/-459 base pair region. Deletion from -459 to -301 base pair resulted in 90% loss of basal Ha-ras-induced AEG-1 promoter activity,

defining a transcription factor-binding element (122). Further analysis uncovered two enhancer-box elements in the -356 to -302 region, that along with other transcription factor binding sites regulate AEG-1 promoter activity. Although AEG-1 is predominantly localized in ER and perinuclear region (121), its membrane topology still remains incompletely defined.

AEG-1 is a lysine-rich, highly basic protein that contains 582 amino acids, with a predicted molecular weight of 64 kDa and isoelectric point of 9.3, which can be altered by post-translational modifications to regulate its intracellular localization (**Fig. 1.5**) (121). It contains one transmembrane domain (TMD) spanning amino acids 51-72. In addition, AEG-1 also contains N-terminal "LXXLL" domain that is used by co-activators to interact with transcriptional factors (123). It has three putative nuclear localization signals (NLS) at amino acids 79-91, 432-451, and 561-580 (**Fig. 1.5**). Later on, targeting of AEG-1 to different sub-cellular compartments was attributed to the presence of intrinsic NLS and ubiquitination. Mutation and deletion studies of AEG-1 NLS indicated that extended sequences outside of canonical NLS (exNLS) were important AEG-1 localization determinants. Nuclear localization of AEG-1 is directed by extended NLS-3 (amino acids 546-582), whereas, extended NLS-1 (amino acids 78-130) mediates its nucleolar localization. The extended NLS-2 (amino acids 415-486) is modified by addition of ubiquitin that retains AEG-1 in the cytoplasm (summarized in **Fig. 1.5**).

AEG-1 exerts multiple functions with the aid of various binding domains that recruit diverse interacting proteins. Consequently, interactions with several intracellular partners make AEG-1 a broad-spectrum effector protein (**Fig. 1.5**).

1.4.4 AEG-1 in HAND

Astrocyte AEG-1 in HAND

Despite being a significantly upregulated gene in astrocytes following HIV-1 infection and/or TNF-α treatment (110), AEG-1 has not been investigated thoroughly in the context of HAND. In addition to high levels of AEG-1 in HIV+ patient brains (120), AEG-1, by its physical interaction with HIV-1 Gag protein, the main structural protein driving virion assembly and release from infected cells, and by the incorporation into the viral particles and cleavage by viral proteases, suggests a plausible role in HAND pathogenesis (124). Although there is no direct evidence to unequivocally confirm that AEG-1 facilitates HIV-1 viral entry into the CNS, recent inferences for AEG-1 in dengue virus-associated endothelial cell malfunction suggest that AEG-1 may regulate BBB permeability (125). In this study, AEG-1 expression on human endothelial cells mimicked dengue virus non-structural protein 1, thereby facilitating the targeting of endothelial cells for destruction by dengue virus auto-antibodies and increasing vascular permeability (125). Following entry of the virus *via* BBB, HIV-1 replicates in the primary sites of productive viral replication *i.e*., CD4+, chemokine receptor CXCR4 and CCR5 expressing brain resident microglia, peripheral macrophages and infiltrating monocytes (126). In the current ART era, the hippocampus, along with the surrounding entorhinal and temporal cortexe, are the main sites for HIV-associated neuroinflammation (127). A parallel analysis of AEG-1 expression in these distinct brain regions following viral BBB cross over are thus warranted to investigate whether increased astrocyte AEG-1 expression is a consequence of direct HIV-1 infection and/or HIV-associated neuroinflammation or an indirect downstream outcome. AEG-1 is a late onset gene in

human astrocytes, with induction at days 3-7 post-HIV-1 exposure (110), indicating AEG-1 could be largely regulated by downstream signaling events and early onset genes induced by HIV-1. In attempt to address this issue, *in vitro* short-term studies confirmed that astrocyte AEG-1 induction is mainly driven by neuroinflammation, but can be exacerbated by the presence of HIV-1 (120).

AEG-1 and Reactive Gliosis

Prior work by our lab on reactive astrocytes revealed a fundamental role for AEG-1 in regulating proliferation and migration of astrocytes, which is important for post-injury/inflammation recovery to homeostasis (128). As discussed above, in the context of cancer, elevated AEG-1 levels enable successful proliferation, migration and metastasis of cancer cells in a variety of tissue environments. These properties, in the context of HAND, translate into the phenomenon of reactive gliosis. Although AEG-1 expression, *per se*, was not significantly altered in injury-induced reactive astrocytes, intracellular localization changed dramatically. AEG-1 was localized to the nucleolus in a wound healing scratch model and hydrogen peroxide (H_2O_2) exposure. Differential trends of AEG-1 intracellular distribution were identified in HIV-1 or TNF-α treated astrocytes. AEG-1 remained in the cytoplasm in HIV-1-exposed astrocytes, whereas AEG-1 translocated to the nucleus in response to TNF-α treatment (120). AEG-1 intracellular localization is a critical determinant of its cellular function. While AEG-1 nuclear localization is symbolic of its transcriptional role in response to inflammation, its retention in the cytoplasmic is indicative of its role as a scaffolding protein, regulating signaling events in the cell. These observations validate differential regulation of astrocyte AEG-1 in response to various stimuli and are testimonial to its diverse roles in

the context of inflammation, oxidative stress and/or physical injury.

AEG-1 in HAND-associated Neuroinflammation

AEG-1, with its pleotropic functions and influences on inflammation, likely plays a central role in regulating HAND-associated neuroinflammation. One study demonstrated an early indication for AEG-1 involvement in regulating inflammatory cellular responses in U937 human promonocytic cells, wherein lipopolysaccharide induced AEG-1 and prostaglandin E2 production (129). Endogenous AEG-1 stable interaction with HIV-1 Gag protein, its incorporation into HIV-1 viral particles and cleavage by viral protease (124) is also an indicator of AEG-1 manipulation as a host factor to enhancing viral infectivity. This is supported by the fact that the AEG-1 and Gag interaction is also conserved for other retroviruses, such as murine leukemia virus and equine infectious anemia virus, and inhibition of this interaction leads to decreased viral infectivity (124). Therefore, analysis of AEG-1 expression and function in primary cellular targets of HIV-1 in the brain, including dendritic cells, microglia, macrophages and leukocytes, in addition to astrocytes, is necessary. In parallel with its direct interaction with HIV-1 viral proteins, previous work from our lab illustrate that AEG-1 also regulates signaling events in astrocytes post-HIV-1 exposure (120). We suggest that this is mainly orchestrated by AEG-1 functioning as a co-activator to facilitate protein-protein interactions based on its spatiotemporal availability at distinct intracellular locations. AEG-1 interaction with the HIV-1 Gag protein has been speculated to influence astrocyte inflammatory responses as the binding domain for HIV-1 Gag overlaps with binding domains for NF-κB and BCCIPα. In such, HIV-1 Gag creates a competitive binding environment, which ultimately influences cellular

proliferation and cytokine/chemokine production through AEG-1. Astrocytes, the most abundant cells in the brain, respond to the inflammatory challenge and undergo reactive gliosis, a process that involves a graded continuum of progressive alterations in molecular expression, hypertrophy, proliferation and, in severe cases, scar formation. Reactive astrocytes secrete a wide variety of neurotropic factors, pro- and anti-inflammatory molecules that can potentially mediate neuroprotective or neurotoxic effects, depending upon the severity of the insult. IL-1β and TNF-α are major pro-inflammatory cytokines in the brain and both stimulate astrocyte AEG-1 changes and subsequent inflammatory responses. Increased AEG-1 levels in reactive astrocytes, as noted in HAND brain tissues, signifies a positive correlation to increasing degree of neuroinflammation (120). Moreover, AEG-1 modulation of astrocyte inflammatory responses was demonstrated *via* regulation of NF-κB signaling, a master regulator of inflammation. AEG-1 interacts with the p65 subunit of NF-κB and alters its nuclear availability in response to inflammatory stimuli. Further, AEG-1 transcriptionally induces the expression of the upstream activator of NF-κB, IκB kinase (IKK) β (120). As NF-κB is a significant regulator of numerous cytokines and chemokines, AEG-1 modulation of NF-κB and IKKβ in the context of HIV-1-induced inflammation is an important finding with far-reaching implications. Astrocytes can also facilitate immune cell extravasation into the CNS by secreting chemokines and by regulating the neuro-vasculature (130). Astrocytes enhance T cell activation by modulating innate immune cells *i.e.*, microglial activity (131, 132). Moreover, in malignant glioma, astrocyte AEG-1 induction in response to neuroinflammation demonstrated transcriptional regulation of CXCL8, a neurotophil chemoattractant (133) that also mediates productive HIV-1 infection in

monocyte-derived macrophages and microglia (134). In response to injury or neuroinflammation, hypertrophic reactive astrocytes upregulate cytoskeletal proteins, such as glial fibrillary acidic protein (GFAP), vimentin and nestin, which are important for wound healing and homeostasis (135). AEG-1 may also modulate astrocyte cytoskeletal protein expression, as AEG-1 regulates the epithelial-mesenchymal transition proteins: vimentin, snail and snug, in cervical cancer, non-small cell lung cancer and HCC (136-139). All together, AEG-1 plays a central role in orchestrating HAND-associated neuroinflammation.

AEG-1 at Astrocyte Intracellular Crossroads

Astrocyte intracellular signaling molecules, in addition to cytoskeletal proteins, are also heavily implicated in the molecular mechanisms of gliosis during neuroinflammation; *viz.* increased expression of NF-κB, cAMP, oligodendrocyte transcription factor 2, mammalian target of rapamycin, SOX9, *etc.* (140-142). Besides NF-κB, AEG-1 also physically interacts with a number of transcription factors and to regulate downstream pathways. AEG-1 interacts with β-catenin to influence Wnt/β-catenin signaling in colorectal carcinoma (143), which is critical for cytoskeletal changes that recruit astrocytes to the injury site (144). Consequent to injury/neuroinflammation, reactive astrocytes can induce rapid activation of microglial cells and release of inflammatory mediators like IL-1β and TNF-α, triggering a cascade of autocrine signaling events to amplify the neuroinflammatory barrage (145, 146). Identification of AEG-1 as a downstream target of IL-1β and TNF-α, along with its regulation of NF-κB signaling, implies that AEG-1 is a key component of autocrine inflammatory signaling in astrocytes. Astrocyte production of IL-17A, IL-6, IL-12 and CXCL10 induce significant

neuroglial activation (147, 148). Disrupting astrocyte-derived inflammatory signaling and cytokine production in a rat model of neuroinflammation, resulted in attenuated neuro/glial activation (149). Similarly, AEG-1 knockdown in astrocytes exposed to IL-1β and TNF-α reduced NF-κB activation and signaling, suggesting that AEG-1 can be utilized as a target to enhance astrocyte-mediated neuroinflammation (120). This observation is further supported by a recent phosphoproteomics study conducted in breast cancer cells, wherein it was demonstrated that AEG-1 is a direct target of IKKβ at serine 298 and that AEG-1 phosphorylation is essential for IκBα degradation, as well as, NF-κB-dependent gene expression (150).

AEG-1 in HAND-associated Excitotoxicity

The functional outcome of neuroinflammatory cascades, consequent to reactive astrogliosis and traumatic CNS injuries or infections, is neurodegeneration. Neurodegeneration is characterized by rapid or gradual progressive decline in neuronal structure or functions, ultimately leading to neuronal loss, and manifests as cognitive, behavioral or motor deficits (151). As the more resilient counterpart to neurons in the brain, the principle role of astrocytes is to protect and nourish neurons. In such, neurodegenerative disorders are consequences of astrocyte functional failures. Astrocytes affect neurodegeneration over a graded continuum comprising of initial, immediate effects on neuronal survival post-injury; followed by delayed, effects, which ultimately influence neuronal recovery and function long-term.

Efficient removal of the neurotransmitter glutamate from the synapse is a critical function of astrocytes, which is often dysregulated during neuroinflammation and leads to excitotoxic neuronal damage. Prime examples are HIV-1-associated

neuropathies, which develop gradually over time, due to excitotoxic damage to neurons, owing to persistent chronic low-level neuroinflammation (152). With an increase in the number of reactive astrocytes at various spatiotemporal locations of the brain, in response to neuroinflammation, there is significant clustering of glutamate transporters, which are responsible for efficient clearance of extracellular glutamate. Failure to clear glutamate in the astroglial-neuronal tripartite synapse results in seizures and neurodegeneration implicated in HAND (74, 153, 154). The principle regulator of extracellular glutamate in the brain is the transmembrane sodium and potassium coupled transporter, EAAT2, which is expressed mainly on astrocytes (155). Thus, EAAT2 expression and activity are important determinants of excitotoxicity in the neuronal microenvironment. In studies on glioblastoma, which express high levels of AEG-1, implicated AEG-1 in downregulating EAAT2 promoter activity, and thus, in mediating glioblastoma-associated necrosis and neurodegeneration (156). Previous work by our lab demonstrated AEG-1-mediated EAAT2 downregulation and reduced glutamate clearance in primary human astrocytes treated with IL-1β, a prominent cytokine regulating astrocytes during HAND (120). AEG-1 is, thus, also implicated in HAND-associated neurodegeneration. Further, in glioblastoma, it was revealed that AEG-1 interacted with transcriptional repressor yin and yang (YY) 1 to downregulate EAAT2 (156). Cultured human astrocytes overexpressing AEG-1, displayed induction of YY1, thereby implying that AEG-1 interaction with YY1 may be able to regulate EAAT2 levels. Additionally, studies described EAAT2 expression as highly regulated at the translational level (157), indicating that, despite changes in EAAT2 transcription, the resultant EAAT2 protein levels may or may not resemble mRNA transcription. In this

regard, AEG-1 regulation of protein translation has been demonstrated in certain cancer types; *viz.* AEG-1-facilitated association of multi-drug resistance gene 1 (MDR1) mRNA to polysomes to induce MDR1 protein translation (158), and microRNA processing as a component of RNA-induced silencing complex in HCC (159). However, AEG-1-mediated regulation of EAAT2 translation needs to be investigated. Yet another unidentified important indicator for AEG-1 regulation of extracellular glutamate is its genomic location on chromosome 8q22, flanking the migraine susceptibility gene and in the vicinity of the glutamate regulatory element, plasma glutamate carboxypeptidase, which increases extracellular glutamate concentrations by cleaving N-acetyl-L-aspartyl-L-glutamate into N-acetyl aspartate and glutamate (113, 114).

Impaired glutamate transport and dysregulation of the glutamate-glutamine cycle are critical elements of most neurodegenerative disorders. Enhancing glutamate uptake by astrocytes would thus be an effective strategy to overcome resultant neuronal malfunction. Upregulation of astrocyte glutamine synthetase is linked to protection in ischemic stroke with reduced infarct size and plays a role in ischemic intolerance induced by inhibition of succinate dehydrogenase (160, 161). Taking into account AEG-1 regulation of astrocyte glutamate uptake, analysis of the effect of AEG-1 on glutamine synthetase activity in astrocytes is warranted. Similarly, ablation of astrocyte glutamate transporter EAAT2 significantly influenced neural stem cell population, neuronal regeneration and synaptic recovery post-injury (162), suggesting that astrocyte glutamate clearance capacities can dictate neurogenesis.

Accumulation of extracellular glutamate, an important excitatory neurotransmitter in the brain (163), was observed in several neurodegenerative

disorders like dementia, multiple sclerosis, AD, amyotrophic lateral sclerosis, Parkinson's disease, alcoholism, epilepsy and HD (164). Excessive glutamate in the neuronal microenvironment allows high levels of calcium influx into the neurons, thereby activating phospholipases, endonucleases and proteases like calpain that damage the neuronal cytoskeleton, membrane and DNA (165, 166). Since, EAAT2 expression and function in astrocytes is an important determinant of neurotoxicity, identification of AEG-1 as a negative regulator of astrocytic glutamate clearance *via* EAAT2 downregulation is an important finding that implies AEG-1 controls the level of neurodegeneration associated with neuroinflammation in HAND. Taken together, AEG-1 regulates several HIV-1-mediated mechanisms in astrocytes and might represent as therapeutic target during HIV-1 CNS infection (**Fig. 1.6**).

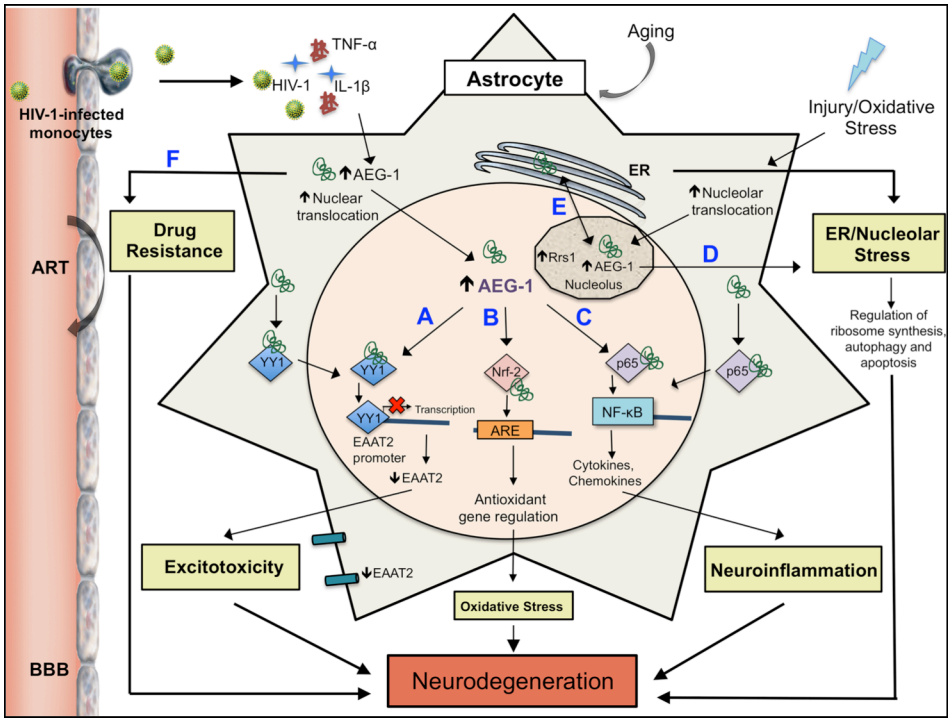

Figure 1.6. Collective perspectives on AEG-1 regulated pathways in HAND and aging. Increased AEG-1 levels and/or its nuclear-nucleolar translocation occur in astrocytes during inflammation, oxidative stress and injury, which contribute towards neurodegeneration in HAND and aging. AEG-1 exerts a multifactorial impact on these outcomes, through mechanisms including but not limited to: EAAT2 dysregulation leading to glutamate-induced excitotoxicity (A), Nrf-2 mediated oxidative stress responses (B), enhanced neuroinflammation through NF-κB (C), ER/nucleolar stress following altered sub-cellular localization (D-E, respectively), and increased antiretroviral drugs efflux at the BBB (F). AEG-1 thus bears the prospects of being an attractive therapeutic target for the not so distant future.

Objectives of the present study

Despite ART, the prevalence of cognitive dysfunctions in HIV-1 positive patients remains high. Notably, though astrocytes are susceptible to HIV infection, they do not develop productive infection. Thus, the pathogenesis of HAND is multifaceted, and increasing evidence indicates that persistent glial-mediated inflammation plays a major role in neurodegeneration. In balance, astrocytes represent an intriguing therapeutic target for re-establishing CNS homeostasis or for promoting neuroprotective behaviors in the context of HAND. In this study, we attempt to highlight astrocytic dysfunctions and their roles in HIV-1 neurotoxicity, which potentially contribute to HAND. We hypothesize that astrogliosis and ER/nucleolar stress contribute to HIV-associated neurodegeneration. The following studies will be addressed in this proposal:

First, we will delineate role of β-catenin signaling in regulating reactive astrogliosis during HIV-associated inflammation.

Second, we will investigate astrocyte ER stress responses and downstream neurotoxic mechanisms in HAND

CHAPTER 2

Role of β-catenin pathway in reactive astrocytes during injury and HIV-1-associated inflammation

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(In Preparation for PlosOne)

2.1 Abstract

Reactive astrogliosis is prominent in most central nervous system (CNS) infections. Besides ongoing low-level CNS viral replication, glial-induced inflammation and oxidative stress contribute to the pathogenesis of HIV-1-associated neurocognitive disorders (HAND); however, the molecular mechanisms causative to neurotoxicity have not yet been fully understood. In this study, first we identified HIV-1-mediated inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α to induce critical mediators of classical wingless type (Wnt) signaling including β-catenin, and lymphoid enhancer-binding factor (LEF)-1 expression in astrocytes. Next, we investigated the role of β-catenin pathway on astrocyte proliferation and migration, primary aspects of reactive astrogliosis. Our results confirmed that β-catenin knockdown altered astrocyte proliferation and migration, as determined by BrdU incorporation and wound healing assay. Not much is known about the consequences of β-catenin signaling activation in neuroinflammation. Therefore, we evaluated the effect of Wnt/β-catenin signaling on HIV-associated inflammation. We found that cytokine-mediated chemokine CXCL8 production was accompanied by GSK-3β phosphorylation. Particularly, β-catenin knockdown diminished NF-κB p65 expression, and IL-1β-induced CXCL8 levels in astrocytes, suggesting a mutual regulation between these two pathways during HIV-1-associated inflammation. In summary, our study shows HAND-relevant stimuli increased β-catenin expression, and activated reactive astrocyte responses including migration, proliferation, and inflammation. Therefore, further investigation into the role of β-catenin signaling is warranted as a possible therapeutic target for HAND.

2.2 Introduction

More than 30% of the human immunodeficiency virus-1 (HIV-1) positive patients develop disorders associated with the central nervous system (CNS) (1, 167). HIV-1 affects brain despite antiretroviral therapy (ART), and causes neurological impairments, commonly referred as HIV-associated neurocognitive disorders (HAND). Even in the post-ART era, low-level viral replication, HIV-1-associated CNS neuroinflammation and oxidative stress contribute to neuropathogenesis (168, 169), which emphasizes the significant need for identifying indirect HIV-1 host regulated mechanisms for HAND adjunctive therapy.

Astrocytes, the most abundant cells in the brain that maintain CNS homeostasis and blood-brain barrier (BBB) integrity (170, 171). Reactive astrogliosis is a process where astrocytes respond to CNS insults, including infection, trauma, ischemia and neurodegeneration. The severity of HAND is strongly associated with astrocyte dysfunction and immune activation. Reactive astrocytes secrete a wide variety of pro- and anti-inflammatory factors that can potentially mediate neuroprotective or neurotoxic effects depending upon the severity, type, context and duration of the insult (172). Inflammation mediates CNS damage and is a major contributor to HAND in HIV-infected individuals. Furthermore, HIV-1 infected microglia, together with activated astrocytes release various inflammatory and death factors. Several inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 are elevated in CNS or cerebrospinal fluid of HAND individuals (42, 173-175). Elevated proinflammatory cytokines such as TNF-α, and IL-1β, activate CNS resident glial cells to release excitatory amino acids and other inflammatory mediators, leading to neuronal

dysfunction and death (176). However, the precise molecular mechanisms of HIV-1-associated neuropathogenesis resulting from astrocyte activation have not yet been fully understood.

The canonical wingless type Wnt (Wnt/β-catenin) signaling pathway is an important intracellular signal transduction pathway that promotes cell survival, differentiation, proliferation and communication. It is implicated in several neurodegenerative diseases including Alzheimer's disease, Parkinson's and neuroAIDS (177-180). The extracellular Wnt ligands trigger three different pathways: the canonical Wnt/β-catenin, and non-canonical Wnt/planar cell polarity and Wnt/Ca^{2+} pathways. Wnt ligands are specifically upregulated in spinal cord dorsal horns of HIV+ patients with chronic pain, suggesting a critical role in pathogenesis of HIV-associated pain (181, 182). A hallmark of Wnt/β-catenin pathway is the stabilization of cytosolic β-catenin, which translocates to the nucleus and activates the transcription of Wnt target genes (183). Glycogen synthase kinase (GSK)-3β is highly active, that phosphorylates and degrades β-catenin through proteosomal degradation. GSK3 has constitutive kinase activity, which is often negatively regulated through posttranslational modifications. In contrast, inactivation of GSK-3β activity inhibits β-catenin degradation. GSK-3β, upstream regulator of β-catenin has been known to play major role in neuroinflammation in several neurodegenerative diseases (184-186).

Canonical β-catenin signaling regulates the activation of astrocytes and is dysregulated in astrocytoma (144). Yet, the role of β-catenin pathway in reactive astrocytes during HIV-1-associated neuropathogenesis has not been well known. In the

present study, we investigated the intrinsic mechanisms regulated by Wnt/β-catenin pathway in reactive astrocytes in response to injury and HIV-1-associated inflammation.

2.3 Materials and Methods

Isolation, cultivation and treatment of primary cultured human astrocytes

Human astrocytes were isolated from first- and early second-trimester aborted specimens, obtained from the Birth Defects Laboratory, University of Washington, Seattle, WA in full compliance with the ethical guidelines of the NIH, Universities of Washington and North Texas Health Science Center. The Birth Defects Laboratory obtained written consent from all tissue donors. Human astrocytes were isolated as previously described (187). Briefly, human foetus brain tissues were dissected and mechanically dissociated. Cell suspensions were centrifuged, suspended in media, and plated at a density of 20×10^6 cells/150 cm^2. The adherent astrocytes were treated with trypsin and cultured under similar conditions to enhance the purity of replicating astroglial cells. The astrocyte preparations were routinely >99% pure as measured by immunocytochemical staining for glial fibrillary acidic protein (GFAP). Astrocytes were treated with IL-1β (20 ng/ml, R&D Systems), and TNF-α (50 ng/ml, R&D Systems) for 8 or 24 h at 37^{o}C and 5% CO_2.

Transfection of astrocytes

Human astrocytes were transfected with no siRNA (mock), ON-TARGETplus® siRNA specific to β-catenin (siβ-catenin), scrambled non-targeting siRNA (siCon, Dharmacon), using the Amaxa™ P3 primary cell 96-well nucleofector kit (Lonza, Walkersville, MD, USA). Briefly, 1.6 million astrocytes were suspended in 20 μl nucleofector solution containing siCon or siβ-catenin (200 nM), and transfected using a Nucleofector/Shuttle

(Lonza) device. Transfected cells were supplemented with astrocyte media and incubated for 30 min at 37°C prior to plating. Cell were then cultured in 25 cm^2 flasks and allowed to recover for 48 h prior to experimental use.

***In vitro* scratch assay**

A well-established *in vitro* wound-healing model to study migration of astrocytes was used (188). Briefly, mock, siCon and siβ-catenin transfected human astrocytes were plated at a density of 2.0×10^6cells per 6-well tissue culture plate, and grown to confluence for 48 h. The medium was then aspirated and a thin stretch of the confluent monolayer was scraped to create a "scratch or injury" using sterile 10 µl pipette tip. Fresh astrocyte medium was added and the wound was allowed to heal. The culture plates were examined periodically at 8, 24, 36 and 48 h and then returned to resume incubation. Live images of migrating cells were obtained using a phase contrast microscope (Zeiss Invertoscope 40C) (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA).

Measures of cell viability and proliferation

Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. Absorbance was measured using a microplate reader (Molecular Devices, USA) at 490 nm.

Cell proliferation was measured by BrdU (5-bromo-2′-deoxyuridine) incorporation assay (BrdU Cell Proliferation Assay Kit, Cell Signaling Technology, USA) as per manufacturer's recommendations.

Quantification of proinflammatory mediators by ELISA

CXCL8 protein levels (ng/ml) were determined using culture supernatants by ELISA

(R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol.

RNA extraction and gene expression analyses

RNA was isolated as previously described (189) from astrocytes after 8 h treatment. RNA was reverse-transcribed into cDNA as per the manufacturer's instructions (Thermo Fisher) and gene expression was quantified by TaqMan® 5′ nuclease real-time RT-PCR in 50 μl reactions, using a StepOnePlus sequence detection system according to the manufacturer's protocol (Thermo Fisher). The 25 μl reactions were carried out at 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in 96-well optical, RT^2-PCR plates (Thermo Fisher). The TaqMan® gene expression assays for AEG-1, cyclinD1, β-catenin, LEF-1, and GAPDH (ThermoFisher) were used. Transcripts were quantified by the comparative ΔΔCT method, and represented as fold change of control.

Western blot analyses

Astrocytes were cultured as adherent monolayers in 25 cm^2 flasks at a density of 4 × 10^6 cells/flask and allowed to recover for 24 h. Following recovery, cells were treated for 24 h with varying concentrations of H_2O_2 and whole cell or cytoplasmic and nuclear protein extracts were isolated using mammalian extraction buffer (Thermo Fisher) or nuclear and cytoplasmic extraction kit (NE-PER, Thermo Fisher Scientific, Pittsburgh, PA). Cells were collected by scraping in sterile ice-cold PBS to avoid alteration of protein expression on surface of cell membranes. Cytoplasmic and nuclear protein extracts (15 μg) were boiled with 4X NuPAGE lithium dodecyl sulfate loading sample buffer at 100°C for 5-10 min, resolved by Bolt 4-12% bis tris gel and subsequently transferred to nitrocellulose membranes using i-Blot (Life Technologies). The

membranes were incubated with antibodies against β-catenin (Cell Signaling), GSK-3β(Cell signaling), p-GSK-3β (Cell signaling), or GAPDH (Santa Cruz Biotechnology) overnight at 4°C, washed and then incubated with anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000, Bio-Rad, Hercules, CA) for 2 h at room temperature. The membrane was then developed with SuperSignal west femto substrate (Thermo Fisher Scientific) and imaged in a Flourochem HD2 Imager (Proteinsimple, Santa Clara, CA).

Statistical analyses

Statistical analyses were performed using Prism 7.0 (GraphPad Software, La Jolla, CA), with one-way analysis of variance ANOVA followed by Tukey's post-test for multiple comparisons. Significance was set at $P < 0.05$ and data represents means +/- standard error of the mean. Cumulative data from two or more independent astrocyte donors in multiple replicates is shown as fold changes to control.

2.4 Results

HAND-associated inflammation upregulates critical mediators of classical Wnt signaling

IL-1β and TNF-α are the major proinflammatory cytokines that play an important role in the induction of neuronal injury and severity of HAND (42, 190). Wnt/β-catenin signaling was also identified as a potent proinflammatory regulatory signaling cascade in microglia and astrocytes (191, 192). Wnt can stimulate the transcriptional activation of numerous developmental genes through the activation of β-catenin/LEF-1 complexes in the nucleus. LEF-1 is a crucial transcription factor for Wnt signaling and elevated levels of β-catenin are required for the activation of the LEF-1 promoter (193). However, in

many developmental model systems, LEF-1 expression is induced in a similar fashion to Wnt target genes (194), suggesting that Wnt may regulate LEF-1 gene expression at the transcriptional level. Thus, to evaluate the effect of HIV-1-associated inflammation on Wnt signaling, we assessed IL-1β- and TNF-α-mediated β-catenin and LEF-1 expression in human astrocytes. Primary human astrocytes were treated with IL-1β (20 ng/ml) and TNF-α (50 ng/ml). Following 8 h treatment, the cells were harvested and β-catenin, LEF-1 mRNA levels were evaluated. TNF-α and IL-1β significantly elevated β-catenin mRNA expression in astrocytes (*$p<0.05$, ***$p<0.001$, **Fig 2.1A**). There was also a significant (~2, 2.5 fold) increase in LEF-1 mRNA levels in astrocytes exposed to TNF-α or IL-1β compared to controls (***$p<0.001$, **Fig 2.1B**). Thus, these data show that HAND-relevant inflammatory stimuli induce LEF-1, and β-catenin expression, suggesting regulation of Wnt signaling in human astrocytes.

β-catenin regulates reactive astrocyte migration during wound healing.

To investigate the role of Wnt/β-catenin signaling during HIV-1-mediated neurotoxicity, we transiently silenced β-catenin expression in astrocytes. siRNA targeting β-catenin (siβ-catenin) significantly reduced β-catenin mRNA and protein expression in astrocytes as compared to non-targeting siRNA (siCon) and mock (***$p<0.001$, **Fig 2.2A, B**). Wnt pathway regulates cell proliferation, mobility and differentiation. We investigated the role of β-catenin signaling in astrocytic proliferative and migration responses following injury. For this purpose, mock, siCon and siβ-catenin transfected astrocytes were plated, and after adherence, a scratch wound was made to induce astrocytes migration to the injured site (**Fig 2.2C1-E4**). Wound sites were observed at defined time points to measure morphological changes and migration of astrocytes. At 24 h, many mock and

siCon cells, adjacent to the wound area migrated to the site of the injury (**Fig 2.2C2, D2**). On the other hand, very few cells migrated with β-catenin knockdown (**Fig 2.2E2**). Migration of astrocytes to the wound site from both sides of the injury was detected at 24 h (**Fig 2.2C3, D3**). β-catenin knockdown reduced the migration of astrocytes to the injury site at 24 and 32 h compared to mock and siCon transfected astrocytes (**Fig 2.2E2, E3**). Scratched monolayer was completely closed after 48 h in mock and siCon injured astrocytes, but little gaps were observed in the wound sites of siβ-catenin astrocytes (arrows, **Fig 2.2C4, D4, E4**). Therefore, knockdown of β-catenin altered astrocytes migration capacity and delayed wound healing.

β-catenin regulates astrocyte proliferation

Wnt signaling leads to the activation of T-cell factor/lymphoid enhancer factor (TCF/LEF1) transcription factors and subsequent target genes expression. Several genes are known to be key players in tumorigenesis, such as c-myc, cyclin D1 or survivin. CyclinD1 is an important regulator of the checkpoints allowing cell cycle progression and is one of the critical transcriptional target genes of β-catenin/LEF-1 pathway (195). To study regulation of the Wnt/β-catenin pathway on reactive astrogliosis, particularly proliferation *in vitro*, we measured cyclin D1 mRNA levels and bromodeoxyuridine (BrdU) incorporation in primary cultured human astrocytes following β-catenin knockdown. When β-catenin was transiently knocked down in astrocytes, a significant reduction in cyclin D1 mRNA levels was observed (**p<0.01, **Fig 2.3A**). To confirm β-catenin mediated astrocyte proliferation, BrdU incorporation assay was conducted in parallel. BrdU incorporation was significantly reduced in cells expressing low β-catenin compared to cells expressing normal β-catenin levels (***p<0.001, **Fig**

2.3B). Together, our results showed Wnt/β-catenin pathway regulates astrogliosis *i.e*., migration and proliferation during injury.

Canonical Wnt/β-catenin and NF-κB pathway crosstalk in response to HAND-associated inflammation

Wnt signaling involves inhibition of GSK-3β kinase activity, permitting β-catenin accumulation and subsequent gene expression of Wnt target genes. Since, GSK-3β activity is inhibited by phosphorylation at serine 9, we investigated GSK-3β phosphorylation following IL-1β stimulation. Astrocytes were treated with 20 ng/ml of IL-1β for different time points up to 45 min. IL-1β treatment significantly increased GSK-3β phosphorylation at 15 min through 45 min ($p<0.05$, $p<0.01$, $p<0.001$, **Fig 2.4A**). Data indicate that IL-1β increased the inhibitory phosphorylation of GSK-3β at serine 9, which promotes Wnt signaling by β-catenin stabilization mechanism in astrocytes.

Next, we addressed the regulation of Wnt/β-catenin signaling on NF-κB pathway to better understand the purpose of IL-1β-induced GSK-3β inactivation in astrocytes. Western blot analysis indicated that NF-κB protein was significantly reduced following β-catenin downregulation ($p<0.05$, **Fig 2.4B**). Proinflammatory cytokines such as IL-1β and TNF-α activate astrocytes and enhance CXCL8 production during HIV-1 CNS infection. To investigate the effect of Wnt/β-catenin pathway on inflammation, β-catenin knocked down astrocytes were treated with IL-1β for the induction of inflammatory cytokines expression. IL-1β treatment induced chemokine CXCL8 levels in mock, siCon, siβ-catenin transfected astrocytes ($p<0.001$, **Fig 2.4C**). Though IL-1β treatment in siβ-catenin transfected astrocytes significantly elevated CXCL8 levels, a significant

decrease was observed compared to respective control ($p<0.001$, **Fig 2.4C**). Taken together, these results demonstrate that IL-1β induced GSK-3β phosphorylation, and β-catenin positively regulated NF-κB activity, as well as the expression of proinflammatory chemokines during HIV-1-associated inflammation.

2.5 Discussion

During several neurodegenerative conditions, such as HIV-1-associated neurological impairments, astrocytes are among the first to respond, and exert both beneficial & detrimental effects depending on the severity of insult. Reactive astrogliosis is a hallmark of HIV encephalitis (HIVE) and inflammation. The present work demonstrates the significance of Wnt/β-catenin pathway during reactive astrogliosis in response to injury and HIV-1-mediated inflammation.

Many neurodegenerative diseases, including HAND, are associated with increased inflammation, oxidative stress, glutamate excitotoxicity and endoplasmic reticulum stress. Astrocytes respond to all CNS insults and become “reactive”. It is well established that reactive astrocytes play a key role in the possible neurological complications resulting from HIV-1 infection of the brain (100, 196-198). Reactive astrocytes exhibit migratory phenotype similar to gliomas by extracellular matrix degradation (199). Physiological changes in astrogliomas such as astrocyte behavior and hyperproliferative disorders also resemble reactive astrogliosis (199). The molecular pathways associated with reactive astrocytes and how they contribute to HAND pathogenesis are still unclear.

Proinflammatory cytokines such as IL-1β and TNF-α contribute to the pathogenesis of neurological complications of HIV-1 (200). IL-1β increases glial fibrillary

acidic protein (GFAP) expression and reactive astrogliosis in the cerebral cortex and hippocampus through IL-6 release into the cerebrospinal fluid (CSF) (201). These cytokines have been shown to mediate reactive astrogliosis in neurodegenerative diseases; however, the molecular mechanism remains unclear (201-203). Recent studies showed the critical role of Wnt/β-catenin signaling in the activation of astrocytes, and its dysregulation is implicated in the pathogenesis of astrocytoma (144). Wnt signaling activation includes the β-catenin-TCF-LEF-1 transcriptional complexes. Some studies suggest that Wnt signaling might also regulate LEF-1 gene expression at the transcriptional level (194). β-catenin, a crucial molecule in Wnt signaling is so far understood to be regulated mainly by protein stability. Conversely, other studies identified transcriptional upregulation of β-catenin at the invasion front of colorectal liver metastases (204). In addition, analysis of the β-catenin promoter revealed a number of high-affinity transcription factor binding sites, including activator protein (AP)-1, nuclear factor (NF)-κB and T-cell factor (TCF)-4 sites (205). The NF-κB pathway has been activated primarily in response to IL-1β. High affinity binding of NF-κB to β–catenin promoter is noteworthy, as several findings reported the crossregulation of Wnt and the NF-κB signaling pathways (205). Besides, our data also showed transcriptional activation of β-catenin and LEF-1 in response to these cytokines, suggesting regulation of Wnt/β-catenin signaling during HIV-1-associated inflammation.

At early stages of CNS insults, astrocytes migrate and form barriers at the injured site to restrict the spread of infectious agents or inflammatory cells (140, 206). The Wnt pathway is a well-known signaling cascade that is involved in migration, and proliferation of several cancers. Initially, we investigated if HAND-induced canonical Wnt

signaling is contributing towards reactive astrocyte phenotype *i.e.,* proliferation and migration. Following injury, we identified knockdown of β-catenin altered migration of astrocytes to the wound site. The dynamics of Wnt/β-catenin signaling and its regulation of cell cycle mediators including; cyclin D1, and c-myc expression is well recognized (195, 207). We also demonstrated β-catenin regulation of cyclin D1 expression in astrocytes, which is essential for proliferation. Taken together, these findings indicate the significance of β-catenin in the proliferative and migration capacity of reactive astrocytes during neurodegenerative diseases.

GSK3, a serine/threonine kinase, is a mediator of major signaling pathways including Wnt, NF-κB, phosphatidyl-inositol-3-kinase (PI3K), Notch and Hedgehog signaling. GSK-3β contributes to neuroinflammation in several neurodegenerative diseases (184). HIV-1-associated pro-inflammatory molecules IL-1β and interferon (IFN)-γ initiated GSK-3β phosphorylation in intestinal mucosal inflammation (208, 209). IL-1β is known to activate phosphoinositide 3-kinase (PI3K)/Akt/GSK-3β pathway in several cell types, such as hepatocytes, epithelial cells, airway and colonic smooth muscle cells (208, 210). Inhibitory serine-phosphorylation is the most frequent mechanism that regulates the activity of GSK3. Activated Akt, the product of PI3K pathway inactivates GSK3 through phosphorylation. Wnt pathway activation also inhibits GSK-3β-dependent phosphorylation of β-catenin. Alternatively, there is evidence from our *in vitro* studies that IL-1β triggered β-catenin, LEF-1 expression and GSK-3β phosphorylation, suggesting activation of PI3K or canonical Wnt signaling during HIV-associated inflammation in astrocytes.

Wnt/β-catenin signaling modulate inflammatory responses *via* interaction with NF-κB in murine hepatocytes (211). In human bronchial epithelial cells, depletion of β-catenin lowered lipopolysaccharide (LPS)-induced NF-κB activation and proinflammatory cytokines, including CXCL6, CXCL8, IL-1β, TNF-α and CCL2 expression (212). Moreover, NF-κB promoter regulation by β-catenin in colorectal cancer cells was recognized (213). Here, β-catenin knockdown reduced NF-κB protein levels in astrocytes. NF-κB was identified as a novel transcriptional co-factor for β-catenin/TCF, important regulators of Wnt signaling (214). In mouse chondrocytes, IL-1β-mediated NF-κB activation induced the expression of transcription factor LEF-1, signifying an indirect mechanism for activation of Wnt/β-catenin signaling pathway (215). Therefore, crosstalk or mutual regulation between these two pathways can aggravate HIV-1-induced inflammation. Our study identified β-catenin signaling regulates inflammation in astrocytes that involves direct transcriptional control of NF-κB. CXCL8 colocalizes within reactive astrocytes, but is absent in the normal CNS (216). CXCL8 is primarily released in brain microenvironment by microglia and astrocytes, was found to be elevated in CSF of HIV-infected individuals and promoted neuroinflammation (217-220). The CXCL8 promoter contains a unique consensus TCF/LEF binding site as a direct target of Wnt/β-catenin signaling (221). Simultaneously, our data showed β-catenin knockdown reduced IL-1β mediated CXCL8 expression. Thus, our findings suggest an important role for Wnt/β-catenin pathway in CXCL8 levels regulation in astrocytes.

In summary (**Fig 2.5**), the present study documented Wnt/β-catenin pathway regulation in astrocytes during HIV-1-mediated inflammation. We showed that β-catenin signaling altered reactive astrocyte migration and proliferation during wound healing. Further, β-catenin knockdown downregulated CXCL8 production and NF-κB protein levels. Thus, modulation of β-catenin levels may be a unique mode of regulating NF-κB activity and thus may present novel therapeutic opportunities. Taken together, these findings underscore the complex role of Wnt/β-catenin and NF-κB pathways in the pathophysiology of inflammation and provide innovative therapeutic strategies in HAND.

Acknowledgements:

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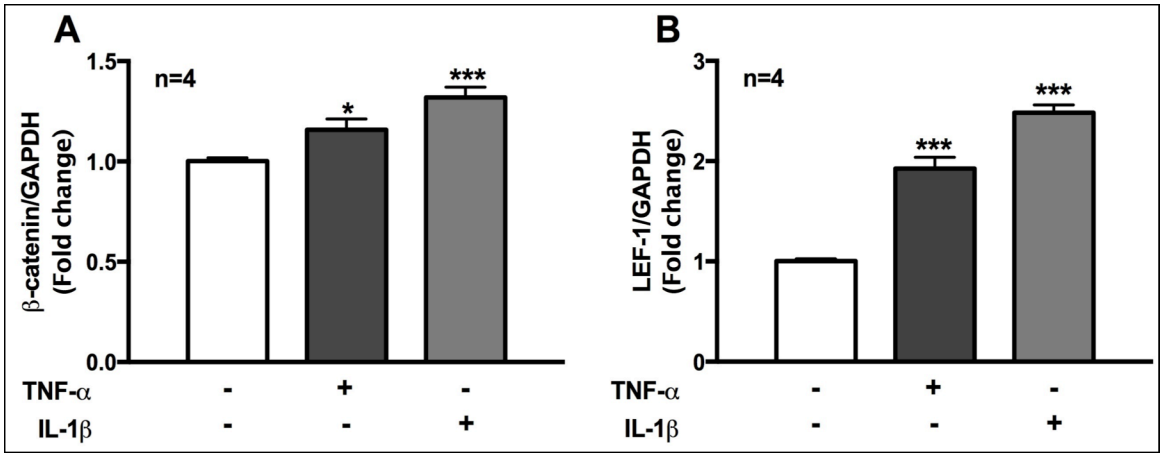

Figure 2.1. HAND-relevant inflammatory stimuli upregulate β-catenin and LEF-1 expression in astrocytes.

Astrocytes were treated with HAND-relevant stimuli, tumor necrosis factor (TNF)-α (50 ng/ml), and interleukin (IL)-1β (20 ng/ml). Total RNA was isolated 8 h post-treatment and **(A)** β-catenin and **(B)** lymphoid enhancing factor (LEF-1) mRNA levels were determined by RT^2-PCR. Data displays mRNA fold change compared to untreated controls. GAPDH was used as normalizing control. Cumulative data represents mean ± SEM from four primary human astrocyte donors. Statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (*$p<0.05$, ***$p<0.001$)

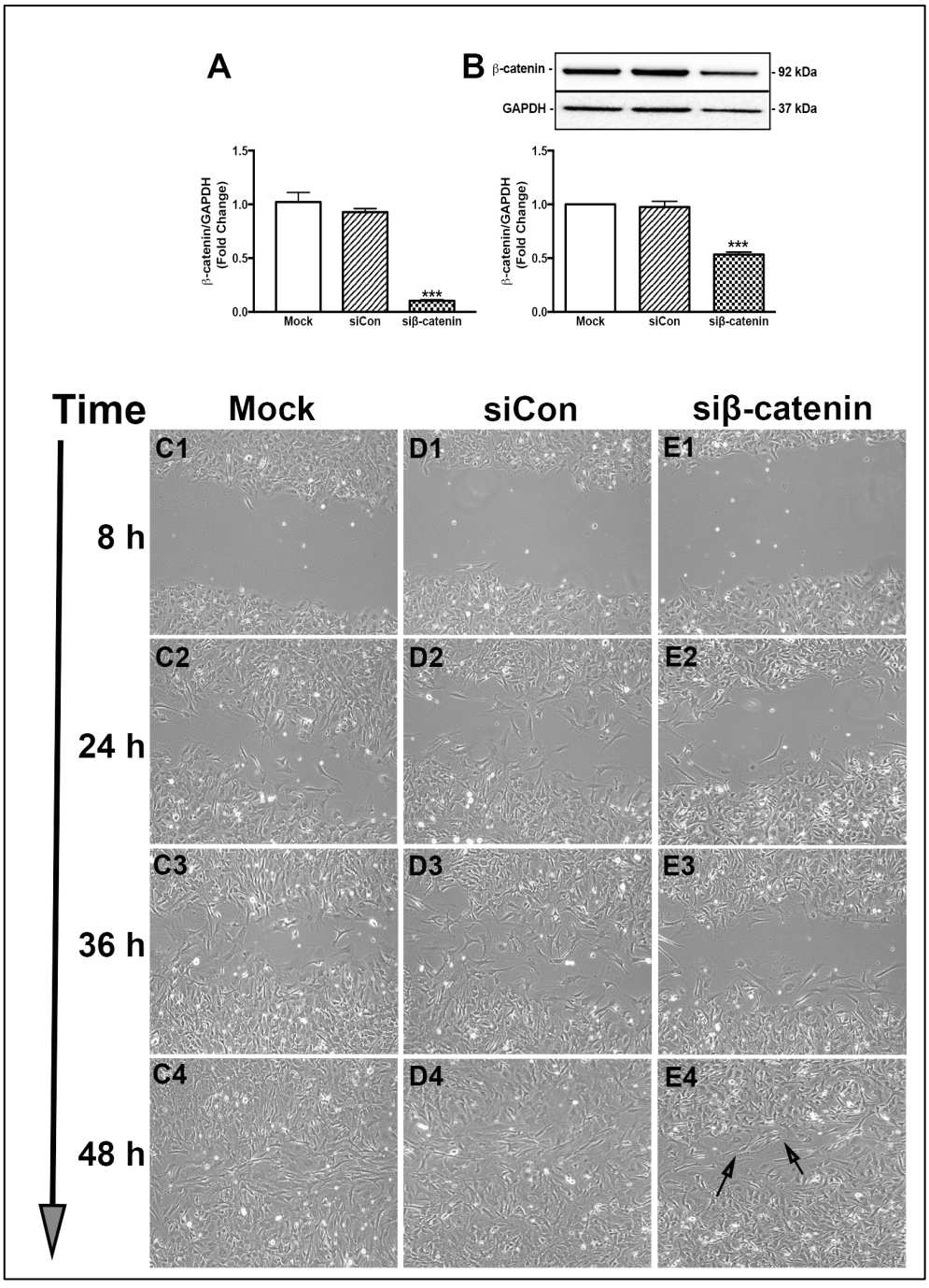
A
B
β-catenin
GAPDH
92 kDa
37 kDa
β-catenin/GAPDH (Fold Change)
1.5
1.0
0.5
0.0
***
Mock
siCon
siβ-catenin
Time
Mock
siCon
siβ-catenin
8 h
24 h
36 h
48 h
C1
D1
E1
C2
D2
E2
C3
D3
E3
C4
D4
E4

Figure 2.2. β-catenin regulates astrogliosis during wound healing.

Astrocytes were transfected with non-targeting siRNA (siCon) or siRNA specific for β-catenin (siβ-catenin) and recovered for 48 h. Mock transfected astrocytes were maintained in parallel. **(A)** β-catenin mRNA levels were determined by RT^2-PCR. GAPDH was used as normalizing control. Graph represents fold change compared to mock. **(B)** Western blot and densitometry analysis of β-catenin in mock, siCon, and siβ-catenin transfected astrocytes is shown. GAPDH was used as loading control. Cumulative data represents mean ± SEM from three primary human astrocyte donors. Statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (***$p<0.001$). In parallel, confluent astrocyte monolayer was scratched and migration of **(C1-C4)** mock, **(D1-D4)** siCon, and **(E1-E4)** siβ-catenin astrocytes to the wound site were monitored for 48 h. Phase contrast images captured at 8, 24, 36 and 48 h are shown. Data is representative of three individual donors.

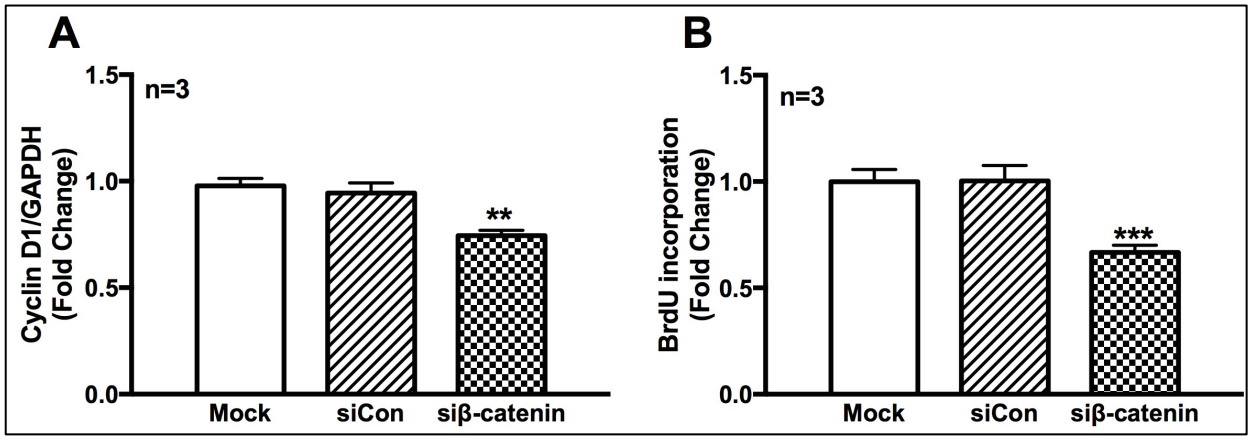

Figure 2.3. β-catenin knockdown reduces astrocyte proliferation.

Human astrocytes were transfected without siRNA (mock) or with non-targeting, scrambled siRNA (siCon) or β-catenin specific siRNA (siβ-catenin) by nucleofection and plated for 48 h. RNA was isolated from transfected astrocytes and cyclin D1 levels were determined by RT^2-PCR. **(A)** Data represents fold change of cyclin D1 gene expression compared to mock. In parallel, 48 h post-transfection, astrocytes were incubated with 10 μM Bromodeoxyuridine (BrdU) for 24 h. Proliferation was measured by BrdU incorporation assay and graph represents relative absorbance at 450 nm fold change compared to mock. **(B)** Cumulative data mean ± SEM from independent experiments of three individual donors (n=3) is shown. Statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (**p<0.01, ***p<0.001).

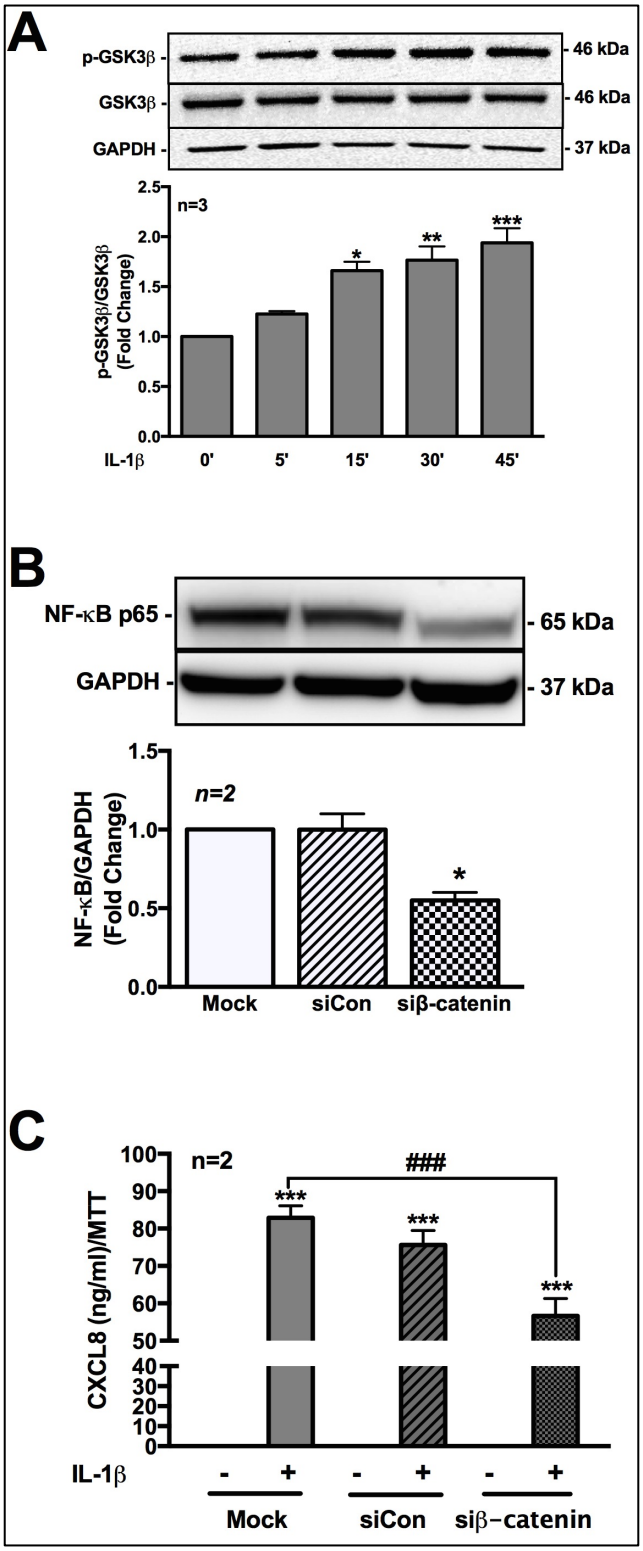
A
p-GSK3β
GSK3β
GAPDH
46 kDa
46 kDa
37 kDa
n=3
p-GSK3β/GSK3β (Fold Change)
IL-1β
0'
5'
15'
30'
45'
B
NF-κB p65
GAPDH
65 kDa
37 kDa
n=2
NF-κB/GAPDH (Fold Change)
Mock
siCon
siβ-catenin
C
n=2
###
CXCL8 (ng/ml)/MTT
IL-1β
Mock
siCon
siβ-catenin

Figure 2.4. IL-1β induces GSK-3β phosphorylation and CXCL8 production *via* NF-κB/β-catenin regulated matter.

Primary human astrocytes were treated with IL-1β (20 ng/ml) for 0, 5, 15, 30 and 45 min. Cell lysates were collected and probed for p-GSK-3β, GSK-3β and GAPDH. **(A)** Densitometry analysis of normalized p-GSK-3β/GSK-3β is presented. GAPDH was used as positive loading control. Cumulative data from three donors is shown (*p<0.05, **p<0.01, ***p<0.001). Whole cell lysates were collected from mock, siCon or siβ-catenin transfected astrocytes at 48 hours post-recovery. **(B)** Lysates were immunoblotted for NF-κB p65 subunit. GAPDH was used as a normalizing control. Cumulative data of two individual donors is presented (*p<0.05). Astrocytes were treated with IL-1β for 24 h. **(C)** Cell supernatants were collected and CXCL8 levels were measured by ELISA. Cumulative data from two independent astrocyte donors each analyzed in multiple replicates is shown as fold changes to respective controls. Statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (***p<0.001, ### p<0.001).

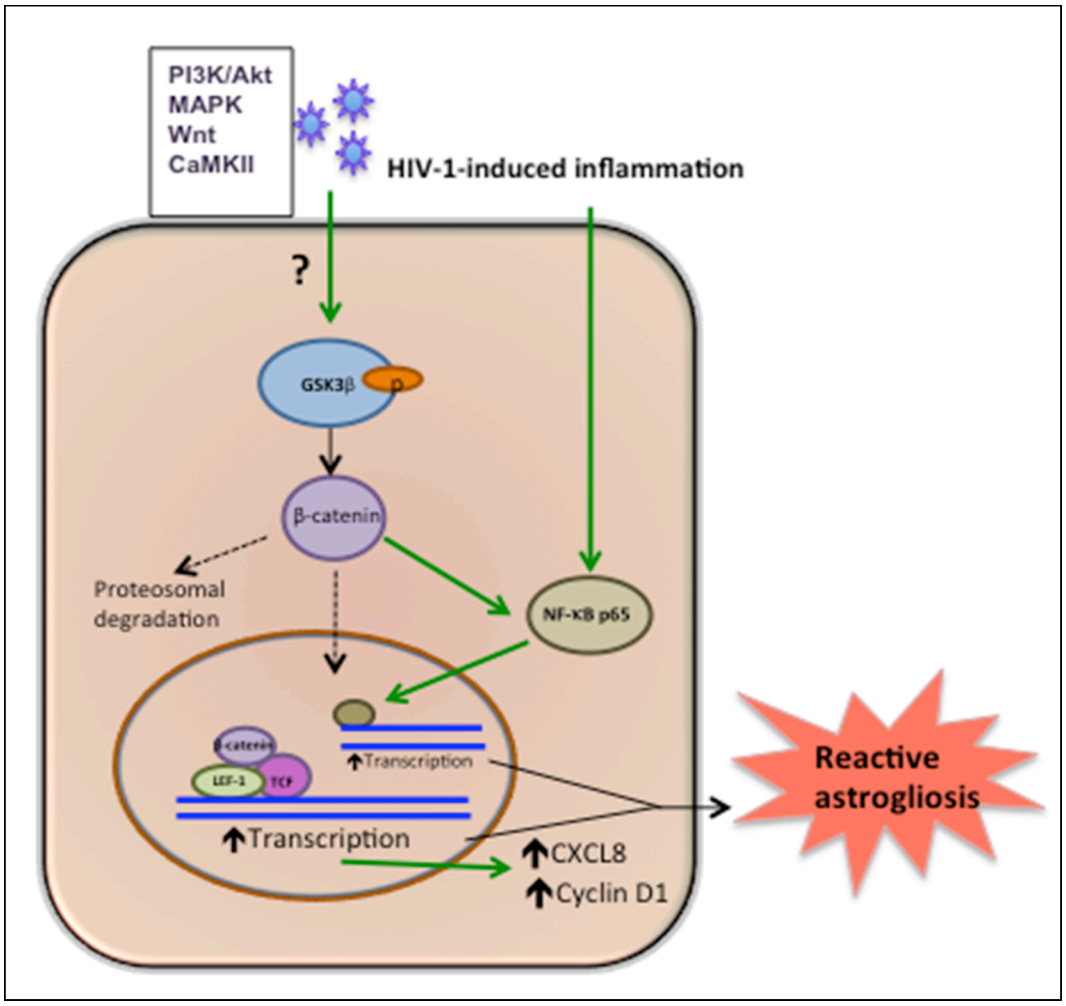

Fig. 2.5. Role of Wnt/β-catenin signaling in reactive astrocytes during HIV-1-mediated neuroinflammation. HIV-1 infection induces molecular mediators of reactive astrogliosis such as pro-inflammatory cytokines, and increases GSK-3β phosphorylation and stabilizes cytosolic β-catenin. The activity of GSK-3β is decreased by phosphorylation, which can occur through several different pathways (PI3K/Akt, MAPK, CaMKII). Upregulated/stabilized β-catenin in response to HIV-1-relevant stimuli regulates cyclin D1, and CXCL8 levels during reactive astrogliosis. On the other side, coordination between NF-κB and Wnt/β-catenin signaling aggravates inflammation during HIV-1 CNS infection.

CHAPTER 3

HIV-1-associated inflammation and antiretroviral therapy regulate astrocyte endoplasmic reticulum stress responses

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(Adapted from Cell Death Discovery (in press))

3.1 Abstract

Antiretroviral therapy (ART) has effectively suppressed the incidence of human immunodeficiency virus (HIV)-associated dementia (HAD) in HIV-1 positive individuals. However, the prevalence of more subtle forms of neurocognitive dysfunction continues to escalate. Recently, endoplasmic reticulum (ER) stress has been linked to many neurological diseases; yet, its role in HIV/neuroAIDS remains largely unexplored. Furthermore, upregulation of astrocyte elevated gene (AEG)-1; a novel HIV-1 inducible gene, along with ER stress markers in Huntington's disease model suggests a possible role in HIV-associated ER stress. The current study is focused on unfolded protein responses (UPR) and AEG-1 regulation in primary human astrocytes exposed to HIV-associated neurocognitive disorders (HAND)-relevant stimuli (HIV-1 virions, inflammation and antiretroviral (ARV) drugs). Interleukin (IL)-1β and the nucleoside reverse transcriptase inhibitor abacavir upregulated expression of ER stress markers in human astrocytes, including binding immunoglobulin protein (BiP), C/EBP homologous protein (CHOP), and calnexin. In addition, IL-1β activated all three well-known UPR pathways: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor (ATF)6, and inositol-requiring enzyme (IRE)1α. AEG-1 upregulation correlated to ER stress and demonstrated astrocyte AEG-1 interaction with the calcium-binding chaperone, calnexin. IL-1β and abacavir enhanced intracellular calcium signaling in astrocytes in the absence of extracellular calcium, illustrating ER-associated calcium release. Alternatively, calcium evoked in response to HAND-relevant stimuli directed to mitochondrial permeability transition pore (mPTP) opening astrocytes. Importantly, IL-1β- and abacavir induced-UPR and mPTP opening were inhibited by the intracellular calcium

chelation, indicating the critical role of calcium signaling in HAND-relevant ER stress in astrocytes. In summary, our study highlights that ARV drugs and IL-1β induced UPR, AEG-1 expression, intracellular calcium, and mitochondrial depolarization in astrocytes. This study uncovers astrocyte ER stress as a novel therapeutic target in the management of HIV-1-associated neurotoxicity and possibly in the treatment of neuroAIDS.

3.2 Introduction

Human immunodeficiency virus (HIV)-1 invades the central nervous system (CNS) during early stages of infection and often leads to neurological complications known as HIV-associated neurocognitive disorders [HAND] (222). While the success of antiretroviral therapy (ART) has dramatically reduced the incidence of the most severe neurological manifestation *i.e.*, HIV-associated dementia (HAD), the prevalence of cognitive dysfunctions in HIV-1 positive patients despite ART remains high (223). In addition to persistent low-grade viral replication and inflammation within the CNS, toxicity of antiretroviral (ARV) drugs also likely contributes to neurological dysfunction in HIV-1-patients (175, 224, 225). Consistent with this notion, withdrawal of ARV drugs in HIV-1 patients resulted into substantial improvement of neurocognitive functioning (226, 227). Thus, ARV drugs clearly have neurotoxic effects that may contribute to HAND, necessitating the identification of underlying mechanisms and development of improved strategies for HAND treatment in the growing HIV-1 patient population.

Endoplasmic reticulum (ER) stress has been implicated in several neurological diseases, including ischemia, brain trauma, Alzheimer's, and Parkinson's disease (228, 229). The ER regulates key cellular functions including protein synthesis and folding, calcium storage and lipid biosynthesis. Physiological and pathological

stimuli, such as serum-starved conditions, high protein demand, viral infections, and inflammatory cytokines; can disrupt ER homeostasis resulting in an accumulation of misfolded proteins (230). To mitigate ER stress, cells activate the unfolded protein response (UPR), which is regulated by three major pathways, each with its own initiator: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor (ATF)-6, or inositol-requiring enzyme 1 (IRE1)-α. The UPR promotes cell survival by attenuating protein translation and by inducing chaperone expression, ER associated degradation (ERAD) of proteins, endogenous antioxidant responses and autophagy. However, prolonged UPR activation can lead to orchestrated cell death *i.e*., apoptosis (231).

Recent studies showed amyloid beta protein accumulation in brains of HIV-1 infected individuals, indicating the potential involvement of protein misfolding and UPR activation in HIV-1 pathogenesis (232, 233). While UPR markers, including binding immunoglobulin protein (BiP) and ATF6 were significantly elevated in the CNS of HIV-1 positive individuals (73), the regulation of ER stress response in HIV-1-induced neuronal damage is not well understood.

Recently, the multi-functional oncogene, astrocyte elevated gene (AEG)-1, was shown to dysregulate glutamate clearance by excitatory amino acid transporter 2 downregulation and HIV-associated neuroinflammation *via* upregulation of NF-κB pathway in astrocytes (121, 128). AEG-1 was also elevated in a Huntington's disease model along with UPR markers such as BiP, C/EBP homologous protein (CHOP) and regulator of ribosome synthesis (RRS)1 (234). These studies suggest the involvement of AEG-1 in ER stress responses during neurological diseases, including HAND.

During HIV-1 infection, several astrocyte-associated mechanisms lead to neurotoxicity, including excitotoxicity, inflammation and oxidative stress (235). These mechanisms have also been linked to ER stress in neurodegeneration (236, 237). In addition, recent studies showed HIV-1 transactivator of transcription (Tat) and glycoprotein (gp)120 protein expression in astrocytes induced ER stress-mediated cytotoxicity, possibly contributing to HIV-associated neuropathogenesis (75, 76, {Fan, 2016 #17973). Nucleolus, a stress sensor, responds to various forms of cellular stress and normal ribosomal DNA transcription, and ribosome assembly is often dysregulated (238, 239). The association between nucleolar dysfunction and neurodegenerative diseases is increasingly being explored (240). Furthermore, AEG-1 nucleolar localization in response to injury and oxidative stress suggests a plausible role of AEG-1 in DNA/RNA processing during HIV-1-associated nucleolar stress. Taken together, astrocytes represent a significant therapeutic target for reestablishing CNS homeostasis. In the present study, we investigated the role of HAND-relevant stimuli [inflammation (IL-1β) and ARV drugs (*i.e.*, NRTI; abacavir)] in the induction of ER stress in astrocytes. We demonstrated that HAND-relevant stimuli increased astrocyte cytosolic calcium, which in turn, triggered ER stress and mitochondrial depolarization. Further, we investigated specific mechanisms through which these events are interlinked thus contributing to neurodegeneration. These findings are significant as prolonged activation of UPR due to severe/chronic ER stress likely plays a critical role in HIV-associated neurodegeneration.

3.3 Materials and Methods

Isolation, cultivation and treatment of primary human astrocytes: Human astrocytes were isolated from first and early second trimester aborted specimens as previously described (187). Tissues were obtained in full compliance with ethical guidelines of the University of Washington, the University of North Texas Health Science Center and the National Institutes of Health. Briefly, brain tissues were dissected and mechanically dissociated. Cell suspensions were centrifuged, washed, suspended in media, and plated at a density of $20x10^6$ cells/150 cm^2. Adherent astrocytes underwent several passages to enhance the purity of replicating astroglial cells prior to experimental use. The astrocyte preparations were routinely >99% pure as measured by immunocytochemical staining for GFAP and microglial marker CD68 to determine possible microglial contamination and contribution of microglia in inflammatory responses. Astrocytes were treated with IL-1β (20 ng/ml, R&D Systems), HIV-1_{DJV} (p24 20 ng/ml), TNF-α (50 ng/ml, R&D Systems), ER stress compounds *i.e*., thapsigargin (0.2 nM, Cell Signaling), tunicamycin (1 µg/ml, Cell Signaling, Baltimore, MD), brefeldin A (0.5 µg/ml, Cell Signaling); ARV drugs *i.e*., abacavir (4 µM, ChemPacific), saquinavir (22.5 µM, ChemPacific), delavirdine (22.5 µM, ChemPacific), lopinavir (0.015 µM, ChemPacific), darunavir (5 nM, ChemPacific), lamivudine (11 µM, ChemPacific), stavudine (2.25 µM, ChemPacific), nevirapine (0.1 µM, ChemPacific), ritonavir (0.1 µM, ChemPacific); and the calcium chelator BAPTA-AM (25 µM, Sigma-Aldrich) at 37°C and 5% CO_2. HIV-1_{DJV} was originally isolated from monocyte cultivation

from HIV-1 demented brain tissue of a patient and was subsequently expanded in culture in peripheral blood monocytes (241).

RNA extraction and gene expression analyses

Astrocyte RNA was isolated 8 h post-treatment as described previously (189). RNA was reverse transcribed into cDNA as per the manufacturer's instructions and gene expression was assayed by RT^2-PCR. Taqman 5' nuclease real-time PCR was performed using StepOnePlus detection system (ThermoFisher Scientific). Commercially available TaqMan gene expression assays were used to measure PERK (cat no. HS00984006_m1), IRE1α (cat no. Hs00176385_m1), ATF6 (cat no. Hs00232586_m1), BiP (Hs00607129_gH), CHOP (cat no. Hs00358796}, calnexin (cat no. Hs01558409_m1), AEG-1 (cat no. Hs00757841_m1) and GAPDH (cat no. 4310884E, ThermoFisher) mRNA. GAPDH, a ubiquitously expressed housekeeping gene, was used as an internal normalizing control. The 25 µl reactions were carried out at 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min in 96-well optical, real-time PCR plates. Transcripts were quantified by the comparative ΔΔCT method, and represented as fold-change to control.

Western blot analyses

Astrocytes were cultured as adherent monolayers in 75 cm^2 flasks at a density of $8x10^6$ cells/flask and allowed to recover for 24 h. Following recovery, cells were treated for various time points and whole cell protein extracts were isolated using mammalian protein extraction buffer (ThermoFisher). Cells were collected by scraping in sterile ice-cold PBS to avoid alteration of protein expression on surface of cell membranes. Whole cell protein extracts (25 µg) were boiled with 4X NuPAGE loading sample buffer at

100^0C for 5 min, resolved by Bolt 4-12% bis tris gel and subsequently transferred to nitrocellulose membranes using i-Blot (ThermoFisher). The membranes were incubated with antibodies against ATF4 (1:1000, Cell Signaling), ATF6 (1:1000, Cell Signaling), XBP-1s (1:1000, Cell Signaling), p-eIF2α and eIF2α (1:1000, Cell Signaling) overnight at 4°C, washed and then incubated with anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000, Bio-Rad, Hercules, CA) or anti-mouse IgG conjugated to horseradish peroxidase (1:10,000, Bio-Rad) for 2 h at room temperature. The membranes were then developed with SuperSignal west femto substrate (ThermoFisher) and imaged using Fluorochem HD2 imager (ProteinSimple). GAPDH (1:2000, Santa Cruz Biotechnology) was used as loading controls.

Immunocytochemistry

Astrocytes were cultured as adherent monolayers at a density of $0.1x10^6$ cells per well. Cultured cells were fixed after 24 h treatment with ice-cold acetone:methanol (1:1) solution for 20 min at 20°C and blocked with blocking buffer (2% BSA in 1X PBS containing 0.1% Triton X-100) for 1 h at room temperature. Cells were then incubated with primary antibodies specific to calnexin (1:500, Cell Signaling), GFAP (1:400, Covance Inc.,) in blocking buffer overnight at 4°C, washed and incubated with AlexaFluor® secondary antibodies, anti-rabbit (488 nm) and anti-chicken (594 nm) (1:100, ThermoFisher). Micrographs were obtained on an ECLIPSE Ti-4 using the NLS-Elements BR. 3.0 software (Nikon).

Transfection of astrocytes

Cultured human astrocytes were transfected with pGP-CMV-GCaMP6s, ultrasensitive protein calcium sensors, a gift from Douglas Kim (Addgene plasmid # 40753)(242)

using the Amaxa^{TM} P3 primary cell 96-well kit, nucleofector and shuttle attachment (Lonza, Walkersville, MD, USA) according to modified manufacturer's instructions. Briefly, 1.6 million astrocytes were suspended in 20 µl nucleofector solution containing GCaMP6s (0.5 µg) and were electroporated using shuttle protocol CL133. Transfected cells were supplemented with astrocyte media and incubated for 30 min at 37°C prior to plating into 6 channel µ-slides (0.4 VI, ibidi, Madison, WI) at 1x10^5 per channel. Cells were allowed to recover for 48 h prior to confocal imaging. Minimum of three were imaged for green fluorescence from each biological astrocyte donor per treatment condition.

Confocal Analyses

For co-localization studies, human non-transfected or transfected astrocytes were cultured on glass bottom 48-well tissue culture plates (MatTek Corp., Ashand, MA, USA) at 1x10^5 cells per well in astrocyte media for 48 h. Cells were then treated for 24h and carefully fixed prior to staining with antibodies specific to AEG-1 (1:200, Life Technologies) and calnexin as described in ICC. Prior to live cell calcium imaging, astrocytes in µ-slides were briefly washed with PBS and supplemented with phenol red-, calcium- and magnesium-free Hank's buffered saline solution at 37°C. Time-lapse micrographs were acquired every 500 milliseconds for six minutes from astrocytes treated with IL-1β (20 ng/ml), abacavir (4 µM), and ionomycin (10 µM) at 10 secs. Micrographs were obtained on Carl Zeiss LSM (Jena, Germany). The objective used was 20x Plan-Apochromat, 0.8NA, 0.55 mm WD. Photomultiplier tube detection was used with an excitation of 450-490 nm and emission of 593-668 nm. Colocalization, live cell video imaging and histogram analyses were performed using FujiFilm, ImageJ

software; Version: 2.0.0-rc-41/1.5d (Fuji ImageJ software). Change in fluorescence was calculated by the following equation: $\Delta F = (F-F_0)/(F_{max}-F_0)$, where F is the fluorescence intensity at any given time, F_0 is the baseline fluorescence intensity and Fmax is the maximum fluorescence intensity when exposed to ionomycin (10 μM).

mPTP

Astrocytes were cultured in 96 well plates at $0.05x10^6$ cells/well. After 24 h treatment, the calcein / cobalt chloride quenching technique was used to elucidate mPTP opening using image-iT LIVE mitochondrial transition pore assay kit (Thermo Fisher). Live cells were imaged with appropriate excitation and emission filters for fluorescein on ECLIPSE Ti-4 using the NLS-Elements BR. 3.0 software (Nikon). In closed mPTP condition, cobalt quenches calcein fluorescence not sequestered in mitochondria; therefore, colocalization of MTR reflected as green/yellow for mitochondria.

3.4 Results

HAND-relevant stimuli initiate ER stress in primary human astrocytes

To mimic HAND-related disease enviornment, astrocytes were treated with IL-1β and HIV-1. As a common mediator of neuroinflammation, IL-1β is primarily released by HIV-1 infected and immune-activated microglia in the CNS (42). Astrocytes are highly responsive to IL-1β and possess an autocrine loop to perpetuate activation (243). Here, primary human astrocytes were incubated with IL-1β (20 ng/ml) and HIV-1 (p24 20 ng/ml) alone or in combination for 8 h and ER stress markers mRNA levels were analyzed by real-time PCR (RT^2-PCR) (**Fig. 3.1a-c**). IL-1β alone and in combination with HIV-1 significantly upregulated three UPR proximal initiators PERK (***p<0.001, **Fig. 1a**), IRE1α (***p<0.001, **Fig. 3.1b**), and ATF6 mRNA levels (***p<0.001, **Fig. 3.1c**).

On the other hand, HIV-1 alone did not alter expression of these markers. In parallel, downstream ER stress markers BiP, CHOP and calnexin were evaluated (**Fig. 3.1d-f**). In astrocytes, IL-1β alone and in combination with HIV-1 significantly increased expression of BiP (***p<0.001, **Fig. 3.1d**), CHOP (*p<0.05, **p<0.01, **Fig. 3.1e**) and calnexin mRNA (**p<0.01, ***p<0.001, **Fig. 3.1f**). However, HIV-1, by itself, did not alter mRNA levels of tested markers as compared to controls. IL-1β was thus identified as a key ER stress inducer in human astrocytes during HAND-associated inflammation.

Nucleoside reverse transcriptase inhibitors (NRTIs) trigger ER stress and AEG-1 expression in astrocytes

Despite the benefits attained with ARV regimen, the 'successful failure' of ART is that it effectively inhibits viral replication while concurrently triggering adverse side effects and toxicities (244, 245). However, ART-associated toxicity in the CNS, particularly in glial cells, remains largely unexplored. To investigate ARV drugs-mediated ER stress, astrocytes were treated with a wide range of ARV drugs including, nucleoside reverse transcriptase inhibitors (NRTI; abacavir, lamivudine and stavudine), non-nucleoside reverse transcriptase inhibitors (NNRTI; nevirapine and delavirdine), and protease inhibitors (PI; darunavir, lopinavir, ritonavir and saquinavir) at therapeutic doses (**Fig. 3.2**). These ARV drugs were being used in the clinic and have CNS penetration effectiveness (CPE) scores ranging from 1-4, with higher scores *i.e.*, 4 reflecting greater likelihood of CNS penetration (246). All concentrations used in the treatments reflect the physiological level of the drugs (247). ER stress markers mRNA levels were evaluated 8 h post-treatment by RT^2-PCR. Levels of PERK (***p<0.001, **Fig. 3.2a**), ATF6 (***p<0.001, **Fig. 3.2b**) and BiP mRNA (**p<0.01, **Fig. 3.2c**) were significantly increased

with abacavir or lamivudine treatments. The NNRTI delavirdine increased mRNA levels by ~1.5 fold, which was not statistically significant for PERK, ATF6, and BiP (**Fig. 3.2a, b, and c**). NRTIs (abacavir and lamivudine), NNRTIs (nevirapine and delavirdine), and PIs (lopinavir and ritonavir) significantly elevated AEG-1 mRNA as compared to respective controls (*p<0.05, **p<0.01, and ***p<0.001, **Fig. 3.2d**). However, stavudine, darunavir and saquinavir did not increase AEG-1 expression. Since AEG-1 mRNA levels were elevated by ARV drugs with greater CNS penetration [*i.e.,* abacavir (3), nevirapine (4), delavirdine (3), and lopinavir (3)], exploring the effects of ART on AEG-1-regulated neurological outcomes *in vivo* is therapeutically relevant. Taken together, of all the ARV drugs tested, only abacavir and lamivudine triggered both UPR markers and AEG-1 expression. Therefore, considering its high CPE and current clinical use, abacavir was used to further study how ARV drugs regulate astrocyte ER stress.

HAND-relevant stimuli activate UPR and promote selective protein translation

Three distinct UPR pathways, *i.e*., PERK, IRE1α and ATF6, can activate transcription of ER stress response element (ERSE) regulated genes, including BiP and CHOP. Therefore, we next focused on identifying which specific UPR mechanism(s) regulate ER stress in astrocytes during inflammation and abacavir exposure. Western blot analysis confirmed increased eIF2α phosphorylation following IL-1β or abacavir treatment (*p<0.05, **Fig. 3.3a**), suggesting that general attenuation of protein translation in astrocytes occurred *via* PERK activation. PERK mediated eIF2α phosphorylation supports translation of ATF4, an ERSE transcription factor, normally suppressed by an inhibitory open reading frame. When active eIF2α is abundant, selective translation of ER stress mitigating genes is promoted (248). Our studies confirmed IL-1β significantly

increased ATF4 levels in astrocytes (***p<0.001, **Fig. 3.3b**). Next, to evaluate IRE1α and ATF6 mediated UPR activation, whole cell lysates were immunoblotted for the spliced form of XBP-1 (XBP-1s) and cleaved ATF6 (**Fig. 3.3c, d**), the respective UPR-associated ERSE transcription factors. IL-1β significantly increased XBP-1s (***p<0.001, **Fig. 3.3c**) and ATF6 (***p<0.001, **Fig. 3.3d**) as compared to controls. Together, these results indicate that HAND-relevant stimuli induce transcriptional regulation during ER stress in astrocytes *via* each of the three UPR pathways: PERK, IRE1α and ATF6.

IL-1β and abacavir transiently induce intracellular calcium in GCaMP6s transfected astrocytes

To investigate IL-1β- and abacavir-induced intracellular calcium ($[Ca^{+2}]_i$) signaling, primary human astrocytes were first transfected with GCaMP6s plasmid, a genetically encoded calcium sensor (242), with an approximate 80% transfection efficiency. Intracellular calcium was visualized by live-cell, fluorescent, confocal imaging of GCaMP6s-transfected astrocytes. Baseline fluorescence was observed initially at 0 sec (**Fig. 3.4a, d**), which robustly increased at 20 sec after IL-1β and abacavir treatment (**Fig. 3.4b, e**). Over time, fluorescence decreased returning to baseline by ~100 sec (**Fig. 3.4c, f**). Changes in fluorescence *i.e.,* calcium transients ($\Delta F= (F-F_0)/(F_{max}-F_0)$, where F is the fluorescence intensity at any given time, F_{max} is the maximum fluorescence intensity and F_0 is the baseline fluorescence intensity) with IL-1β, abacavir, and thapsigargin treatment were plotted from each astrocyte over time (**Fig. 3.4g**). The dynamic changes in calcium transients were quantified using area under the curve (AUC) to assess total change in calcium levels in a given astrocyte and to compare responses across treatments. Interleukin-1β, abacavir and thapsigargin each elicited

significantly higher average $[Ca^{+2}]_i$ than control (***p<0.001, **Fig. 3.4h**, n=40 individual cells/treatment). Thus, these data validate that IL-1β and abacavir triggered transient intracellular calcium increase in astrocytes, comparable to that induced by thapsigargin, a known ER stress inducer.

HAND-relevant stimuli increase ER quality control mechanism in astrocytes

During ER stress, UPR activation increases chaperones such as BiP, calnexin and calreticulin to promote proper protein folding and quality control in the ER (249). HAND-relevant stimuli increased calnexin levels in astrocytes (**Fig. 3.5**). Astrocytes were treated with HIV-1 (**Fig. 3.5b**), abacavir (**Fig. 3.5c**), IL-1β (**Fig. 3.5d**), HIV-1+IL-1β (**Fig. 3.5e**), and abacavir+IL-1β (**Fig. 3.5f**) for 24 h. Cells were fixed and immunostained for GFAP (red), calnexin (green), and DAPI (blue). Untreated astrocytes retained bright glial fibrillary acidic protein (GFAP) with lower perinuclear calnexin staining (**Fig 3.5a**). HAND-relevant stimuli (**Fig. 3.5b-f**) increased calnexin expression in astrocytes.

AEG-1 colocalizes and interacts with calnexin in the context of ER stress

As a pleotropic protein, AEG-1 can localize to the cell membrane, cytoplasm, endoplasmic reticulum, nucleus and nucleolus, and exhibits diverse location-related functions (250). AEG-1 colocalized with calnexin in the ER, which was increased with IL-1β+TNF-α, and thapsigargin treatment (**Fig. 3.5g-i**) when assessed by confocal microscopy. Originally, AEG-1 was identified as an HIV-1 and TNF-α inducible gene in astrocytes. To study how ER stress influences AEG-1, astrocytes were treated with ER stress inducing agents brefeldin A (Bf), tunicamycin (Tm), and thapsigargin (Th) followed by RT^2-PCR analysis (**Fig. 3.5j**). These ER stressors significantly upregulated AEG-1 mRNA levels as compared to controls (**p<0.01, ***p<0.001, **Fig. 3.5j**). Both

tunicamycin and thapsigargin upregulated AEG-1 by 2.5 fold (**Fig. 3.5j**), a feature that is rather uncommon for AEG-1, which primarily exhibits its function *via* subcellular localization and redistribution (250). Moreover, AEG-1 transcripts correlated significantly to BiP (R^2: 0.49, ***p<0.001, **Fig. 3.5k**) and PERK (R^2: 0.92; ***p<0.001, **Fig. 3.5k**) in the ARV drug-exposed astrocytes (shown earlier in Fig. 2). AEG-1 is also known to exhibit diverse functions by interacting with several cellular proteins in cancer (251). Since AEG-1 can function as a transcriptional co-activator and as a scaffolding protein, we examined if AEG-1 directly binds to calnexin (**Fig. 3.5l**). Consistent with the confocal analyses, co-immunoprecipitation data confirmed AEG-1 interacted with calnexin in untreated, IL-1β, abacavir or thapsigargin treated astrocytes (**Fig. 3.5l**). These results establish that AEG-1 expression is elevated during HAND-associated ER stress. Furthermore, a direct interaction with the calcium-binding chaperone calnexin indicates that AEG-1 likely regulates ER quality control and possibly calcium signaling in astrocytes.

HAND-relevant stimuli lead to mitochondrial permeability transition pore (mPTP) opening in astrocytes

Crosstalk between inflammation, oxidative and ER stress neurodegenerative mechanisms increases the severity of many diseases (237). A persistent increase in $[Ca^{2+}]_i$ in response to pathophysiological stimuli can lead to mPTP opening, which compromises mitochondrial function by eliminating membrane electrochemical potential and increasing reactive oxygen species (ROS) (252). Therefore, mPTP opening in response to HAND-relevant stimuli, oxidative stress, and ARV drugs was monitored using a fluorescent live mitochondrial transition pore assay (**Fig. 3.6**). Astrocytes were

stained with the nuclear indicator hoechst (blue), the mitochondrial dye mitotracker red (MTR) and calcein-AM, a membrane permeable fluorophore that diffuses freely into mitochondria and is quenched by cobalt chloride in the cytoplasm. Using live-cell fluorescent imaging, calcein and MTR labeling were measured in astrocytes (**Fig. 3.6a**) treated with H_2O_2 (200 μM, **Fig. 3.6b**), IL-1β (20 ng/ml, **Fig. 3.6c**), HIV-1 (p24 20 ng/ml, **Fig. 3.6d**), TNF-α (50 ng/ml, **Fig. 3.6e**), IL-1β+HIV-1+TNF-α together (**Fig. 3.6f**), abacavir (4 μM, **Fig. 3.6g**), thapsigargin (100 nM, **Fig. 3.6h**) and DMSO (vehicle for thapsigargin, **Fig 3.6i**). Untreated and vehicle-treated astrocytes exhibited calcein (green) and MTR (red) colocalization in mitochondria (yellow, **Fig. 3.6a, i** arrows), reflecting closed mPTP. Calcein fluorescence was decreased in astrocytes treated with H_2O_2 and other HAND-relevant stimuli (**Fig. 3.6b-h**), indicating mitochondrial pore opening (**Fig. 3.6**, arrow head). Together, these data show that HAND-relevant stimuli deleteriously affected astrocytes by promoting mPTP opening and loss of mitochondrial membrane integrity.

Intracellular calcium chelation inhibits abacavir and IL-1β-mediated ER stress and subsequent mitochondrial damage in human astrocytes

To examine the effects of IL-1β- and abacavir-induced calcium release on ER stress and mPTP opening, astrocytes were pre-treated with the calcium chelator, 1,2-Bis (2-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraaceticacid (acetoxymethyl ester) (BAPTA-AM). Consistent with Figs. 1 and 2, BiP was significantly increased with IL-1β and abacavir alone (**p<0.01, ***p<0.001, **Fig. 3.7a**). In parallel, BAPTA-AM pre-treatment successfully decreased IL-1β- and abacavir-induced BiP expression ($^{###}$p<0.001, **Fig. 3.7a**). To identify the consequences of elevated $[Ca^{2+}]_i$ on mitochondrial membrane

integrity, live-cell fluorescent imaging for calcein-AM (green) and MTR (red) was performed. Loss of calcein (green) and increase in MTR (red) fluorescence was observed in HIV-1, IL-1β and abacavir treated astrocytes (**Fig. 3.7d, f, h)**. However, BAPTA-AM pre-treatment preserved mitochondrial integrity as indicated by calcein fluorescence (green) in untreated and HAND-relevant stimuli treated astrocytes (**Fig. 3.7b, c, e, g and i**). These results demonstrate that IL-1β- and abacavir-induced ER stress and mPTP opening are highly calcium-dependent.

HAND-relevant inflammation and AEG-1 overexpression induce nucleolar stress

Nucleolar stress is an emerging component of neurodegenerative process, commonly caused by impaired rRNA transcription, impaired nucleloar activity and altered nucleolar integrity (240, 253-255). To evaluate the role of nucleolar stress in HIV-1-associated neurodegeneration, primary human astrocytes were incubated with IL-1β (20 ng/ml) or HIV-1 (p24; 20 ng/ml) alone or in combination for 8 h. Nucleolar marker; RRS1 mRNA levels were analyzed by real-time PCR (RT^2-PCR, **Fig 3.8a**). IL-1β alone and in combination with HIV-1 significantly upregulated RRS1 mRNA levels (***p<0.001, **Fig 3.8a**). Previous studies from our lab showed astrocyte AEG-1 expression was increased in response to HAND-relevant stimuli. Thus, we next investigated effects of AEG-1 upregulation on nucleolar stress. We utilized AEG-1-GFP overexpression plasmid to increase AEG-1 levels in astrocytes. AEG-1 overexpression alone increased RRS1 levels compared with respective control. However, further treatment with IL-1β or HIV-1 did not significantly changed RRS1 expression (*p<0.05, **p<0.001, **Fig 3.8b**). Taken together, these results showed elevated nucleolar stress during HIV-associated inflammation.

AEG-1 modulate oxidative stress-induced nucleolar stress

Oxidative stress plays an important role in the development of HAND. The cross talk between oxidative stress and nucleolar dysfunction is not well understood. To evaluate oxidative stress-mediated nucleolar stress, astrocytes were treated with different concentrations of H_2O_2. Human astrocytes treated with increasing concentrations of H_2O_2 significantly induced RRS1 mRNA and protein levels in a dose-dependent manner (*p<0.05, **p<0.01, ***p<0.001, **Fig 3.9 a, b**). AEG-1 translocated to nucleolus in response to injury and oxidative stress in astrocytes. AEG-1 nucleolar localization in response to oxidative stress suggests a plausible role of AEG-1 in DNA/RNA processing during oxidative stress. Thus, we evaluated AEG-1-mediated modulation of RRS1 expression that is crucial for pre-ribosomal RNA transcription, processing and ribosome biogenesis in response to H_2O_2 treatment. Astrocytes overexpressing AEG-1 demonstrated an induction in RRS1 mRNA expression, which was further significantly exacerbated upon H_2O_2 treatment (**p<0.001, ***p<0.001, **Fig 3.9C**). Taken together, these data demonstrate the AEG-1 might regulate ribosomal RNA synthesis and processing in response to oxidative stress.

3.5 Discussion

Neurological impairments continue to be a major health concern for HIV-infected patients in the post-ART era (222). Nevertheless, the indirect causes and targetable mechanisms of neuronal degeneration in HAND are not well understood. ER stress is prevalent in several neurodegenerative diseases, though its regulation and contributions to neuropathogenesis have not been well studied in HAND (228, 229). Aiming for a better understanding of HIV-1-induced UPR activation, the present study is focused on

CNS neuroglial cells, *i.e*. astrocytes, with various HAND-relevant stimuli (HIV-1, ARV drugs and inflammation). The present study demonstrates that HAND-relevant stimuli induce ER stress and activate all three UPR pathways: PERK, IRE1α and ATF6 in human astrocytes. Further, astrocyte intracellular calcium is elevated by IL-1β and abacavir *via* ER calcium release, which when prolonged triggers ER stress responses and mitochondrial depolarization. We also document elevated AEG-1 expression that positively correlates with ART-mediated ER stress. AEG-1 colocalization and interaction with calnexin implicate AEG-1 as a scaffolding protein regulating calcium signaling and ER function. We recognize elevated RRS1 expression or impaired nucleolar activity or altered rRNA genes transcription in response to HAND-relevant inflammation and oxidative stress. AEG-1 overexpression further mediated HIV-1 associated nucleolar stress (**Fig 3.10**).

Ongoing low-level CNS viral replication and HIV-1-induced inflammation contribute to HAND (175, 224, 225). Inflammatory cytokines play an important role in pathogenesis of HIV-1-associated neurocognitive impairments. Several cytokines including IL-1β and TNF-α are dysregulated in encephalitic brains of HIV-1-infected patients (42, 256, 257). Many of these elements can initiate ER stress response in astrocytes (230). However, the molecular mechanisms that underlie HAND-relevant stimuli-mediated ER stress in astrocytes remain unknown.

Our findings demonstrate that HAND-associated inflammation induce ER stress in astrocytes. ER stress may be both a trigger and consequence of inflammation in many chronic inflammatory diseases (258). According to recent studies, ER stress-induced UPR signaling is associated with the production of many proinflammatory

molecules (259). All three UPR pathways transcriptionally regulate the expression of inflammatory molecules through nuclear factor (NF)-κB and activator protein (AP)-1 (258, 260, 261). Our study identifies IL-1β as a strong initiator of ER stress and the UPR in astrocytes, highlighting crosstalk between ER stress and inflammation during HIV-1 CNS infection.

Recently, the CNS penetrance of ARV drugs has become increasingly clinically significant as the battle to reduce HIV-1-mediated neurotoxicity (262). However, long-term ARV therapy is associated with a range of adverse effects including neuropathy, hyperbilirubinemia, lipodystrophy, neuropsychiatric disorders, retinal lesions and hypersensitivity (263). ARV drugs triggered adverse side effects and toxicities including, but not limited to: alterations in lipid and protein metabolism, insulin resistance, mitochondrial toxicity, oxidative stress and neuronal damage (244, 245). An *in vitro* study evaluated the neurotoxic effects of several ARV drugs on rat forebrain neuronal cultures. Out of the various ARV classes tested, NRTIs, including abacavir, showed high neurotoxicity (33). Therefore, another possible contributor to HAND is ARV drug related neurotoxicity.

Our results demonstrate increased mRNA levels of ER chaperones, BiP and CHOP, with HAND-relevant stimuli, indicating ER stress in primary human astrocytes. While the UPR elevates BiP and CHOP expression, each play opposing roles in the outcome of the ER stress response. As a pro-survival protein, BiP removes ER malfolded proteins; whereas CHOP promotes apoptosis by favoring mitochondrial depolarization, cytochrome c release and caspase 3 activation (264). Thus, the cell fate depends on the critical balance between BiP and CHOP (265). Initially, HAND-relevant

stimuli significantly upregulated both BiP and CHOP. Whereas, at later time points, BiP expression was decreased (data not shown), indicating that the disrupted balance between these two proteins may lead to cell death or apoptosis. Furthermore, cortical autopsy tissues from HIV-1-infected patients showed upregulated ER stress markers BiP and ATF6 in various CNS cell types, including neurons and astrocytes (73, 266). Therefore, further investigation of ER stress-associated neurotoxic mechanisms of ARV drugs alone or in combination is warranted.

ER stress can induce at least three independent UPR signaling pathways: PERK, IRE1α, and ATF6. Elevation of all proximal sensors and down-stream transcriptional factors suggests that all three UPR pathways regulate HAND-associated ER stress in astrocytes. In addition, IL-1β and abacavir increased eIF2α phosphorylation, indicating general attenuation of protein translation; a strategy to decrease the load of newly synthesized proteins in the ER lumen. However, UPR activation of ERSE binding transcription factors including ATF4, XBP-1s and ATF6 upregulate compensatory expression of ER stress regulatory proteins, including BiP and CHOP.

One study identified astrocyte elevated genes (AEG) expression including, AEG-1, and AEG-9 (calnexin) following HIV-1 infection or treatment with gp120 to investigate potential gene expression changes in HIV-1 infected astrocytes (110). Recently, we identified AEG-1 as a novel modulator of HIV-1-associated neuroinflammation and glutamate clearance in astrocytes, suggesting a significant role in HAND (121, 128). Therefore, we investigated plausible AEG-1 contributions towards HIV-1/ART-induced ER stress in astrocytes. The present study identifies AEG-1 as an

ER stress inducible gene in astrocytes. To our knowledge, this is the first report to recognize AEG-1 as an interacting partner of calnexin, and reinforces AEG-1 as a scaffolding protein regulating ER calcium signaling through formation of multi-protein complexes.

ER harbors an intracellular calcium pool, which regulates essential cellular functions *via* calcium signaling. The present study demonstrates that HAND-relevant stimuli increase intracellular calcium in astrocytes. In addition, other studies have shown that HIV-1 proteins disrupt neuronal calcium homeostasis during HIV-1-encephalitis (267, 268). Many ER calcium binding proteins including calreticulin, calnexin, BiP, GRP94 have large calcium binding capacities and regulate ER calcium homeostasis. Increased calnexin expression supports the theory that HAND-relevant stimuli produce fluctuations in ER calcium in astrocytes. Severe disruption of ER Ca^{2+} homeostasis also triggers ER stress and UPR signaling pathways to upregulate ER stress markers (269). Our study also shows upregulated expression of calcium binding chaperones such as BiP. However, in addition to its chaperone function, BiP also plays an important role in the intraluminal storage of calcium (270).

The ER serves as the principal calcium store in the cell and dynamic calcium transfer through ER-mitochondria contact sites leads to various cellular coordinated responses. Calcium-induced mPTP opening and permeabilization of mitochondrial membrane is a key event in early apoptosis (271). Prolonged mPTP opening plays a crucial role in the pathogenesis of several diseases (272). Intracellular calcium chelation significantly reduced mPTP opening; suggesting that HAND-relevant stimuli-induced calcium influx into mitochondrial matrix causing mitochondrial

dysfunction. Further, increased intracellular calcium can also induce calcium dependent exocytosis, leading to excessive glutamate release from astrocytes (273). Other studies also support this conclusion since buffering of intracellular calcium reduced glutamate release in astrocytes (274). Excessive synaptic glutamate cause excitatory neurotransmission resulting into neuronal damage.

Interestingly, nucleolus senses ER stress and is a central hub to alter protein synthesis machinery *i.e*., ribosome biogenesis. Nucleolar malfunction contributes to the pathology of several neurological diseases. rDNA silencing and ribosomal deficits occurred in early stage of AD pathology (254). Disruption of nucleolar integrity was observed in dopaminergic neurons of PD patients post-mortem brain samples (253). Several studies showed alteration of rRNA genes transcription in HD cases (234, 275, 276). However, HIV-1 induced nucleolar stress is not well known. In addition to inflammation, oxidative stress plays crucial role in the development of HAND. Increased oxidative stress, levels of lipid peroxidation product *i.e*., HNE and oxidized proteins has been documented in HIV-1 demented brain tissues (277, 278). Oxidative stress, which occurs during HIV-1 CNS infection, is also implicated in astroglial and microglia cell death (279). We demonstrated significant elevated expression of RRS1 (regulator of ribosome synthesis); a protein inhibiting transcription of both rRNA and ribosomal protein genes during HIV-1 induced inflammation and oxidative stress in astrocytes. AEG-1 is a pleiotropic protein that translocates across the nuclear membrane and localizes in different cellular compartments such as cytoplasm, endoplasmic reticulum nucleus and nucleolus. AEG-1 nucleolar localization in response to injury, and oxidative stress suggests a plausible role of AEG-1 in DNA/RNA

processing during reactive astrogliosis. Previous studies showed co-localization of AEG-1 with fibrillarin, a DFC marker in reactive astrocytes. This subnucleolar component is crucial site for rDNA transcription, and maturation of pre-RNA transcripts. Thus, we next investigated AEG-1 role in rRNA processing and AEG-1 overexpression enhanced inflammation- and oxidative stress- induced RRS1 expression, *i.e*., nucleolar stress in astrocytes.

Taken together, HIV-1-associated inflammation and ART lead to ER stress and activation of UPR signaling in astrocytes. Our findings in the present study for the first time demonstrate that IL-1β and the NRTI abacavir, induce intracellular calcium dysregulation, ER stress, AEG-1 expression and mitochondrial dysfunction (mPTP opening) in astrocytes (**Fig. 3.10**). We also showed induction of RRS1, which inhibits rRNA transcription, in response to inflammation and oxidative stress that is further elevated by AEG-1 over-expression. In addition to deciphering these outcomes as independent correlates of HAND relevant astrocyte activation, our studies also delineated the temporal order of these events and their concerted roles in astrocyte ER stress as summarized in **Fig. 3.10**. We propose that in the post-ART era, such chronic UPR activation and associated ER stress in astrocytes likely plays a key role in mediating neurotoxicity in HIV-1-infected patients. In particular, these findings uncover astrocyte ER stress as a unique opportunity to explore novel therapeutic targets during HIV-1-associated neuropathogenesis.

Acknowledgements

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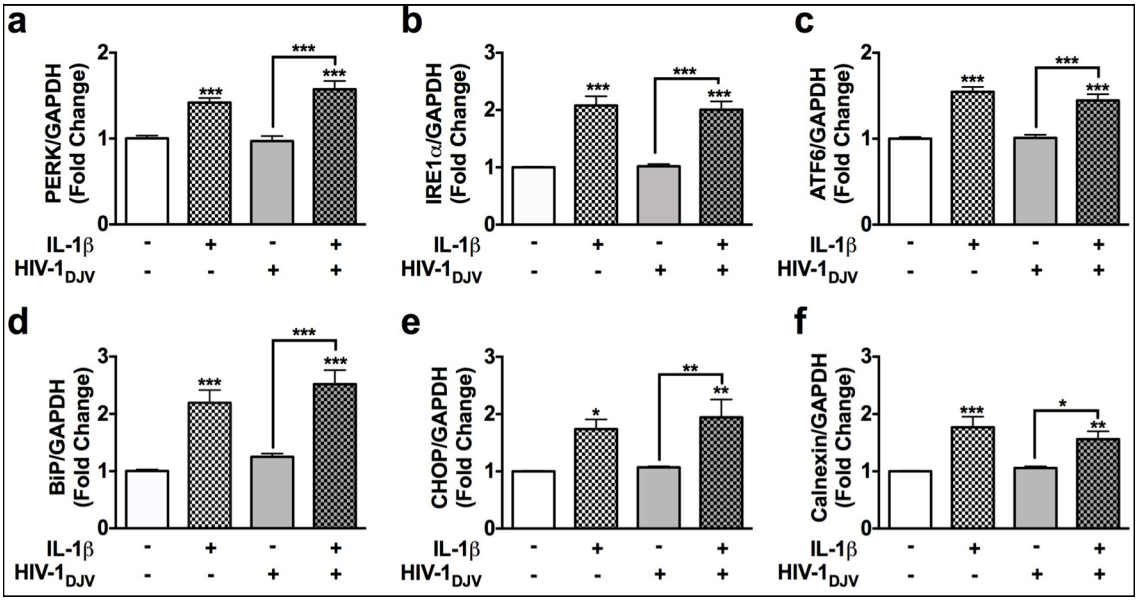


Figure 3.1. IL-1β with or without HIV-1 induces ER stress in astrocytes.

Primary human astrocytes were treated with IL-1β (20 ng/ml) and HIV-1 (at 20 ng/ml p24) alone or in combination and untreated astrocytes were maintained in parallel. After 8 h, total RNA was isolated and PERK **(a)**, IRE1α **(b)**, ATF6 **(c),** BiP **(d)**, CHOP **(e)**, and calnexin **(f)** mRNA levels were analyzed by RT^2-PCR and normalized to GAPDH levels. Data represents mean fold change ± SEM of cumulative data from three independent donors each tested in a minimum of triplicate determinations. Statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (*p< 0.05, **p<0.01, ***p<0.001).

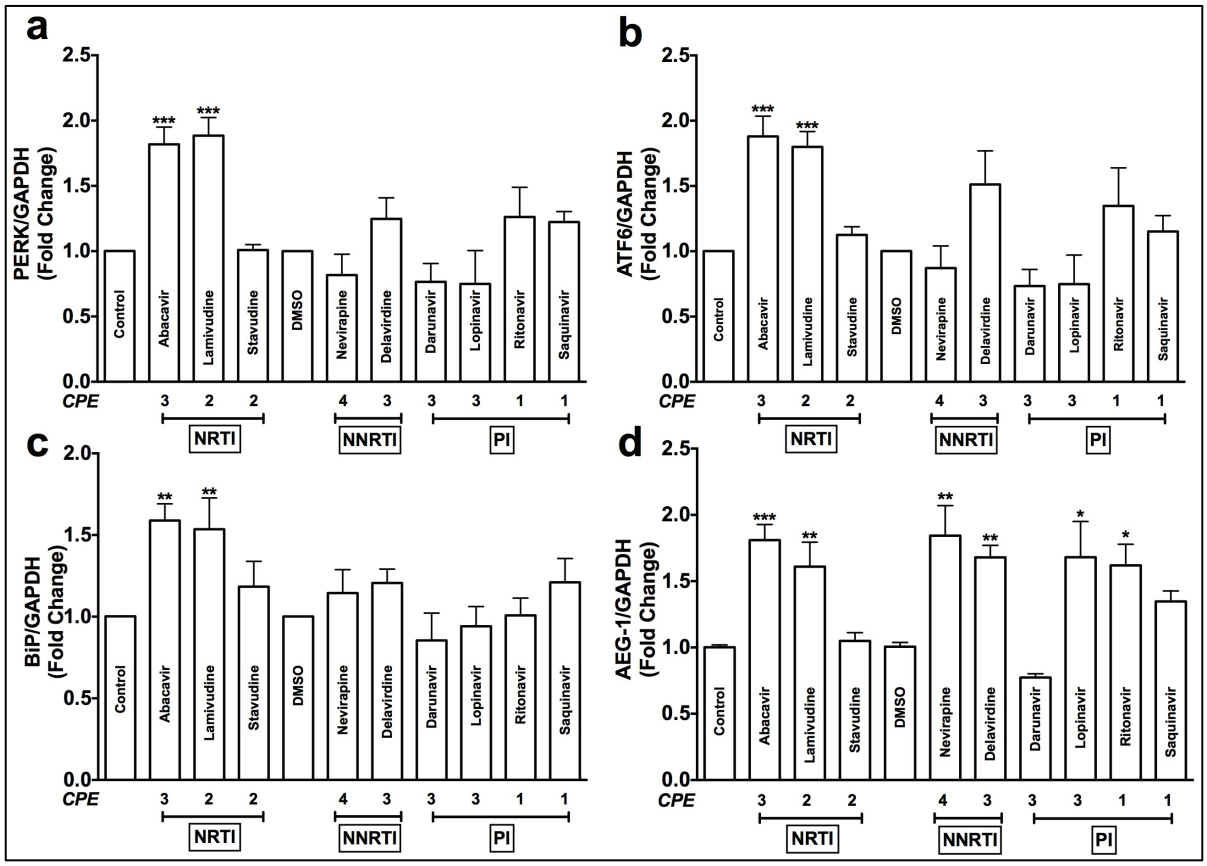


Figure 3.2. Antiretroviral drugs regulate astrocyte ER stress markers and AEG-1 mRNA levels. Astrocytes were treated with a panel of drugs including, nucleoside reverse transcriptase inhibitors (NRTIs - abacavir, lamivudine and stavudine), non-nucleoside reverse transcriptase inhibitors (NNRTIs - nevirapine and delavirdine) and protease inhibitors (PIs - darunavir, lopinavir and ritonavir) with CNS penetration effectiveness (CPE) between 1-4 at therapeutic doses along with vehicle controls for 8 h. PERK (a), ATF6 (b), BiP (c), and AEG-1 (d) mRNA levels were measured using RT^2-PCR. Fold changes to untreated control were calculated using GAPDH as normalizing control. Cumulative data from a minimum of three independent donors is shown. Data represents mean ± SEM and statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001)

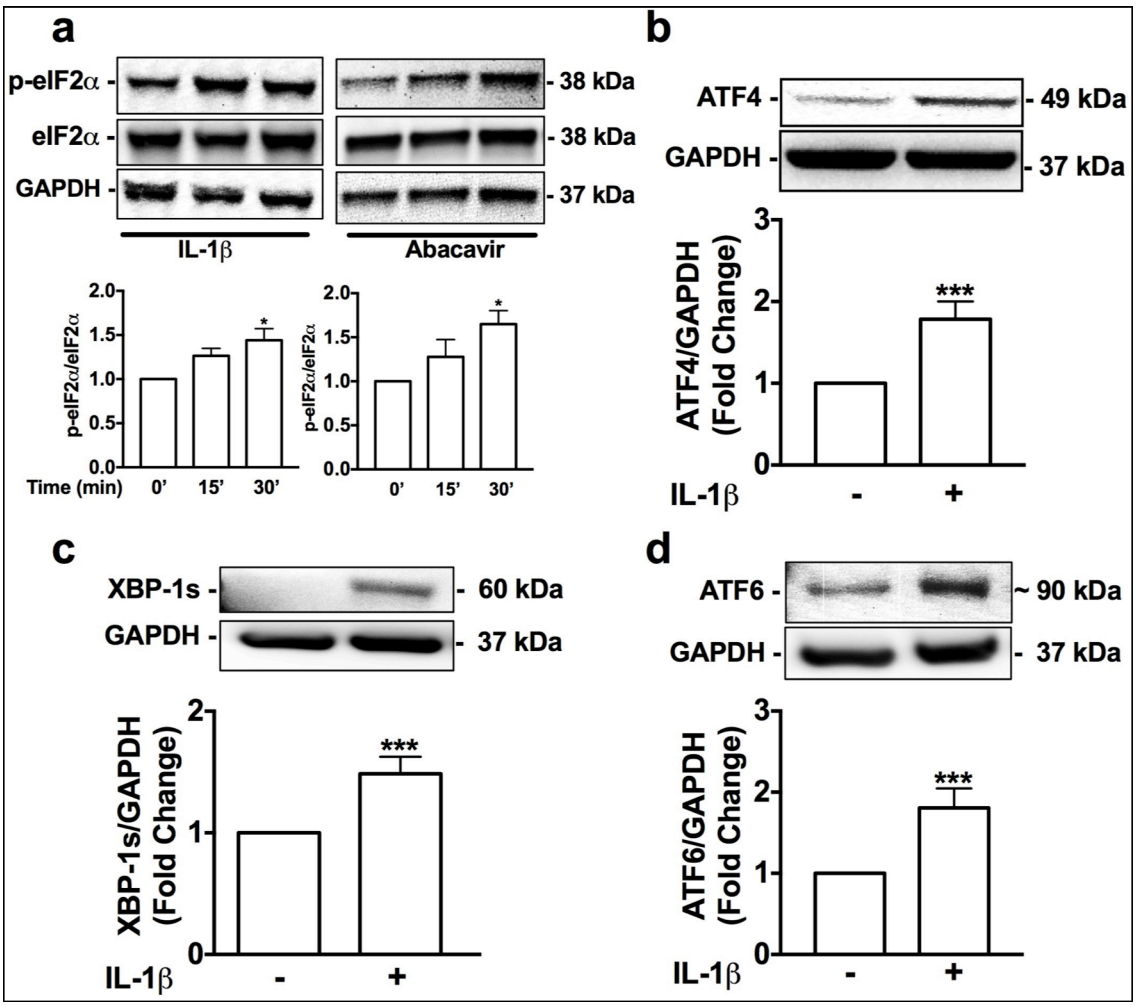

a
p-eIF2α
eIF2α
GAPDH
38 kDa
38 kDa
37 kDa
IL-1β
Abacavir
p-eIF2α/eIF2α
Time (min)
0'
15'
30'
b
ATF4
GAPDH
49 kDa
37 kDa
ATF4/GAPDH (Fold Change)
IL-1β
c
XBP-1s
GAPDH
60 kDa
37 kDa
XBP-1s/GAPDH (Fold Change)
d
ATF6
GAPDH
~ 90 kDa
37 kDa
ATF6/GAPDH (Fold Change)

Figure 3.3. IL-1β and abacavir activate UPR and trigger eIF2α phosphorylation.

Astrocytes were treated with IL-1β (20 ng/ml) or abacavir (4 μM) for 15 and 30 min. Total protein lysates were immunoblotted for p-eIF2α and eIF2α **(a)**. Densitometry analyses were performed to quantify the intensity ratio of p-eIF2α to total eIF2α, what are graphed as fold change to control. One-way ANOVA with Tukey's post-test was used to determine statistical significance (*$p<0.05$). Whole cell lysates of astrocytes treated with IL-1β (20 ng/ml) for 6 h were immunoblotted for ATF4 **(b)**, XBP-1s **(c)**, and ATF6 **(d)**. Statistical analyses were performed by student's unpaired T- test to compare fold change to control (***$p<0.001$). In all panels, representative blots are presented, while the average fold change from three independent donors is graphed. GAPDH was used as a normalizing loading control.

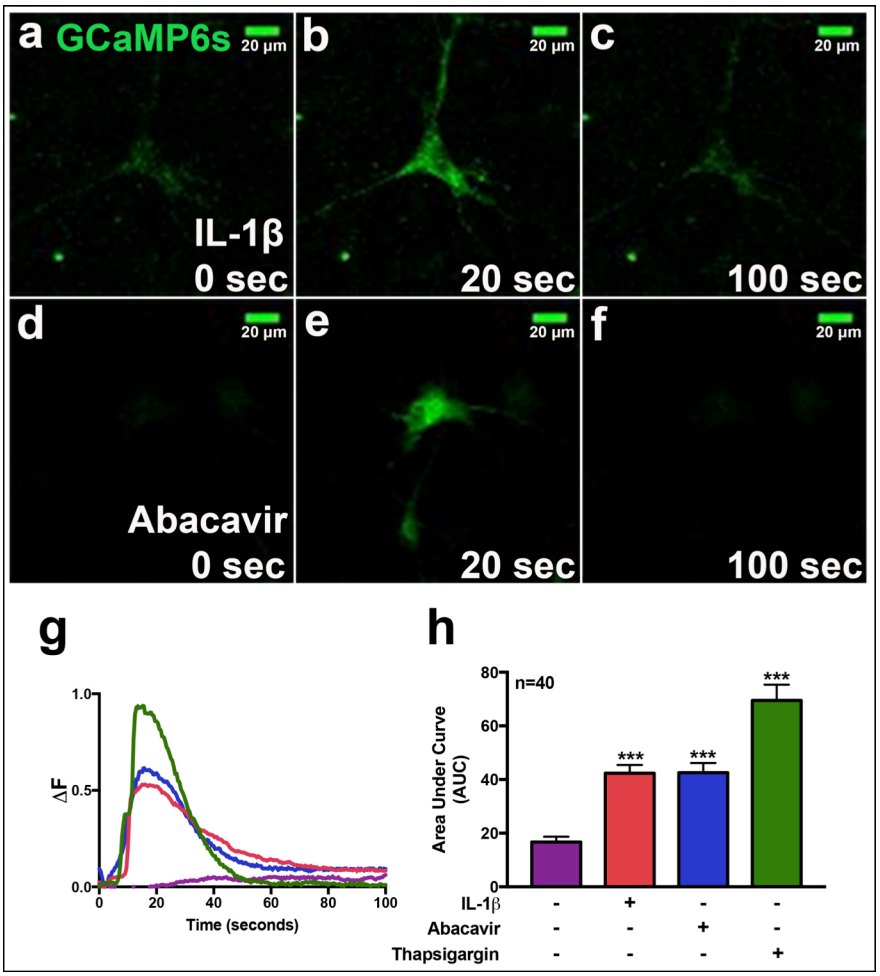

a GCaMP6s
20 µm
IL-1β
0 sec
b
20 µm
20 sec
c
20 µm
100 sec
d
20 µm
Abacavir
0 sec
e
20 µm
20 sec
f
20 µm
100 sec
g
ΔF
1.0
0.5
0.0
0
20
40
60
80
100
Time (seconds)
h
n=40
Area Under Curve (AUC)
80
60
40
20
0
***
***
***
IL-1β - + - -
Abacavir - - + -
Thapsigargin - - - +

Figure 3.4. IL-1β and abacavir induce intracellular calcium signaling in human astrocytes.

For analysis of calcium signaling, primary human astrocytes were first transfected with GCaMP6s, a plasmid expressing an ultrasensitive protein calcium sensor, and allowed to recover for 48 h. Transfected cells were treated with IL-1β **(**20 ng/ml**, a-c)** and abacavir **(**4 μM**, d-f)**. Fluorescence was visualized by confocal microscopy and images were captured every 500 msec. Panels **a-f**, depict fluorescent images taken from a representative cell at time 0 (**a, d,** prior to treatment) and at 20 **(b, e)** and 100 sec **(c, f),** post- IL-1β and abacavir treatment, respectively. The histogram **(g)** shows fluorescence intensity ratio (ΔF) of the representative astrocyte captured over the entire imaging period before and after treatments with HBSS (control, violet), IL-1β (orange), abacavir (blue) and thapsigargin (200 nM, green). Bar graph **(h)** denotes intracellular Ca^{+2} increase, quantified as area under the curve (AUC) in IL-1β, abacavir and thapsigargin treated astrocytes as compared to control. Cumulative data from three individual donors is shown. Data represents mean ± SEM and statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (***$p<0.001$, n=40 individual cells/treatment).

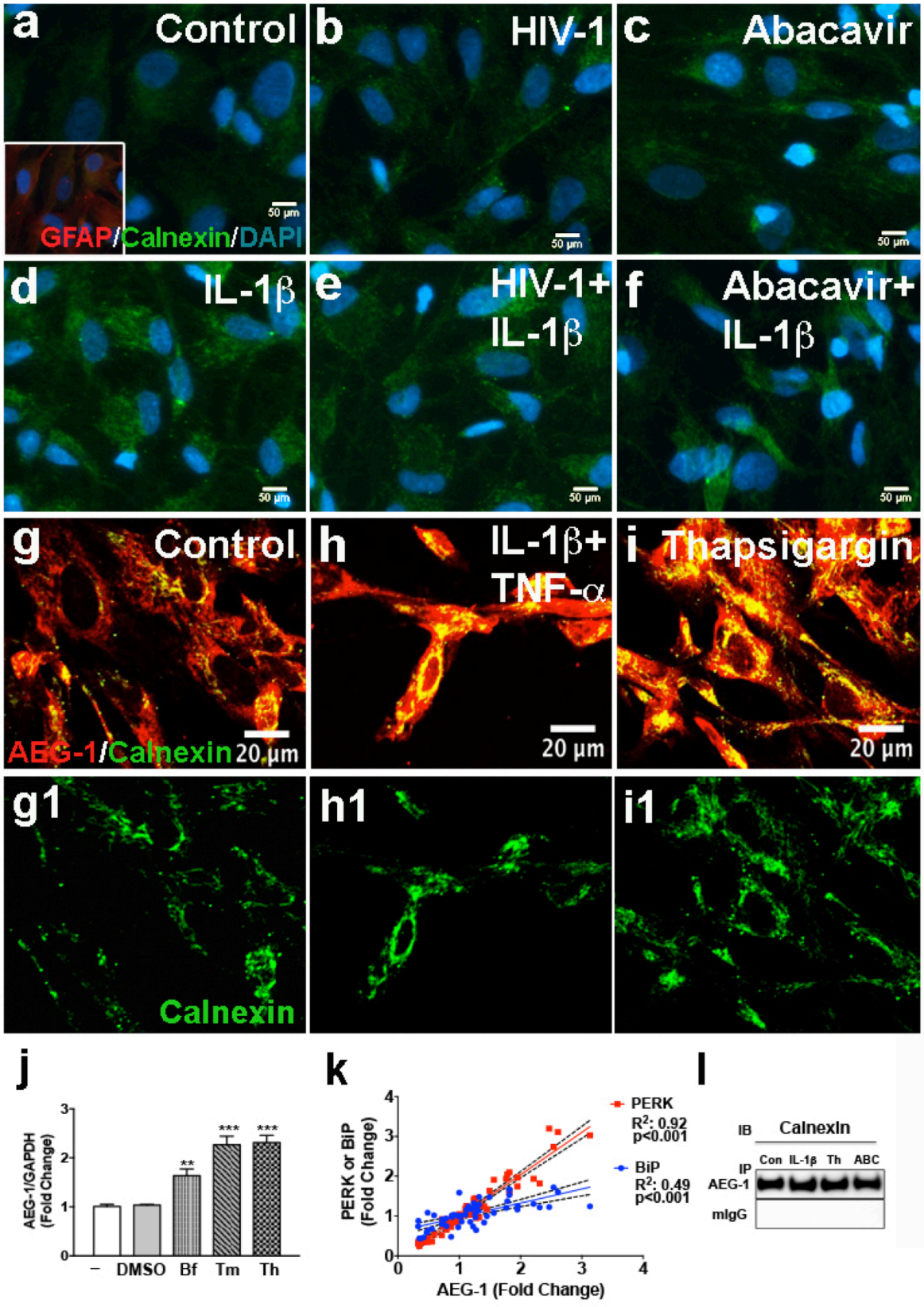

a
Control
GFAP/Calnexin/DAPI
50 μm
b
HIV-1
50 μm
c
Abacavir
50 μm
d
IL-1β
50 μm
e
HIV-1+
IL-1β
50 μm
f
Abacavir+
IL-1β
50 μm
g
Control
AEG-1/Calnexin
20 μm
h
IL-1β+
TNF-α
20 μm
i
Thapsigargin
20 μm
g1
Calnexin
h1
i1
j
AEG-1/GAPDH
(Fold Change)
3
2
1
0
**
***
***
–
DMSO
Bf
Tm
Th
k
PERK or BiP
(Fold Change)
4
3
2
1
0
0
1
2
3
4
AEG-1 (Fold Change)
PERK
R2: 0.92
p<0.001
BiP
R2: 0.49
p<0.001
l
IB
Calnexin
IP
Con
IL-1β
Th
ABC
AEG-1
mIgG

Figure 3.5. HAND-relevant stimuli increase calnexin levels and colocalization with AEG-1 in the context of ER stress.

Astrocytes were treated with IL-1β (20 ng/ml) alone, HIV-1 (p24, 20 ng/ml) ± IL-1β, and abacavir (4 μM) ± IL-1β for 24 h. Representative calnexin (green) and GFAP (red) immunofluorescent images of untreated **(a)**, HIV-1 **(b)**, abacavir **(c)**, IL-1β **(d)**, HIV-1 + IL-1β **(e)**, abacavir + IL-1β **(f)** treated astrocytes were shown. Original magnification 200X. AEG-1 and calnexin (yellow) perinuclear colocalization was confirmed by confocal analysis of astrocytes **(g)** treated with IL-1β + TNF-α **(h),** and thapsigargin **(i)** for 24 h. Cultures were fixed and immunostained for AEG-1 (red) and calnexin (green, **g1-i1**), which are shown as summed Z-stacked images of 12 micrographs taken at 0.5 μm intervals. Original magnification 400X. AEG-1 mRNA expression was assessed by RT^2-PCR **(j)** following 8 h treatment with vehicle or the known ER stressors, brefeldin A (Bf, 1 μg/ml), tunicamycin (Tm, 1 μg/ml) and thapsigargin (Th, 200 nM). Cumulative data represent mean ± SEM and statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (**p<0.01, ***p<0.001). Experiment was conducted in three individual donors. AEG-1 expression positively correlated to PERK (R^2 =0.92, ***p<0.001), and BiP (R^2=0.49, ***p<0.001) **(k)** in the ARV drug treated astrocytes discussed in Figure 2. To evaluate AEG-1 and calnexin interaction, lysates from 24 h untreated, IL-1β, thapsigargin (Th) and abacavir (ABC) treated astrocytes were co-immunoprecipitated with AEG-1 antibody or mouse IgG and immunoblotted for calnexin **(l)**. Representative data is shown from three astrocyte donors that were tested.

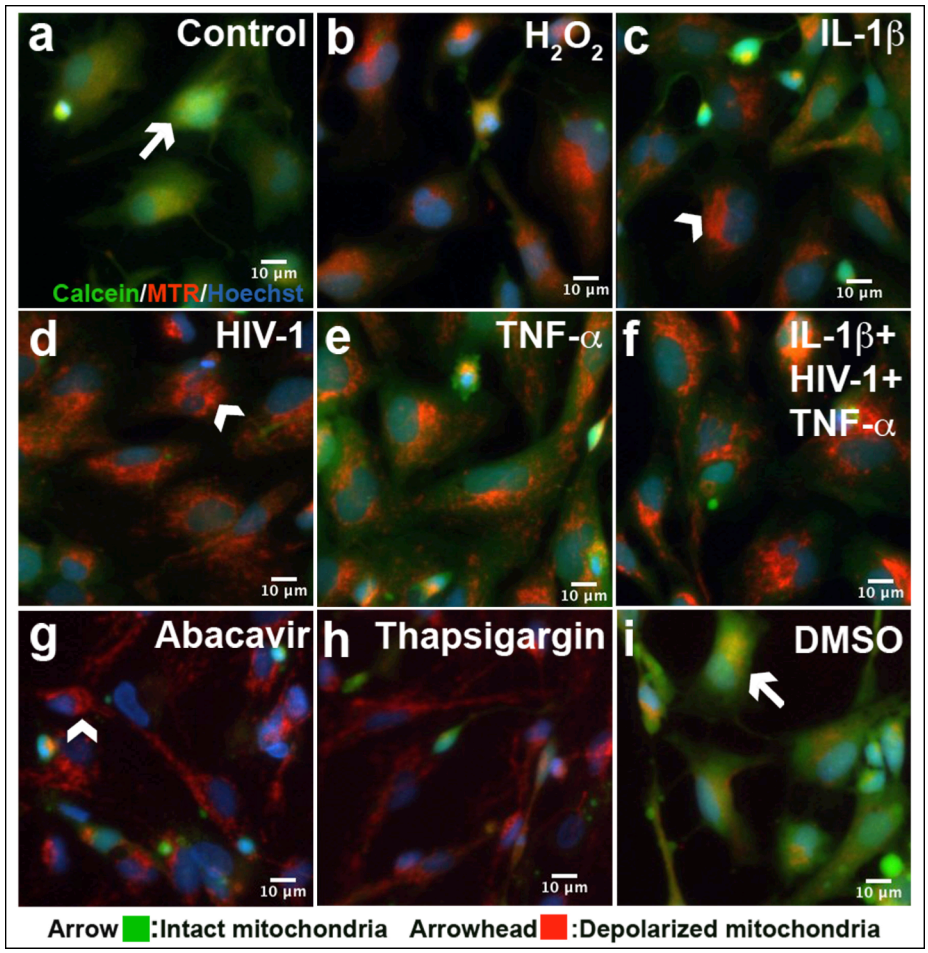

a Control
Calcein/MTR/Hoechst
b H_2O_2
c IL-1β
d HIV-1
e TNF-α
f IL-1β+ HIV-1+ TNF-α
g Abacavir
h Thapsigargin
i DMSO
10 μm
Arrow :Intact mitochondria Arrowhead :Depolarized mitochondria

Figure 3.6. HIV-1, inflammation, oxidative stress and ARV drugs induce astrocyte mitochondrial depolarization.

Astrocytes (untreated **(a)**, and DMSO (vehicle) **(i)**) were treated for 24 h with H_2O_2 (200 μM) **(b)**, IL-1β (20 ng/ml) **(c)**, HIV-1 (p24 20 ng/ml) **(d)**, TNF-α (50 ng/ml) **(e)**, IL-1β + HIV-1 + TNF-α **(f)**, abacavir (4 μM) **(g),** and thapsigargin (200 nM) **(i).** Live-cell imaging was performed using calcein/cobalt chloride quenching assay to monitor mitochondrial permeability transition pore (mPTP) opening. Calcein (green) colocalization with mitotracker red (MTR, red) represents closed mPTP (arrow, green; yellow), while loss of green fluorescence represents mPTP opening (arrowhead, red). Representative images from three donors are shown. Original magnification 200X.

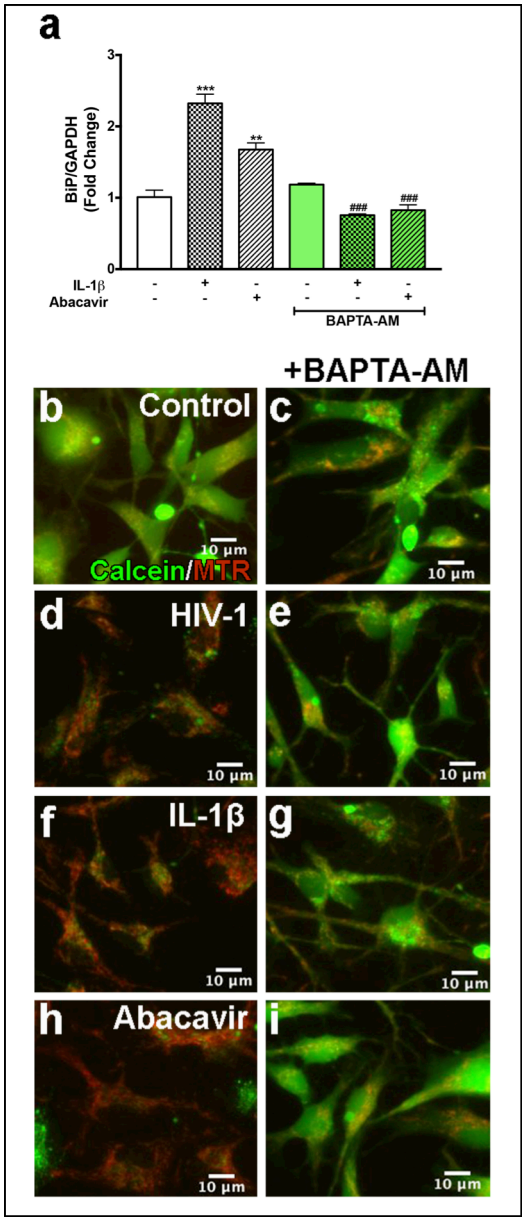

a
BiP/GAPDH (Fold Change)
3
2
1
0
***
**
###
###
IL-1β
Abacavir
BAPTA-AM
+BAPTA-AM
b Control
Calcein/MTR
c
d HIV-1
e
f IL-1β
g
h Abacavir
i
10 μm

Figure 3.7. Chelating intracellular calcium induced by IL-1β and abacavir reduces ER stress and mitochondrial depolarization.

Astrocytes were treated with IL-1β (20 ng/ml), HIV-1 (p24 20 ng/ml), and abacavir (4 μM) for 8 or 24 h with or without pretreatment with BAPTA-AM (25 μM) for 1 h. Total RNA was extracted after 8 h and BiP mRNA expression, as a measure of ER stress, was determined by RT^2-PCR **(a)**. Cumulative data from three individual donors is shown. Data represents mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey's post-test for multiple comparisons (**p<0.01, ***p<0.001, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001), where # represents statistical significance for treatment ± BAPTA-AM comparisons. Panels **b-i** depict live-cell fluorescent images for mPTP detection by calcein/cobalt chloride assay. Representative calcein (green) and MTR (red) fluorescence images from one of three donors are shown from untreated **(b)**, BAPTA-AM pre-treated **(c)**, HIV-1 **(d)**, HIV-1 + BAPTA-AM **(e)**, IL-1β **(f)**, IL-1β + BAPTA-AM **(g)**, abacavir **(h)** and abacavir + BAPTA-AM **(i)** treated astrocytes. Original magnification 200X.

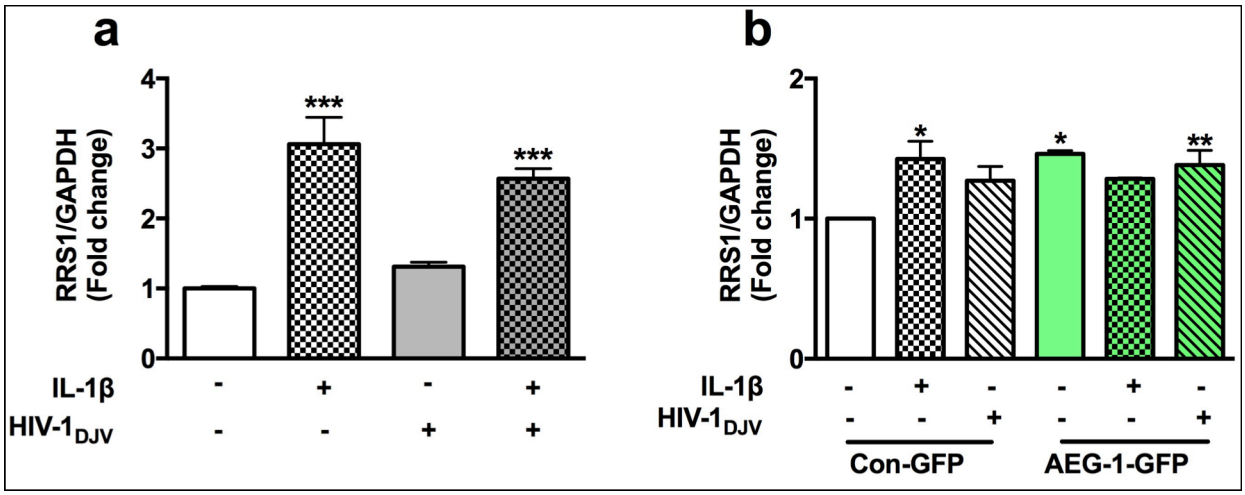


Figure 3.8. HIV-1-associated inflammation and AEG-1 overexpression promoted RRS1 expression

(a) Primary human astrocytes were treated with IL-1β (20 ng/ml) and HIV-1 (p24, 20 ng/ml) alone or in combination and untreated astrocytes were maintained in parallel. **(b)** In parallel, after 48 h recovery, astrocytes transfected with con-GFP and AEG-1-GFP overexpression vectors were treated with IL-1β and/or HIV-1. After 8 h, total RNA was isolated and RRS1 mRNA levels were analyzed by RT^2-PCR. GAPDH was used as normalizing control. Data represents mean ± SEM. Statistical analyses were performed using one-way ANOVA with Tukey's post-test for multiple comparisons (*p< 0.05, **p<0.01, ***p<0.001)

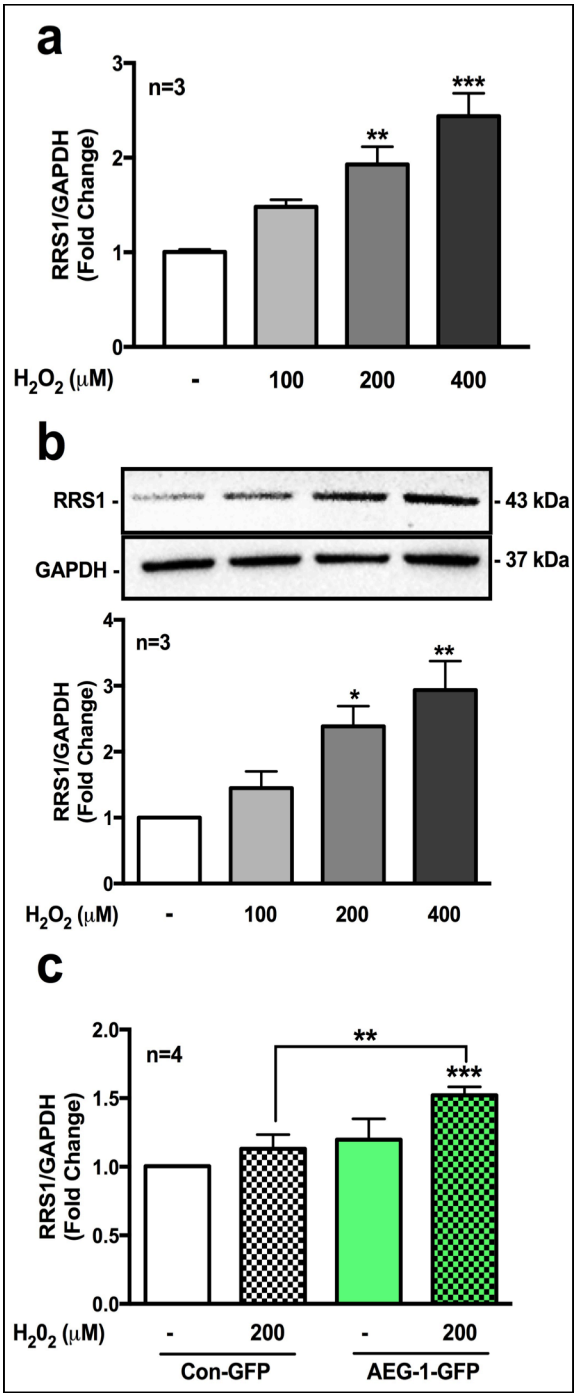

a
n=3
RRS1/GAPDH (Fold Change)
**
***
H2O2 (μM)
-
100
200
400
b
RRS1
43 kDa
GAPDH
37 kDa
n=3
RRS1/GAPDH (Fold Change)
*
**
H2O2 (μM)
-
100
200
400
c
n=4
RRS1/GAPDH (Fold Change)
**
***
H2O2 (μM)
-
200
-
200
Con-GFP
AEG-1-GFP

Figure 3.9. AEG-1 upregulated H_2O_2-mediated RRS1 expression

Astrocytes were treated with increasing concentrations of H_2O_2 (100 μM, 200 μM and 400 μM). **(a)** After 8 h incubation, RNA was isolated and RRS1 mRNA levels were analyzed. **(b)** Total cell lysates were probed for RRS1 and GAPDH following 24 h. GAPDH was used as loading control. Representative blot is presented, while the average fold change from three independent donors is graphed. **(c)** After 48 h, astrocytes transfected with AEG-1-overexpression construct (AEG-1-GFP) or vehicle control (Con-GFP) were treated with H_2O_2 (200 μM) for 8 h. Total RNA was isolated and RRS1 mRNA levels were analyzed by RT^2-PCR. Collective data from minimum three individual donors is shown. Data represents mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey's post-test for multiple comparisons (***$p<0.001$)

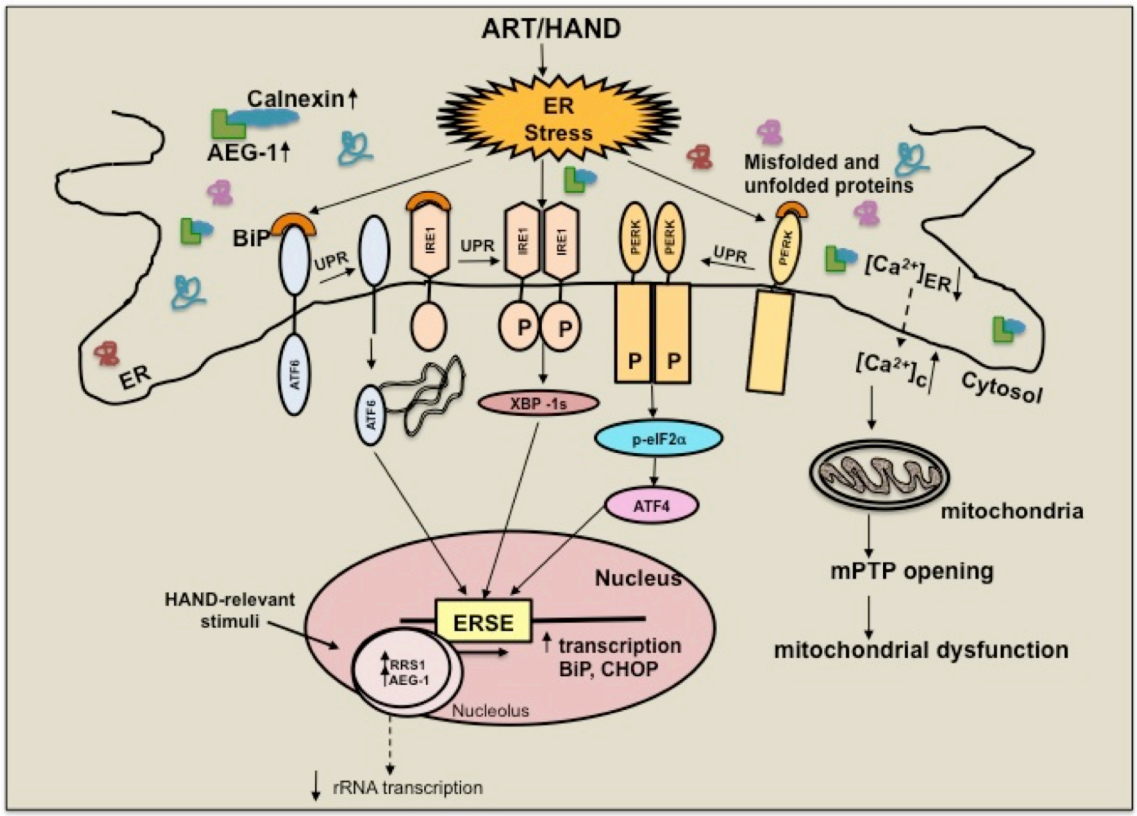

ART/HAND
ER Stress
Calnexin↑
AEG-1↑
Misfolded and unfolded proteins
BiP
UPR
IRE1
PERK
ATF6
XBP -1s
p-eIF2α
ATF4
[Ca2+]ER
[Ca2+]c
Cytosol
ER
mitochondria
mPTP opening
mitochondrial dysfunction
Nucleus
ERSE
transcription BiP, CHOP
RRS1 AEG-1
Nucleolus
HAND-relevant stimuli
rRNA transcription

Figure 3.10. HIV-associated inflammation and therapy induce ER stress and unfolded protein response signaling in astrocytes.

HAND-relevant inflammatory stimuli induce ER stress and trigger UPR activation in astrocytes. Increased ATF4, XBP-1s and ATF6 levels result in the transcriptional activation of ERSE regulated genes such as BiP and CHOP. IL-1β and abacavir increase expression of calnexin, a calcium binding, ER chaperone. Concurrently, AEG-1 expression is augmented, this coupled with its interactions with calnexin possibly acting as a scaffolding protein, implicating it in regulation of ER stress and calcium regulation. In addition, AEG-1 elevated oxidative stress-induced RRS1 expression. Here, we show that IL-1β- and abacavir-mediated increases in intracellular calcium trigger ER stress and mitochondrial dysfunction. Since calcium chelation blocks IL-1β- and abacavir-mediated changes in BiP and mPTP opening, calcium appears to be a critical regulator HAND-relevant ER stress responses. Taken together, perturbations in astrocyte calcium regulation, ER and mitochondrial function by ART and HAND-relevant inflammation may play significant mechanistic roles in HIV-1-associated neuropathogenesis.

CHAPTER 4

Concluding Remarks

4.1 Overall Discussion

Despite HAART and successful virological control, approximately 18-50% of HIV-infected individuals develop neurological consequences of HIV infection. In order to develop effective adjunctive therapies for HAND, we must investigate and target indirect pathological mechanisms and consequences of HIV infection that persist in the CNS, even in the post-ART era. Persistent low-level immune activation, and inflammation contribute to neuronal damage and one of the principal CNS cell types facilitating these events is astroglia. However, the mechanisms linking inflammation to HIV-associated neurotoxicity are still incompletely resolved. We hypothesized that HIV-relevant neurotoxic or inflammatory mediators alter many signaling components of cellular metabolism within astrocytes. Specifically, we focused on Wnt/β-catenin pathway and ER stress responses, which regulate neuroinflammation. This work begins to address the roles of these signaling mechanisms in astrocyte-mediated inflammation/neurotoxicity following HIV- infection that can be potential targets for drug development in HAND.

Glial cells are major targets of HIV-1; nevertheless HIV-1-infected neurons have rarely been observed. While astrocytes are not capable of *de novo* HIV-1 viral replication, they are considered as a significant HIV-1 reservoir in the post-ART era (85, 280, 281). Crosstalk between microglia and astrocytes leads to augmentation of HIV-1-associated inflammation. Astrocytes become “reactive” to a variety of CNS insults and one of the major cellular manifestations of HIV-associated neuroinflammation is

astrogliosis. It is a complex process that involves morphological and functional changes including hypertrophy, upregulation of intermediate filaments, such as GFAP, and increased proliferation (172, 282). Extensive progress has been made in identifying mechanisms of reactive astrogliosis and in determining the roles of astrocytes in CNS disorders.

Together with microglia, astrocytes are important players in neuroinflammatory processes. Activated astrocytes undergo rapid proliferation, enhanced migration towards the site of inflammation and make effort to alleviate collateral damage. At the same time, reactive astrocytes also release cytokines and other factors that mediate inflammatory responses, thus suggesting both beneficial and detrimental roles in HIV-associated brain pathology (283-285). It is clear that several inter- and intracellular signaling pathways regulate morphological, molecular and functional changes in reactive astrocytes. Different characteristics of reactive astrogliosis such as GFAP upregulation, hypertrophy, proliferation, and pro- or anti-inflammatory effects are associated with several regulatory mechanisms including signal transducer and activator of transcription 3 (STAT3), NF-κB, suppressor of cytokine signaling 3 (SOCS3), cyclic adenosine monophosphate (cAMP), and Olig2 (140, 286).

The Wnt/β-catenin pathway induces epithelial and mesenchymal transition, cell proliferation and oncogenesis in multiple cancers (287-289). In this context, we were particularly interested in investigating the role of Wnt/β-catenin signaling in astrocytic proliferative and migration, and inflammatory responses during wound healing and HIV-1-relevant neuroinflammation. We reported for the first time that Wnt/β-catenin signaling regulated reactive astrocyte responses during HIV-1-associated inflammation. Wnt

signaling initiation leads to the activation of T-cell factor/lymphoid enhancer factor (TCF/LEF1) transcription factors and subsequent target genes expression. In particular, knockdown of β-catenin diminished both the response of astrocytes to injury and induction of inflammation. Our data showed upregulation of critical mediators of Wnt signaling including β-catenin and LEF-1 in response to HIV-associated inflammation, implicating Wnt/β-catenin pathway regulation in astrocytes. The expression of well-recognized cell proliferation marker cyclin D1 was measured after β-catenin knockdown to determine if β-catenin pathway regulates proliferation in reactive astrocytes. HIV-1 induced cyclin D1 expression in infected podocytes, implying HIV-1 activates cyclin D1-dependent cell-cycle mechanisms to promote proliferation of infected renal epithelium (290). Our investigation identified significant decrease in cyclin D1 and proliferation with β-catenin knockdown in astrocytes, signifying β-catenin signaling during reactive astrogliosis. We also showed β-catenin knockdown delaying astrocytes migration in response to injury. Taken together, the present work identifies the critical role for β-catenin signaling in astrocyte injury responses.

GSK-3β contributes to neuroinflammation in several neurodegenerative diseases (184). It is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation (291). It has been clearly established that phosphorylation at serine 9 position correlates with the inhibition of kinase activity (292). GSK-3β also positively regulated NF-κB activity without changing IκBα degradation or NF-κB nuclear translocation in mouse embryonic fibroblasts (MEFs) (293). HIV-1-associated proinflammatory molecules IL-1β and interferon (IFN)-γ initiated GSK-3β phosphorylation in intestinal mucosal inflammation (208, 209). Consistent with other

studies, we also showed IL-1β induced GSK-3β phosphorylation in astrocytes. Thus, IL-1β, may activate other signaling pathways along with NF-κB to enhance inflammation during HIV-infection. NF-κB is a key pathological mediator in HAND. Synergistic and cooperative functions of Wnt and NF-κB signaling in tumor-triggered inflammation is also identified (294). The effector molecule responsible for activating TCF/LEF-responsive genes is β-catenin, which serves as a transactivator that binds to DNA-bound TCF/LEFs. Interestingly, β-catenin:TCF/LEF complex has been shown to bind to promoters of NF-κB target genes, such as CXCL8, C-reactive protein (CRP*)* and marix metalloproteinase (MMP)13, and positively regulate gene transcription in concert with NF-κB (221, 295, 296). We also demonstrated reduction of NF-κB p65 expression with β-catenin knockdown in astrocytes. CXCL8 is upregulated in the brains and CSF of HIV-1 infected individuals suggesting its role in HIV-1 associated neuroinflammation (297, 298). Based on the literature, we were excited to investigate if β-catenin signaling *via* NF-κB regulates CXCL8 levels in astrocytes during HIV-1 inflammation. We also explored mechanisms by which β-catenin influences CXCL8 production and found that β-catenin knockdown decreased transcription factor NF-κB expression, suggesting that proinflammatory cytokines/chemokine production is regulated through β-catenin/NF-κB-dependent mechanism. Our findings showed that elevated CXCL8 levels in response to HIV-associated inflammation is related to both NF-κB and β-catenin signaling. Therefore, this study provides accumulating evidence that β-catenin pathway also plays a inflammatory role in astrocytes during HIV-infection.

Over the last decade, the UPR has been addressed in an increasing number of studies on neurodegeneration. More recently, by inhibiting the function of specific mediators of the ER stress, the UPR was documented as a target for drug therapy for treatment and prevention of neurodegeneration. However, the effect of HIV infection on UPR has scarcely been investigated. Thus, we also examined the role of HIV-relevant neuroinflammation on ER stress and activation of the UPR. IL-1β and TNF-α are prototypical mediators of neuroinflammation, primarily released by HIV-1-infected and immune-activated microglia in CNS (42, 299). Moreover, astrocytes are highly reactive to IL-1β and possess an autocrine loop to enhance CNS inflammation (243). Therefore, initially astrocytes were treated with TNF-α, and IL-1β to assess UPR activation. We studied mRNA expression of UPR pathway initiators, PERK, IRE1α and ATF6. Robust changes were observed with IL-1β compared to TNF-α treatment.

Three UPR pathway regulators PERK, IRE1α and ATF6 induce ERSE containing promoter activity, including classical proteins BiP, an ER resident molecular chaperone, and CHOP, an apoptotic regulator. Recently, a significant increase in BiP expression was also detected in CNS of HIV-1 infected individuals (73). Therefore, next, BiP and CHOP expression was studied in response to HAND-relevant stimuli. Astrocytes treated with IL-1β alone significantly increased ER stress markers BiP and CHOP. Our studies also showed activation of all three UPR pathways in response to inflammation. Thus, we identified IL-1β as a strong initiator of ER stress in astrocytes, highlighting mutual regulation between ER stress and inflammation during HIV-1 CNS infection.

The neurotoxic side effects of ARV drugs are among several contributing factors to the continued prevalence of HAND. However, ARV drug-mediated toxicity in CNS, particularly in glial cells remains largely unexplored. Here, we aimed to investigate ARV drugs induced ER stress in primary human astrocytes. Taken together, of different classes of ARV drugs tested, only NRTIs, specifically abacavir significantly upregulated ER stress markers in astrocytes. One recent *in vitro* study showed that neurotoxicity was associated with abacavir, efavirenz, etravirine, nevirapine, and atazanavir (33). Our data provide initial evidence that ARV drugs induce astrocyte ER stress that may be useful for the development of treatment strategies that potentially reduce the risk of antiretroviral neurotoxicity.

HIV does not infect neurons, but HIV-1 proteins induce Ca^{2+} dysregulation, indicated by abnormal and excessive Ca^{2+} influx and increased intracellular Ca^{2+} release that in turn elevate cytosolic free Ca^{2+} levels. Such alterations in intracellular Ca^{2+} homeostasis significantly disturb normal functioning of neurons, and induce dysregulation, injury, and death of neurons or non-neuronal cells, and associated tissue loss in HIV-vulnerable brain regions. In addition, other studies have shown that HIV-1 proteins disrupt neuronal calcium homeostasis during HIV-1-Encephalitis (267, 268). Cellular dysfunctions as well as increased oxidative stress and dysregulation of calcium homeostasis involving specific subcellular compartments, such as mitochondria and ER, are emerging as crucial players in the pathogenesis of neurodegeneration. The ER serves as main storage organelle for calcium. HIV proteins gp120 and tat disrupted neuronal calcium homeostasis by modulating calcium-regulating systems in the plasma membrane and endoplasmic reticulum. We showed

IL-1β and abacavir treatment dysregulated astrocyte intracellular calcium levels, and elevated the expression of calcium-dependent chaperones. Excessive increase of astrocyte intracellular calcium can be toxic, and could lead to neuronal dysregulation, injury and death.

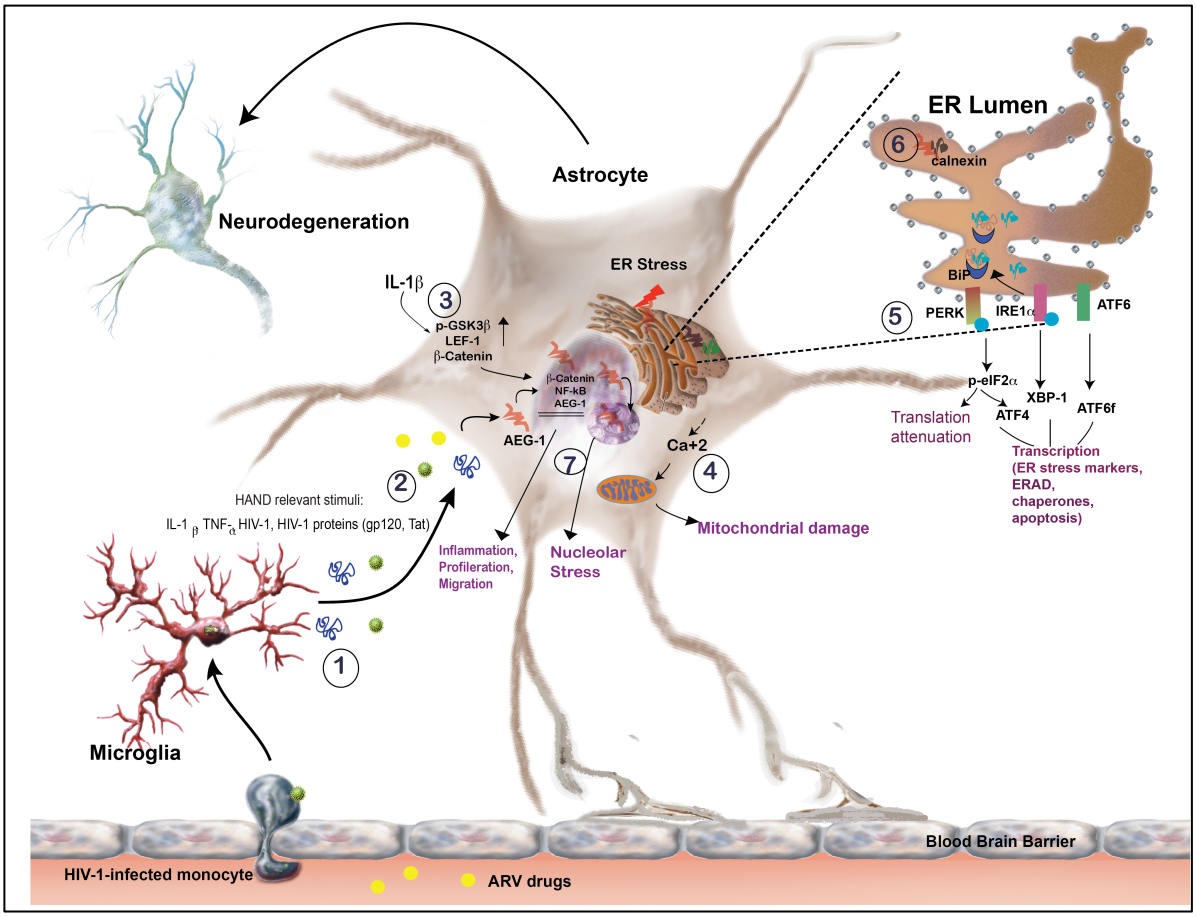
Pretreatment with the calcium chelators, BAPTA-AM also reduced HAND-associated ER stress, suggesting calcium dysregulation is upstream of ER stress in astrocytes. Mitochondrial dysfunction has been associated with both HIV infection and exposure to ART(300, 301). Among all ARV drugs, NRTIs are mainly responsible for mitochondrial dysfunction. ER and mitochondria interact closely with each other, sustained ER stress and calcium release can induce mitochondrial stress and mPTP opening (302). Prolonged mPTP opening plays a crucial role in the pathogenesis of several diseases (272). Our studies demonstrated intracellular calcium chelation significantly reduced mPTP opening; suggesting that HAND-relevant stimuli-induced ER stress/calcium influx into mitochondrial matrix causing mitochondrial dysfunction.

AEG-1 was identified as a novel modulator regulating astrocyte responses to injury, HIV-1-associated neuroinflammation and glutamate-mediated excitotoxicity, suggesting its role in HAND. Therefore, we investigated a plausible AEG-1 contribution towards HIV-1/ART-induced ER stress in astrocytes. In this study, we identified AEG-1 as an ER stress inducible gene. To our knowledge, this is the first report to recognize AEG-1 as an interacting partner of calnexin, which supports AEG-1, may act as a scaffolding protein to regulate ER calcium signaling through formation of multi-protein complexes. Due to various intracellular localization sequences, increased AEG-1 may act as sensor, regulating UPR signals between the ER and nucleolus. Nucleolus, a

stress sensor, responds to various forms of cellular stress and normal ribosomal DNA transcription, and ribosome assembly is often dysregulated (238, 239). The association between nucleolar dysfunction and neurodegenerative diseases is increasingly being explored (234, 240, 253, 254, 275, 276). However, HIV-1-induced nucleolar stress is not well known. Since AEG-1 localizes to nucleolus in response to injury and oxidative stress, AEG-1 may play a role in DNA/RNA processing during HIV-1-associated nucleolar stress. We demonstrated significant elevated expression of RRS1; a protein inhibiting transcription of both rRNA and ribosomal protein genes during HIV-1 induced inflammation and oxidative stress in astrocytes. AEG-1 overexpression enhanced inflammation- and oxidative stress- induced RRS1 expression, *i.e*., nucleolar stress in astrocytes.

In conclusion (**Fig 4.1**), we report here that HIV-relevant inflammation *via* various pathways contribute to HIV-associated neurotoxic effects. HAND-relevant stimulus, IL-1β activated β-catenin pathway to enhance inflammation and also elevated intracellular calcium to induce ER stress, UPR pathway activation and mitochondrial toxicity/dysfunction in astrocytes. HIV-associated inflammation and oxidative stress also initiated nucleolar stress in astrocytes, supporting ER stress and protein overload in astrocytes during HIV-1 infection. Our work also revealed that AEG-1 occupied a unique niche in HIV-associated ER and nucleolar stress, signifying its contribution towards neurodegeneration. AEG-1 being versatile and playing a central role in multicellular compartments including nucleus, mitochondria, ER and the nucleolus may be an effective therapeutic target. Accordingly, further studies are definitely warranted to identify AEG-1-mediated regulation in multifaceted mechanisms during HIV infection.

Taken together, the work presented in the thesis provides a framework for further elucidation of role of astrocytes in HIV-1 neuropathogenesis and develop novel therapeutics aiming at astrocyte-mediated neurotoxic mechanisms in HAND.



Scheme 4.1 Overall summary

1. HIV-1 infected/activated CNS cells release HIV-1 proteins or proinflammatory cytokines such as TNF-α and IL-1β.
2. Astrocytes become reactive in response to HAND-relevant stimuli.
3. β-catenin pathway regulates astrocyte proliferation, migration and CXCL8 production in response to IL-1β in reactive astrocytes in association with NF-κB mediated mechanism.
4. IL-1β- and abacavir-mediated increases in intracellular calcium initiate ER stress and mitochondrial dysfunction.

5. Elevated ER stress triggered three UPR pathways PERK, IRE1α, and ATF6 and resulted into upregulation of ER chaperones BiP and calnexin.
6. HAND/ART-mediated AEG-1 upregulation is correlated with ER stress and interaction with the calcium-binding chaperone calnexin, signifying its role in calcium signaling.
7. AEG-1 overexpression also elevated HAND-associated RRS1 expression, suggesting AEG-1 mediated regulation of nucleolar stress during HIV-1 infection.

4.2 Future Directions

The aim of this dissertation was to investigate elusive astrocytic responses during HIV-1 CNS infection. We conclude from our overall results that HAND-relevant stimuli provoked the following changes in astrocyte functions: 1) the expression of CXCL8 *via* Wnt/β-catenin signaling is increased; 2) intracellular Ca^{2+} concentration is elevated, 3) the expression of ER stress markers is upregulated, 4) mitochondrial function is disturbed and 5) nucleolar stress is induced. Since, we reported activation of Wnt/β-catenin signaling for enhanced HIV-1-associated inflammation, it is logical to further investigate the mechanism by which β-catenin influences inflammatory cytokines/chemokines production. However, the Wnt effector molecules upstream of β-catenin signaling need to be deciphered to fill in the picture of cellular mechanism in astrocytes. Further studies need to be performed to elucidate the upstream (Wnt, NF-κB, PI3K, Notch and Hedgehog) signaling leading to the high levels of phosphorylation of GSK-3β at serine 9. As we examined differences in NF-κB expression with altered Wnt/β-catenin signaling, it will be effective to investigate crosstalk regulatory mechanisms, as NF-κB signaling plays crucial role during HIV-associated inflammation.

Our studies revealed induction of ER stress in response to ARV drugs and HIV-1-mediated inflammation. However, persistent ER stress also provokes aberrant inflammatory signaling and facilitates cell death. Consequently, further examination of this interplay could provide further insights into HIV-associated inflammation and neurotoxicity, and reveal novel therapeutic targets. β-catenin mediating growth inhibition

via induction of ER stress in multiple myeloma cells, suggests a possible link between Wnt signaling and ER stress (303). Therefore, it will be very interesting to elucidate if activated β-catenin signaling also mediates HIV-associated ER stress. In this study, we identified elevated intracellular calcium in response to HAND-relevant stimuli in astrocytes. These astrocytic calcium variations can increase the release of excitatory neurotransmitter, glutamate that likely lead to neurotoxicity (304). Considering this, it is very significant to explore if the HAND-elevated calcium modulates synaptic transmission, and neuronal excitability. Since we showed induction of RRS1 expression during inflammation, and oxidative stress, it is logical to explore modulated pre-rRNA transcription, processing, and ribosomal RNP assembly in HIV-infected astrocytes. Our previous studies showed injury/oxidative stress-induced AEG-1 co-localization with fibrillarin, pre-RNA processing protein in astrocytes. Consistent with these studies, we also showed AEG-1 overexpression elevated RRS1 expression during inflammation/oxidative stress. Therefore, it is critical to examine AEG-1 role in pre-RNA processing and ribosomal synthesis during HIV-1 infection. This study evaluates the multi-functions of AEG-1 and designates the major molecular alterations regulated by AEG-1 in HIV-1 CNS infection, indicating its key role and potential as a biomarker or significant target for the therapy of HAND. This dissertation highlights series of changes in astrocyte functions during HIV-1 infection including, but not limited to, calcium dysregulation, inflammation, ER stress and mitochondrial depolarization. Taken together, we postulate further deep insights into HIV-associated astrocyte dysfunctions and therefore modulation of these mechanisms may provide novel therapeutic strategies in HAND.

Recently, HIV-reservoirs of latently infected cells were discovered despite effective ART. The clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system is developing gene-editing technique to eradicate HIV-integrated genomes from multiple HIV reservoirs that could result into wide-ranging cure of neuro-AIDS. Therefore, further studies are warranted on gene editing using CRISPR/Cas9 for complete elimination of HIV DNA and AIDS.

4.3 Appendix

Model characterization: All experiments were conducted using primary human astrocytes that we culture in our own laboratory. Isolation, and cultivation of astrocytes are described in detail in the materials and methods. However, it is noteworthy that given that each donor we use is a biological replicate in itself, we treat each donor as a unique read-out and obtain comparative trends for key common features of astrocytes prior to any new experiments. Following variables are evaluated for each donor prior to setting up any experiment: **1.** Cellular morphology, **2.** Proliferative ability, **3.** GFAP expression, **4.** Conversion to prototypical reactive phenotype when exposed to IL-1β (**Fig. 4.2**), **5.** CCL2 & **6.** CXCL8 chemokines production in response to inflammatory stimuli, and **7.** Baseline ability to clear extracellular glutamate that is diminished by ~40% in response to IL-1β. The quantitative ranges of responses vary from donor to donor, thus, statistically significant trends in comparable directions for these known variables tested, provide a unified validation for the individual donor with a baseline for interpretation of new experimental data.

In order to have consistency in our results, taking into account variability between preparations or lots of fetal bovine serum (FBS), each new lot of FBS is

characterized following the same procedures as described for new astrocyte donors. Several lots of FBS are assessed in parallel using the same donors and compared to previous lots of FBS. The specific lot of FBS is chosen based on its consistency with the previous lot, and sufficient FBS is purchased to 1-2 years of experiments.

The potential pitfalls for using FBS in our cell culture conditions are similar to any cell culture system in which undefined media is used. There will be lot-to-lot variability between FBS lots that cannot be effectively defined. Similarly, for conditioned media, the specific cytokines present are also not defined. Furthermore, the amount of conditioned media is not sufficient to be used across multiple experiments, whereas FBS can be. For this reason, we characterize the FBS in using the same procedure as used for characterizing new donors in order to provide consistent results across FBS preparations. In addition, as astrocytes are not exposed to serum in the brain, it could be argued that they may respond differently in media containing FBS versus serum-free. There is ongoing discussion about this issue in the literature and while the jury is still out, in our studies as described earlier, we confirmed their well-established *in vivo* responses such as ability to clear glutamate and changes in response to inflammation, in the cultured conditions as a guiding framework for our new investigations.

The goal of *in vitro* cell culture based experiments is to characterize the responses of individual, or groups, of cells under specifically controlled conditions. The rigor and reproducibility of these experiments is also vital for ensuring validity of the results. While extrapolating our cell culture-based results to a physiological system may be limited by the controlled conditions of *in vitro* system, the goal is to mimic the *in vivo*

microenvironment stimuli relevant to disease and interpreting new results in context of confirmed, well-established cellular responses.

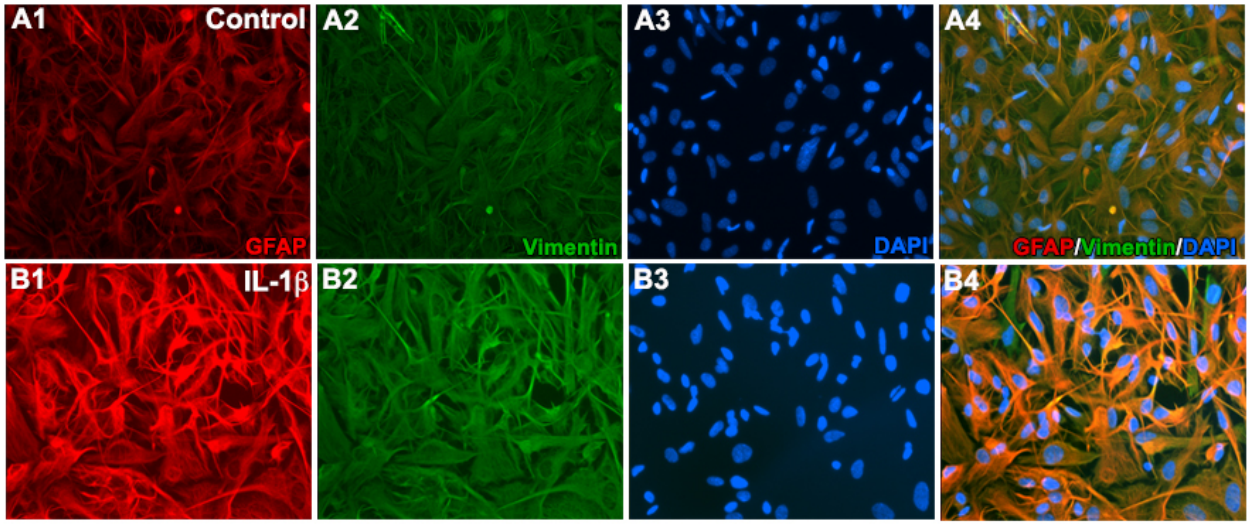


Figure 4.2. IL-1β induces reactive phenotype in astrocytes

Primary human astrocytes (passage 2) were untreated or treated with IL-1β and incubated for 24 h. Fixed cells were immunostained with (GFAP, red), vimentin (green) and counter-stained with DAPI (blue) to identify nuclei.

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