THE ROLE OF HIV PROTEINS IN MEDIATING NEURONAL MECHANISMS IMPLICATED IN AGE-RELATED COGNITIVE DYSFUNTION

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ABSTRACT

While the proportion of aged individuals is increasing across populations, therapeutic advances have resulted in a particular increase amongst aged persons living with HIV, who maintain an elevated risk for developing HIV-Associated Neurological Deficits (HANDs). To determine the neurobiological mechanisms of HAND, the current experiments assessed if mechanistic insights from Alzheimer's Disease, such as oxidative stress and protein quality control (i.e. stress granules, BAG chaperones), may similarly contribute to neuronal dysfunction in response to HIV proteins Tat and Nef. Although data indicate limited effects on stress granules, Tat and Nef facilitated elevations in intraneuronal oxidative stress, decreased BAG1 transcription and suppressed BAG3 levels (i.e. protein and RNA), while these disrupted mechanisms coincided with impaired electrophysiological firing capacities in neurons. Oxidative stress, in the form of H_2O_2, exacerbated the reduction in BAG3, induced an upregulation of BAG1 and dysregulated key mitochondrial proteins. Such inverse BAG chaperone ratios were maintained in two animal models which express viral proteins in the CNS, the doxycycline-inducible Tat (iTat) and Tg26 mouse. Interestingly, the inhibition of oxidative stress in primary neurons was capable of partially preserving electrophysiological functioning and BAG3 levels otherwise altered by HIV viral proteins. Results suggest oxidative stress induced by HIV proteins or other factors (i.e. aging) may dysregulate neuronal PQC, particularly BAG chaperones, and contribute to the neurobiological mechanisms underlying HAND.

In Loving Memory of Michael P. Duggan and Grace P. Duggan

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CHAPTER 1

INTRODUCTION

1.1 HIV-Associated Neurological Disorders (HANDs)

Human immunodeficiency virus (HIV) infection, as well as its associated neurological and behavioral consequences, remain a consistent public health concern, particularly in the context of our aging HIV+ population. In 2019, an additional 1.7 million people were infected with the virus, bringing the total number of people living with HIV to 38 million worldwide (UNAIDS, 2020). Despite improved patient outcomes due to enhanced diagnostic testing and targeted therapies, there remains no effective vaccine for HIV, or a permanent cure for HIV-infected individuals (Davenport et al., 2019). Moreover, HIV continues to be a leading cause of morbidity across the globe (World Health Organization, 2018). However, the implementation of combined antiretroviral therapies (cART) has transformed this once fatal disease into a chronic, manageable condition (Deeks, Lewin, & Havlir, 2013; Saylor et al., 2016). Following its introduction in the mid-1990s, cART has cut mortality rates in half and substantially increased the life expectancy of the HIV population, which is now approaching that of the general population (Fauci & Marston, 2015; Trickey et al., 2017). These trends have resulted in a precipitous increase among aged persons living with HIV, as well as an infected individual's susceptibility to age-related neurocognitive dysfunction.

In the cART era, decreased mortality rates and increased life expectancies have resulted in approximately half of HIV+ individuals reaching at least 50 years of age (Goodkin et al., 2001). Although current treatments can control HIV infection, infected individuals maintain increased risks for numerous chronic comorbidities, including HIV-

Associated Neurological Disorder (HAND) (Sengupta & Siliciano, 2018). For instance, there is equivocal evidence that viral burden in the central nervous system (CNS) is associated with the incidence and degree of cognitive impairments (McArthur et al., 2003; Robertson et al., 1998). Amongst HIV infected individuals, HAND is estimated to afflict up to half of patients, even if cognitive deficits are not self-reported or if viral loads are suppressed to undetectable levels (Alford et al., 2019; Cassimjee & Motswai, 2017; Heaton et al., 2010; Simioni et al., 2009). Moreover, aged persons living with HIV maintain significantly greater risk for developing HAND compared to uninfected, age-matched controls as well as younger, infected individuals (Fazeli, Crowe, et al., 2014; Joska et al., 2012; Pasipanodya et al., 2019; Sacktor et al., 2016; Valcour et al., 2004; Vance, Fazeli, Ball, Slater, & Ross, 2014; Xiao et al., 2020).

Broadly, these detriments can reflect impairments in several cognitive domains, including executive functioning (e.g. impulsivity, problem solving), memory (e.g. immediate retrieval, working memory) and attention (e.g. sustained attention, strategy shifting) (Clifford & Ances, 2013). According to recently adopted criteria, HAND can further be delineated into asymptomatic neurocognitive impairment, mild neurocognitive disorder or dementia, depending on the severity of performance deficits and subsequent impact on daily functioning (Antinori et al., 2007). Along with clinical presentation of cogntive symptoms, transgenic mouse models of HIV routinely display significant performance deficits in cognitively demanding tasks, particularly working memory and spatial memory paradigms (Carey, Sypek, Singh, Kaufman, & McLaughlin, 2012; Putatunda et al., 2019). Similarly, rat models of HIV maintain signifciant detriments in spatial memory performance as well as detriments across mutiple operant-based behavioral tasks (e.g. discrimination learning, extradimenstional set shifting) (Lashomb, Vigorito, & Chang, 2009; McLaurin, Li, Booze, & Mactutus, 2019; Moran, Booze, & Mactutus, 2014; Vigorito, LaShomb, & Chang, 2007). Thus, these neurocognitive impairments remain a persistent challenge amongst HIV infected individuals, including those who adhere to therapeutic regimens and maintain long term viral suppression (i.e. aged HIV+ individuals).

In the cART era, the prevelance and clinical presentaiton of HAND has shifted. Specifically, findings from the comprehensive CHARTER cohort indicated an 80% reduction in the most severe forms of cognitive impairment since cART implementation, although milder cognitive deficits were found to persist at comparable rates. While only 2% of virally suppressed individuals are now estimated to display severe deficits (i.e. compared to 10-15% pre-CART), more than half (52%) maintain detectable cognitive impairments upon neuropsychological assessment (McArthur, Steiner, Sacktor, & Nath, 2010). Subsequent reports disputed these findings and suggested the rates of HAND might be substantially lower, in part due to inconsistent testing criteria (Gisslén, Price, & Nilsson, 2011; McDonnell et al., 2014). Despite active debate surrounding the prevalence of HAND, it is evident HIV infected individuals in the cART era can maintain significantly greater risk for the development and progression of neurocognitive deficits compared to the general population (Grant et al., 2014; Schouten et al., 2016). Furthermore, such likelihoods remain elevated even if alternative neuropsychological criteria used to assess HIV infected persons (De Francesco et al., 2016; Su et al., 2015).

Along with aging, several key variables render HIV+ individuals at increased risk for HAND, including co-infections, drug use and antiretrovirals themselves. Several studies have also shown that HIV patients coinfected with other viruses or bacteria have increased risks for developing cognitive impairments (Cherner et al., 2005; Hestad et al., 2019; Marra et al., 2013; Ryan et al., 2004). Illicit drug use, including methamphetamine and cocaine, is also known to be significantly higher amongst HIV patients who develop cognitive impairments compared to those who do not use illegal substances (Ayuso-Mateos et al., 2000; Byrd et al., 2011). However, it remains unclear if this association is mediated by pre-existing substance use disorders, which themselves can result in cART interruption and increase risk for HAND (Kamal et al., 2017; Meyer, Althoff, & Altice, 2013). Adherence to antiretroviral treatment itself may also increase risk for HAND in aging; for instance, long term cART can induce metabolic changes (e.g. central obesity, dyslipidemia, and insulin resistance) which are themselves considered risk factors for dementia (Behrens et al., 1999; Falutz, 2007; Narciso et al., 2001; Whitmer et al., 2008). Indeed, some data suggest discontinuation of long-term cART treatment may improve performance in HIV patients upon neuropsychological assessment (Robertson et al., 2010).

Although HAND's prevalence has shifted and risk factors have been identified, the continued growth of the HIV population and ensuing development of neurological impairments has increased focus on the neurobiological underpinnings of HAND. For instance, the implementation of a variety of neuroimaging techniques (e.g. fMRI, PET) has elucidated structural and functional alterations in specific brain regions (e.g. frontal cortex, hippocampus) that are associated with HAND (Ances & Hammoud, 2014; Sanford et al., 2017). However, the molecular mechanisms that impair neuronal homeostasis and functioning underlying HAND require further elucidation. Given the increased risk of HAND in the aging HIV population, mechanistic insights from Alzheimer's Disease (AD) research (i.e. oxidative stress, protein quality control) has lead neurovirologists to question if similar aberrant mechanisms may contribute to age-related neuronal dysfunction in HAND.

1.2 Aging and Alzheimer's Disease (AD)

The neurological complications that accompany our aging population is a growing concern across economic, societal and health indices (Prince, Comas-Herrera, Knapp, Guerchet, & Karagiannidou, 2016). This mounting challenge is due to an unprecedented shift in aging demographics across the globe: by midcentury, the percentage of aged individuals (i.e. >60 years old) will exceed 21% (an increase from 10% in 2010), and, for the first time in human history, the number of aged persons will exceed the number of young (i.e. 10-24 years old) (World Health Organization, 2015). In turn, there are increasing numbers of individuals vulnerable to age-dependent neurological dysfunction, including increasing susceptibility to sporadic AD and other neuropathological detriments, such as HAND. For instance, between 2015 and 2030, global estimates of the number of years lost in full health due to AD suggest an increase of 36%, and, amongst the total number of deaths due neurological disorders, the proportion of fatalities due to AD are projected to increase by 9% (World Health Organization, 2006). In the United States, the roughly 5.5 million individuals suffering from age-associated cognitive detriments is expected to rise dramatically to 13.8 million by midcentury (Alzheimer's Association, 2018).

Furthermore, unlike other leading causes of death (e.g. cancer), where advances in treatment have alleviated symptomology and decreased mortality rates, no FDA approved therapy can stop or reverse the progression of AD (Herrmann, Lanctot, & Hogan, 2013). Meanwhile, the drug development process is largely unsuccessful, with the vast majority of preclinical results failing to translate into clinical benefits (Cummings, Morstorf, & Zhong, 2014; Sarter, Hagan, & Dudchenko, 1992a). For example, of those medicinal compounds advanced from preclinical to clinical trials to treat AD, a failure rate of greater than 99% is observed (Cummings et al., 2014; Sarter et al., 1992a; Sarter, Hagan, & Dudchenko, 1992b). Although progress in monoclonal immunotherapies provide optimism (e.g. aducanumab), it remains unclear if this pharmacological class will prove effective in large scale, AD cohorts (Bullain & Doody, 2020). Moreover, the costs of caring for patients remains similarly unsettling; after adjusting for inflation, calculations suggest an annual expenditure of over thirty-six thousand USD for the care (e.g. medical services, prescription costs, nursing home dues) of a single patient with AD; what's more, the indirect costs of patient management (e.g. unpaid care provided by family members, psychological burden on relatives) are not readily quantified and render the need to combat such pathologies that much more apparent (World Health Organization, 2006). As the solvency of health care systems is increasingly unstable, in conjunction with the increasing frequency of elderly individuals who require health services and the increasing cost of such services, addressing the age-related neurobiological mechanisms responsible for these impairments has never been more urgent.

The cognitive decline observed in AD is most commonly characterized by deficits in behavioral indices of information processing, reasoning, and most notably, in the domains of attention as well as memory (Glisky, 2007; Hedden & Gabrieli, 2004; Levy, 2005; Perry & Hodges, 1999). Interestingly, these neurocognitive impairments in AD are reminiscent of the decline in performance observed amongst HAND individuals upon neuropsychological assessment (Canet et al., 2018; Overton et al., 2013; Wendelken & Valcour, 2012). Such deficits are thought to be due to the compromised neural circuity that is primarily responsible for these higher-order cognitive functions, the basal forebrain cholinergic system and its principal innervations (i.e. prefrontal cortex (PFC) and hippocampus (HPC)) (Ballinger, Ananth, Talmage, & Role, 2016; Bishop, Lu, & Yankner, 2010; Hullinger & Puglielli, 2017). Progressive inefficiencies in these capacities have indeed been observed in AD and HAND, although the degree of impairment across specific domains can similarly vary in other age-related neurodegenerative pathologies, including, but not limited to, Lewy Body Dementia (LBD), and frontotemporal lobe dementia (FTLD) (N. Cairns et al., 2007; McKeith et al., 2005; McKhann et al., 2011). Furthermore, such deficits are also displayed amongst those age-related neurodegenerative pathologies that primarily afflict motor capabilities, including Parkinson's Disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's Disease (HD) (Emre et al., 2007; Paulsen, 2011; Phukan, Pender, & Hardiman, 2007).

As the extent of age-related functional changes can vary greatly between individuals, this suggests distinct underlying biological mechanisms that may contribute varying degrees of neuropathogenesis (Ebaid & Crewther, 2020; Verny, Moyse, & Krantic, 2015; R. Wilson et al., 2002). For instance, some older individuals retain intact cognitive functioning, or display only minimal decline, while others show significant cognitive impairments and can eventually develop AD as well as other forms of dementia (Fortenbaugh et al., 2015; Habib, Nyberg, & Nilsson, 2007; Verhaeghen & Cerella, 2002). Conversely, some individuals display profound deficits that progress at significantly elevated rates in comparison to age-matched peers (McDonough, Wood, & Miller, 2019; McMurtray, Clark, Christine, & Mendez, 2006). Interestingly, similar observations have been made in aging HIV individuals (Saloner et al., 2019). Meanwhile, resiliency to these impairments in aging and HIV can be observed among those who display certain epidemiological factors (i.e. healthy diet, aerobic exercise, increased cognitive stimulation) (Fazeli, Woods, et al., 2014; Frain & Chen, 2018; Kaur, Dendukuri, Fellows, Brouillette, & Mayo, 2020; Mapstone et al., 2013; Ngandu et al., 2015; Saloner et al., 2019; C. Zhao et al., 2018). While the neurobiological underpinnings responsible for this variation in age-related cognitive dysfunction have been subjected to much interrogation, it is increasingly relevant to determine if emerging mechanisms in AD may similarly contribute to adverse neuronal functioning associated with HAND.

1.3 Emerging Trends in HAND and AD

The postulation of common mechanisms underlying AD and HAND is spurred by their similar pathological manifestations, as well as contemporary trends in neuroscience and neurovirology. Across clinical and preclinical models, AD and HAND are associated with increased risk for impaired performance across similar cognitive domains (e.g. memory, attention) as well as similar neuropathological hallmarks (Dickens et al., 2017; Esiri, Biddolph, & Morris, 1998; Gisslén et al., 2009; D. R. Green et al., 2005; Putatunda et al., 2019; Webster, Bachstetter, Nelson, Schmitt, & Van Eldik, 2014). Moreover, each is associated distinct overlapping mechanisms in the brain (e.g. oxidative stress, protein quality control) that are age-dependent and contribute to impaired neuronal functioning (Brew, Crowe, Landay, Cysique, & Guillemin, 2009; Canet et al., 2018). Additionally, recent debate among AD researchers surrounding the amyloid-cascade hypothesis has encouraged examination of alterative disease mechanisms, including those implicated in HAND; meanwhile, accumulating data suggesting an increased risk for AD following CNS infections suggest the underlying pathways of neuronal dysfunction in HIV may be similar to pathogenic mechanisms in AD.

As the amyloid-cascade hypothesis of AD remains debated, other mechanisms have been subject to increased focus and may provide insights to HAND (i.e. oxidative stress, protein quality control) (Selkoe & Hardy, 2016). At the molecular level, AD is indeed commonly distinguished by its amyloid-beta (Aβ) plaques and hyperphosphorylated tau (pTau) neurofibrillary tangles (i.e. paired helical filaments) (Ittner & Götz, 2011; Selkoe & Hardy, 2016). However, much dispute surrounds the precise proportional combination, temporal dynamics and regional specificity of these particular polypeptides that are involved in AD (DeTure & Dickson, 2019; Ittner & Götz, 2011; Selkoe & Hardy, 2016). Some researchers argue the variation in age-induced cognitive deficits occurs independent of known pathological manifestations (Boyle, Fau, Wilson, Schneider, & Bennett, 2013; James et al., 2016; Negash et al., 2013; L. Yu et al., 2015). Indeed, early reports indicated that roughly a fifth of individuals remain cognitively sound despite meeting pathological criteria for Alzheimer's Disease (AD); while later evidence confirmed this finding, it was also suggested that the probability of insoluble plaque or neurofibrillary tangle absence in demented patients is comparable the probability of their presence in non-demented patients, despite displaying equivalent behavioral symptoms (Bennett et al., 2006; Davis, Schmitt, Wekstein, & Markesbery, 1999; Esiri et al., 2001; Jack et al., 2014; Jansen, Ossenkoppele, Knol, & et al., 2015; Nelson et al., 2012).

The rekindled debate surrounding the pathological contributions of Aβ and pTau has encouraged examination of potentially alterative biological underpinnings of cognitive decline and AD, such as aberrant oxidative stress and variation in protein quality control (Boyle, Wilson, et al., 2013; Ricciarelli & Fedele, 2017). Specifically, researchers have suggested these polypeptides may instead be a single factor in a spectrum of dynamic variables that interact to precipitate cognitive dysfunction in neurodegenerative conditions (Bu, Jiao, Lian, & Wang, 2016; Deary et al., 2009; Rahimi & Kovacs, 2014). As many of these variables have similarly been implicated in HAND (i.e. oxidative stress, protein quality control), such mechanisms may constitute common mediators of neuronal dysfunction, particularly in aging.

Consistent with this postulation, accumulating data indicate increased risk for AD following viral infections in the brain, suggesting the underlying pathways of neuronal dysfunction in HIV may be similar to pathogenic mechanisms in AD. Neurotropic viruses, such as HIV, pose a particular risk given their capacity to penetrate the blood-brain-barrier (BBB), infect cells of the central nervous system (CNS) (e.g. neurons, glia) and persist at low levels as latent infections (Hotta, 1997; Lévêque et al., 2014). Although each maintains unique genetic materials (e.g. single stranded, double stranded; RNA, DNA) encapsulated by one or more distinctive capsid proteins, the persistence of neurotropic viruses can be attributed in part to the integration of viral genomes with the host or their maintenance as extrachromosomal DNA (Gilden & Lipton, 2012). Latent reactivation of neurotropic viruses can then be triggered across an individual's lifespan by a variety of factors, including disruptions in antiviral therapeutics, environmental toxins, psychological stressors and co-infection with other pathogens (Baldwin & Cummings, 2018; Palmer et al., 2018). By utilizing gene regulatory mechanisms to then reproduce infectious particles, these microbes can disrupt various cellular functions and potentially contribute to pathological mechanisms (Gilden & Lipton, 2012; Ludlow et al., 2016).

While the role of pathogens in AD has been debated for decades, a series of seminal studies reporting HSV-1 DNA in plaques of AD patients suggested potential similar disease mechanisms in AD and neuroinfection (Jamieson, Maitland, Wilcock, Craske, & Itzhaki, 1991; Jamieson, Maitland, Wilcock, Yates, & Itzhaki, 1992; Sigurdsson, 1954; Sjogren, Sjogren, & Lindgren, 1952; Wozniak, Mee, & Itzhaki, 2009). Further studies indicated these viruses are more abundant in the brains of individuals displaying age-related cognitive decline and AD, particularly those with AD risk factors (e.g. APOE4) (Itzhaki et al., 1997; Wozniak et al., 2009). For instance, aged individuals previously infected with CMV (i.e. for an average of 5 years) display increased risk for AD and faster rates of decline in global cognition during follow-up assessment (L. Barnes et al., 2015). Such retrospective cohort studies also indicate increased likelihoods for developing dementia following VZV or HSV (HSV 1 or 2) infection, while this risk is considerably reduced amongst individuals receiving antiviral therapies (V. Chen et al., 2018; Tsai et al., 2017; Tzeng et al., 2018).

Although investigators have noted a variety of fungal and bacterial associations with AD, the particular mechanisms of these neurotropic viruses suggest their contribute to neurodegenerative disease progression (Gilden & Lipton, 2012; Ludlow et al., 2016).

Specifically, neurotropic viruses are thought to induce subacute neurobiological alterations that interact with environmental and/or genetic factors to progressively compromise functioning over time (Harris & Harris, 2018; Itzhaki, 2014). Recently, genomic analyses of comprehensive brain tissue collections, as well as preclinical examinations of Aβ's antimicrobial responses, have supported this claim (Allnutt et al., 2020; D. Cairns et al., 2020; Eimer et al., 2018; Readhead et al., 2018). Meanwhile, data now indicate Aβ's inherent antimicrobial properties, including its highly conserved polypeptide sequence and pathogen-relevant structural bioactivity (e.g. oligomerization, fibrilization), constitute innate immune responses to neurotropic infection that may become dysregulated over time and contribute to AD (Luna, Cameron, & Ethell, 2013; Moir, Lathe, & Tanzi, 2018; Yount, Bayer, Xiong, & Yeaman, 2006). Such subliminal interactions and chronic alterations in host cells may reflect an adaptive viral response in the CNS, provided that acute, severe infections in the immune-privileged brain might otherwise trigger significant immune responses (e.g. meningitis, encephalitis) and compromise the viability of host cells necessary for viral reproduction (Gilden & Lipton, 2012; Hotta, 1997). Taken together, the increased risk for AD associated with neurotropic infection indicates the neurobiological mechanisms in AD may similarly contribute to adverse neuronal functioning associated with HAND.

CHAPTER 2

MECHANISMS IN HAND

2.1 HIV Neuropathogenesis and Viral Proteins

Although HIV was initially thought to target peripheral immune cells (T-lymphocytes, monocytes/macrophages, dendritic cells), subsequent findings illustrated CNS immune cells are also susceptible to infection (Gartner et al., 1986; Koenig et al., 1986; Pope et al., 1994; Weissman, Li, Orenstein, & Fauci, 1995). While the exact mechanisms of infiltration remain debated, the virus is thought to cross the blood-brain-barrier (BBB) within weeks of systemic infection through infiltrating monocytes or infected CD+ T lymphocytes (Spudich & Gonzalez-Scarano, 2012; Valcour et al., 2012). Once in the CNS, microglia and perivascular macrophages serve as persistent and latent viral reservoirs, provided that they are the only resident cells in brain parenchyma that have been shown to support productive HIV infection (N. Chen, Partridge, Sell, Torres, & Martín-García, 2017; Crowe, Zhu, & Muller, 2003; K. Williams et al., 2001). Conversely, while limited evidence suggests neurons and other non-neuronal cells (e.g. astrocytes) may also be infected, it remains unclear if such cell types can support constitutive viral replication (Bissel & Wiley, 2004). Nonetheless, data illustrate HIV viral proteins secreted by actively infected cells within the CNS can be rapidly internalization by proximal CNS cells, particularly neurons, and compromise functioning in recipient cells (Y. Liu et al., 2000; Saribas et al., 2018).

Although its mechanisms remain actively debated, evidence indicates the adverse effects of specific viral proteins may ultimately contribute to HAND (Ellis, Langford, & Masliah, 2007; Avi Nath, 2002). The HIV genome is itself composed of nine genes that encode fifteen viral proteins (Frankel & Young, 1998). *Gag, pol,* and *env* code for structural proteins (MA, CA, NC), enzymes (Pro, RT, IN, RNase H), and envelope proteins (gp120, gp41), while the remaining genes code for regulatory (Tat, Rev) and accessory proteins (Vif, Vpr, Vpu, and Nef). Notably, these HIV proteins can be detected in excreted extracellular vesicles from HIV infected individuals, including Gp120, Vpr, Nef and Tat (Anyanwu et al., 2018). In addition to modulating viral replication, these proteins maintain a plethora of roles, including the regulation of host-cell gene expression, metabolic modifications, and alterations in intracellular signaling cascades (Frankel & Young, 1998; Swanson & Malim, 2008; Wyatt & Sodroski, 1998).

As suggested by initial investigations of gp120, the neurobiological mechanisms underlying HAND may be due the adverse consequences of HIV viral proteins (D. Barnes, 1987). Indeed, gp120 can dramatically influence HIV neuropathogenesis, particularly at the BBB, while exposure to gp120-containing extracellular vesicles can double the rate of infection in naive tissue (Arakelyan, Fitzgerald, Zicari, Vanpouille, & Margolis, 2017; Kanmogne, Kennedy, & Grammas, 2002; Louboutin & Strayer, 2012). Gp120 has also been shown to disrupt ion regulation and glutamate homeostasis amongst neurons and glia, which can subsequently contribute to contextual memory impairments *in vivo* (Fernandes, Edwards, Ng, & Robinson, 2007; Holden, Haughey, Nath, & Geiger, 1999; Z. Wang et al., 2004). Additionally, gp120 induces the production of proinflammatory cytokines, such as IL-1β, TNFα, and IL6, that subsequently elevate rates of apoptosis in both neuronal and non-neuronal cells (R. Cheung, Ravyn, Wang, Ptasznik, & Collman, 2008; W. Li, Galey, Mattson, & Nath, 2005; Yeung, Pulliam, & Lau, 1995). Interestingly, such deteriorated neuronal variability could be inhibited by the application of antioxidants, suggesting this viral protein's induction of oxidative stress might also contribute to compromised neuronal functioning (Louboutin, Agrawal, Reyes, van Bockstaele, & Strayer, 2012; Reddy, Agudelo, Atluri, & Nair, 2012). Along with oxidative stress, disrupted autophagic mechanisms are found in response to gp120, while data from primary neuronal culture and transgenic models suggest this viral protein's effects may be attributed to its dysregulation of mTOR-dependent signaling cascades (Fields et al., 2013; S. Liu et al., 2019).

Along with gp120, investigations of the HIV viral protein R (VpR) further suggest the neurotoxic effects of HIV viral proteins may contribute to neuronal dysfunction underlying HAND. Across a variety of primary cell models, including human hippocampal neurons as well as rat cortical and striatal neurons, VpR is neurotoxic (M.-B. Huang, Weeks, Zhao, Saltarelli, & Bond, 2000; C. A. Patel, Mukhtar, & Pomerantz, 2000; Piller, Jans, Gage, & Jans, 1998; Sabbah & Roques, 2005). Such compromised viability could be due to direct consequences in neuron's themselves, given that VpR can impair the functional capacitates of neuronal mitochondria while also inhibiting axonal stability (Kitayama et al., 2008; Y. Wang et al., 2017). Conversely, the consequences of this viral protein may be equally aversive across all CNS cell types; for instance, it can disrupt calcium regulation and induce pro-inflammatory cytokine expression in both neuronal and non-neuronal cells alike (Mamik et al., 2017; Na et al., 2011; Rom et al., 2009). Moreover, *in vivo* expression of VpR in the rodent brain can result in significantly decreased performance across long-term, spatial memory tasks as well as short-term, working memory tasks (Torres & Noel, 2014). While the adverse consequences of HIV viral proteins are hypothesized to facilitate neurobiological mechanisms underlying HAND, two viral proteins have been subject to particular focus, namely Tat and Nef.

2.1.1 The Trans-Activator of Transcription (Tat)

The Trans-activator of transcription (Tat) is a 14-16 kDa polypeptide that has extensively studied in the context of HAND, given its essential role in viral transcription (Jeang, 1996). After reverse transcription and integration into the host genome, proviral DNA of HIV stimulates the production of the Tat protein, which then binds the TAR element in the 5' LTR of HIV-1 RNA; subsequent recruitment of host cell elongation factors then significantly enhances rates of HIV transcription and enables viral replication (Conrad, Jeng, & Ott, 2013). While its proline and cysteine rich domains are thought to modify its diverse functions in target cells, Tat's arginine and glutamine rich domains facilitate its binding to cell surface receptors as well as subsequent cellular entry and exit (Spector, Mele, Wigdahl, & Nonnemacher, 2019). Interestingly, Tat's nucleic acid binding capacity, along with its net positive charge, low hydrophobicity and low sequence complexity, resembles the aggregate-prone polypeptides that can hinder neuronal functioning and PQC amongst age-related neurodegenerative pathologies, including AD (Kunihara, Hayashi, & Arai, 2019). This binding capacity may account for *in vitro* observations of Tat's capacity to enhance aberrant protein aggregation of pathologically relevant polypeptides, such as Aβ (Hategan et al., 2017; Rempel & Pulliam, 2005).

As the first viral protein to be transcribed and translated from integrated HIV proviral DNA, Tat is essential for the production of high viral loads and HIV pathogenesis (Arya, Guo, Josephs, & Wong-Staal, 1985). Consistent with its primary function in viral transcription, Tat strains which lack its basic domain are incapable of precipitating HIV replication by failing to recruit elongation factors, inhibiting viral mRNA transport and inhibiting reverse transcriptase (M. Lin et al., 2015; M. Lin et al., 2012; Meredith, Sivakumaran, Major, Suhrbier, & Harrich, 2009; Rustanti et al., 2017). Conversely, Tat mutants that maintain alternative expression systems but impaired TAR binding domains can nevertheless reestablish viral replication by improving promoter activity and activating transcription (Das, Harwig, & Berkhout, 2011).

In addition to its roles in HIV pathogenesis, evidence also indicates a potential role for Tat in the HAND development (Bagashev & Sawaya, 2013). Since the early 1990s, its role in facilitating neurotoxicity has been well documented (Hayman et al., 1993; Magnuson, Knudsen, Geiger, Brownstone, & Nath, 1995; Tardieu, Héry, Peudenier, Boespflug, & Montagnier, 1992). Such a role of Tat in facilitating HAND was specifically suggested by the reliable detection of extracellular Tat in the CNS of HIV infected individuals, despite adherence to cART and clinical aviremia (Henderson et al., 2019; Johnson et al., 2013). Furthermore, *in vitro* and *in vivo* examinations of Tat have replicated the disrupted homeostatic processes otherwise observed in the CNS of HAND individuals, such as impaired neuronal viability, disrupted synapse formation and elevated neuroinflammatory profiles (Ajasin & Eugenin, 2020; H. J. Kim, Martemyanov, & Thayer, 2008; A. Nath, Conant, Chen, Scott, & Major, 1999; Sabatier et al., 1991). For instance, by decoupling mitochondrial membrane potentials, reducing mitochondrial fusion and triggering apoptosis pathways via the permeabilization of mitochondrial membranes, Tat mimics the disruptions in neuronal metabolism implicated in HAND (Lecoeur et al., 2012; Rozzi, Avdoshina, Fields, & Mocchetti, 2018). Additionally, Tat-containing exosomes produced from a variety of cell types (i.e. primary mouse astrocytes, CD4+ T cells, 293T, U373) can impair neural cell viability and blunt neurite elongation (Rahimian & He, 2016a, 2016b). In replicating the persistent effects of Tat expression otherwise observed in the CNS of HIV+ individuals, chronic exposure to this viral protein in human neurons can also impair the regulation of cytoskeletal maintenance and anchoring proteins, along with reducing intrinsic excitability (Gurwitz et al., 2017). Despite this viral protein's diverse consequences on neuronal homeostasis, the pharmacological modulation of PQC or application of antioxidant reagents has been shown to mitigate Tat's neurotoxic effects (Fan & He, 2016a, 2016b; Zou et al., 2007).

Furthermore, a substantial body of evidence from preclinical models has illustrated both the exogenous application or endogenous expression of Tat in the CNS results in cognitive impairments reminiscent of HAND (Langford et al., 2018). Indeed, mouse models expressing Tat in the CNS display impaired contextual learning (i.e. conditioned place preference, fear conditioning), elevated levels of anxiety (i.e. open filed, marble burying, social interaction) as well as disrupted inhibitory control (i.e. Go/No-Go task) (Hahn et al., 2016; Hahn et al., 2015; Jacobs et al., 2019; Paris, Carey, et al., 2014; Paris, Fenwick, & McLaughlin, 2014; Paris, Singh, Ganno, Jackson, & McLaughlin, 2014). Similarly, these models maintain deficits across multiple measures of working memory (i.e. Y maze, Novel Object Recognition, Novel Location Recognition) and spatial memory (i.e. Barnes Maze, Morris Water Maze) (Carey et al., 2012; Kesby, Markou, & Semenova, 2016; Nookala et al., 2018). In addition, the cognitive deficits induced by Tat are not restricted to mice, provided that similar deficiencies are observed in rat models (Fitting, Booze, & Mactutus, 2008; Harricharan, Thaver, Russell, & Daniels, 2015; McLaurin et al., 2019; Repunte-Canonigo et al., 2014). Along with behavioral detriments, the *in vivo* expression of Tat is consistently associated with neuropathological measures observed in HAND (i.e. reduce gray matter volumes, dysregulated neuronal excitability, synaptic and axonal damage, BBB disruption and infiltrating peripheral immune cells), while many of these pathological effects can be exacerbated by advancing age (Carey et al., 2013; Dickens et al., 2017; B. Kim et al., 2003; Leibrand et al., 2017; Schier et al., 2017; Xiaojie Zhao, 2020). Along with these neurotoxic effects, emerging data suggest Tat may facilitate neurobiological dysregulation through two other pathogenic processes, namely oxidative stress and variations in cellular autophagy.

2.1.2 The Negative Regulatory Factor (Nef)

The Negative Factor (Nef) protein is a multifunctional, 27– 34-kDa polypeptide that has historically been understudied in the context of HAND (Carroll & Brew, 2017; V. Rao, Ruiz, & Prasad, 2014). A combination of X-ray crystallography and nuclear magnetic resonance has characterized Nef's three-dimensional structure, which consists of a folded core, with flexible N-terminal and C-terminal domains, as well as a central flexible loop within the folded core (Arold et al., 1997; Geyer & Peterlin, 2001; C.-H. Lee, Saksela, Mirza, Chait, & Kuriyan, 1996). While such structural features may account for Nef's abundant interactions with host cell proteins, its myristoylated N-terminus in particular allows for its association with the cytosolic face of cellular membranes and is required for Nef's cellular entry/exit (Fackler et al., 1997; Geyer, Munte, Schorr, Kellner, & Kalbitzer, 1999).

As its name implies, Nef was initially considered an inhibitor of viral genome transcription, but studies have since shown that Nef is essential for the maintenance of high viral loads, and promotes disease progression to AIDS (Gorry et al., 2007; Hanna et al., 1998; Thompson et al., 2003). For example, HIV particles produced in the presence of Nef are ten-times more infectious than particles produced in its absence (Delassus, Cheynier, & Wain-Hobson, 1991; Kestler et al., 1991). Conversely, HIV strains that lack a functional Nef protein result in delayed disease progression; here, strains with deletions in the Nef gene, due to truncated 3'-LTRs, result in normal CD4 counts and lower viral loads up 14 years post-infection, even in the absence of cART treatment (Deacon et al., 1995; Learmont et al., 1999).

In addition to its roles in HIV pathogenesis, evidence also indicates a potential role for Nef in the HAND development. This postulation was spurred by initial post-mortem analyses of brain tissue from HIV infected individuals; while only half of all patients maintained detectable Nef-positive cells, this rate increased to ~85% amongst those individuals who met behavioral criteria for dementia (Ranki et al., 1995). Similar to Tat, it can be reliably detected in patients despite suppressed viremia, while its mitigation after antiretroviral implementation is minimal compared to other HIV gene products (Fischer et al., 2004; Fischer et al., 2008; Raymond et al., 2011; Thomas et al., 2017). Along with altered concentrations of Nef within the CNS, HIV individuals displaying cognitive deficits maintain specific structural subtypes of Nef, compared to patients who remain cognitively stable. Following structural bioinformatics, computational modeling and proteomic analyses, findings suggest the Nef protein in the brains of individuals suffering from HAND maintain distinct structural alignments that potentially alter its conformational transitions and subsequent binding potentials (Lamers, Poon, & McGrath, 2011). Moreover, the Nef structural signatures associated with HAND, including modifications to SH3, AP-2 and cytokine bindings domains, were found to be specific to the CNS, further suggesting a role for Nef in contributing to neuronal dysfunction in the HIV brain (Lamers et al., 2018).

Nef may promote HAND through a number of complementary processes. Amongst neuronal and non-neuronal cell types, exposure to the Nef protein results in significantly decreased metabolic activity and increased rates of cell death (Trillo-Pazos, McFarlane-Abdulla, Campbell, Pilkington, & Everall, 2000). *In vivo* expression of Nef not only recapitulates such neuronal loss, but impairs locomotor activity and triggers a robust neuroinflammatory response, including an upregulation of IP-10; furthermore, such increased expression of IP-10 has also been detected in the brains of HIV individuals suffering from severe cognitive deficits (van Marle et al., 2004). Neural cell lines exposed to Nef-containing exosomes display several abnormalities, including enhanced amyloid precursor protein (APP) expression as well as elevated production and section of Aβ; similarly, compared to exosomes from cognitively sound individuals, exposure to Nef-containing exosomes from individuals diagnosed with HAND induces increased levels of toxic Aβ (Khan et al., 2016). Furthermore, Nef-containing exosomes secreted by primary human astrocytes induce elevated oxidative stress following reuptake by proximal primary neurons, while such exposure to Nef can also impair functional efficiencies in neurons, as indicated by blunted action potential frequency and decreased spiking activity (Saribas et al., 2018).

Along with direct impairments on neuronal functioning, Nef may contribute to cognitive impairments by inducing the recruitment of peripheral immune cells into the CNS (Koedel et al., 1999). Specifically, transplantation of Nef-expressing glia into the rat brain results in increased recruitment of peripheral macrophages, as well as neuronal apoptosis and a neuroinflammatory profile; in turn, these animals maintain significantly decreased performance on a variety of cognitive measures, including spatial and non-spatial memory tasks (Chompre et al., 2013; Mordelet et al., 2004). Such Nef-induced recruitment of peripheral immune cells and ensuing behavioral impairments may in part be due to Nef's capacity to increase expression of CCL2 in the brain, a chemoattractant for circulating monocytes (Chompre, Martinez-Orengo, Cruz, Porter, & Noel, 2019; Michael Herbert Lehmann, Jonas Michael Lehmann, & Volker Erfle, 2019). Similar to Tat, emerging data suggest Nef may compromise neuronal viability through two other pathogenic processes, namely oxidative stress and cellular autophagy.

2.2 Oxidative Stress

Evidence indicates oxidative stress may contribute to the mechanistic underpinnings of age-related neuronal dysfunction, particularly in HAND (Mollace et al., 2001). Here, oxidative stress refers to an imbalance between reactive oxygen species (ROS) generation and degradation that lead to its accumulation and ensuing consequences on cellular functioning (Pizzino et al., 2017). ROS are a group of molecules which include the more reactive superoxide, peroxynitrate and hydroxyl radicals as well as the less reactive hydrogen peroxide and nitric oxide (Jones, 2008; Navarro-Yepes et al., 2014). As their name implies, the electrostatic properties of these species allow them to react with a multitude of cellular components and influence a diverse range of homeostatic processes (Murphy et al., 2011). The inherent properties of the CNS render it particularly susceptible to the accumulation of ROS, further suggesting their pathological contributions to cognitive dysfunction. Along with the post-mitotic origin of neurons, the brain maintains less antioxidant defenses compared to other organs, while its increased energetic demand, in conjunction with decreased energetic reserve, is compensated by a high degree of vascular turnover, which itself can generate ROS due to increased heavy metal exposure (e.g. Fe, Mg) (Halliwell, 1992, 2006; Salim, 2017).

Indeed, abnormal ROS regulation and oxidative stress can induce variation in a diverse range of homeostatic mechanisms that can compromise neuronal functioning, including intracellular signaling cascades, gene transcription and ion regulation (Coyle & Puttfarcken, 1993; Devi & Satpati, 2017; Tonnies & Trushina, 2017). Given their diverse effects on cellular functioning, ROS production is not surprisingly accompanied by a number of enzymatic antioxidant mechanisms. For instance, superoxide dismutase (SOD 1,2,3) degrades O_2^- to H_2O_2, while catalase (CAT) and glutathione peroxidase (GPx) are responsible for degrading H_2O_2 to H_2O and O_2 (Nimse & Pal, 2015). In addition, non-enzymatic inhibition upon the part of nutrients can mitigate ROS levels (Vitamin E, Vitamin C) (B. Yu & Chung, 2006). However, the CNS in aging and HIV is commonly correlated with imbalances in reactive oxygen species (ROS), while the resulting oxidative stress is associated with impaired neuronal functioning (Bhat et al., 2015; Finkel & Holbrook, 2000; Uzasci, Nath, & Cotter, 2013).

The increased H_2O_2 in aging is particularly unique given its lack of an unpaired electron, which allows it to be more abundant and maintain the longest half-life compared to other ROS, in addition to its capacity to permeate lipid bilayers (Giorgio, Trinei, Migliaccio, & Pelicci, 2007; Halliwell, 1992). Given its membrane permeability, increases in H_2O_2 can migrate throughout the neuronal cytosol where it is capable of driving further ROS production (i.e. hydrogen peroxide generates the hydroxyl radical through fenton reactions, and generates peroxynitrate through reactions with nitric oxide) and contributing to the generation in aggregate-prone proteins (Murphy et al., 2011). For instance, through deamidation, α-amidation, β-scission and hydrogen abstraction, H_2O_2 can facilitate the cleavage of polypeptide backbones and expose hydrophobic residues that are highly aggregate prone (Chiti & Dobson, 2006; Stadtman & Levine, 2006). In addition, H_2O_2 modifications to amino acid sidechains, including oxidation, carbonylation and carbomylation, can decrease the activation energy required for crosslinking between proteins and increase the likelihood for ensuing polymerization into proteinaceous assemblies (Gorisse et al., 2016; Mirzaei & Regnier, 2008; Stadtman & Levine, 2006; Tanase et al., 2016).

The mechanistic relevance of ROS can be exhibited through its modulation of neuronal circuits implicated in cognitive decline in aging, specifically its capacity to blunt NMDA receptor functionality and subsequent calcium dyshomeostasis in the HPC (Oh, Simkin, & Disterhoft, 2016; Thibault, Gant, & Landfield, 2007). Initially, accumulated ROS can induce the formation of disulfide bonds on extracellular cysteine residues of NMDARs and drive the release in Ca^+ from intracellular stores (T. Foster, 2007; Hopp et al., 2015; Kumar & Foster, 2004; Sama & Norris, 2013; Yang et al., 2010). Within CA1, this blunted NMDAR functioning results in an abnormally increased threshold for depolarization (T. Foster, 2012; Kumar, Bodhinathan, & Foster, 2009;

Thibault et al., 2007). In turn, proportional increases in stimulation, via L-type voltage gated calcium channels (VDCCs), are required to allow sufficient Ca^+ influx for depolarization, which begets VDCC binding to intracellular ryanondine receptors, inducing more Ca release; in concert with enhanced calcium dependent K^+ channel slow burst afterhyperpolarization (sAHP), such effects further a cell's hypoexcitabile state and inhibit the encoding of the to be recalled stimuli in aging (Gant et al., 2015; Oh, Oliveira, & Disterhoft, 2010; Tombaugh, W., & Rose, 2005). Moreover, growing evidence indicates dysfunction in similar synaptic mechanisms within the prefrontal cortex and basal forebrain (Griffith et al., 2014; Guidi, Kumar, & Foster, 2015; McQuail et al., 2016). Thus, such adverse effects on neuronal signaling mechanisms highlight the potential relevance of abnormal ROS in facilitating age-dependent neuronal dysfunction.

2.3 Oxidative Stress in Aging and Alzheimer's Disease

Accumulating levels of oxidative stress within the brain are associated with age-related neurocognitive deficits and may provide important insights for the mechanisms underlying HAND (Bukrinsky et al., 1995; Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013). As indicated previously, the CNS is uniquely suspectable to the consequences of such variations compared to other organs (i.e. decreased anti-oxidant defenses, increased energy demand etc.) (Finkel & Holbrook, 2000). While the aging CNS is correlated with elevations in oxidative stress, such imbalances are associated with compromised neuronal homeostasis and impaired neuronal functioning, including in AD (Barnham, Masters, & Bush, 2004; Bhat et al., 2015; Giorgio et al., 2007; Tonnies & Trushina, 2017). Furthermore, this accumulation can be exacerbated due to a number of age-dependent variables, including chronic microglial activation, decreased mitochondrial efficiency, increased permeability of the blood-brain-barrier (BBB) and further compromised antioxidant mechanisms (Balaban, Nemoto, & Finkel, 2005; Finkel & Holbrook, 2000; Von Bernhardi, Eugenín-von Bernhardi, & Eugenín, 2015).

Notably, these sources of ROS in aging may potentiate further dysregulation in oxidative stress within the brain. For instance, microglia reactivity facilitates ROS generation, particularly under conditions of chronic activation (i.e. aging) (Pawate, Shen, Fan, & Bhat, 2004; Qin, Li, et al., 2005). As one mechanism, microglial NOX generate oxidative stress (i.e. superoxide) as a byproduct of degrading aberrantly folded proteins (Qin, Block, et al., 2005; Qin, Liu, Hong, & Crews, 2013; Qin et al., 2004). Similarly, microglia contain a number of other extracellular proteases that produce oxidative stress as a byproduct of receptor mediated phagocytosis of Aβ, including neprilysin and insulin-degrading enzyme (C. Y. Lee & Landreth, 2010). Directly, such oxidative stress can interact with intracellular signaling cascades to elevate the production and distribution of proinflammatory cytokines, which further generates oxidative stress (D. Brown & Griendling, 2015; Kierdorf & Prinz, 2013; Mrak & Griffin, 2005). Indirectly, oxidative stress can cause the aberrant processing of lipids, proteins and nucleic acids which in turn may impair neuronal functioning and/or sustain microglia as damage-associated-molecular-patterns (DAMPs) (Rubartelli & Lotze, 2007; Tang, Kang, Coyne, Zeh, & Lotze, 2012; Venegas & Heneka, 2017; Yamaguchi et al., 2020). Thus, while the dynamic regulation of oxidative stress is characteristic of the aging CNS, its capacity to compromise neuronal functioning suggests its role as an important regulator of neuronal homeostasis, such as in HAND.

2.4 Oxidative Stress in HAND

Given their capacity of alter a diverse range of homeostatic functions in host cells, including neurons and glia, studies have assessed if the ROS dysregulation in aging and AD may also contribute the neural mechanisms in HAND. Indeed, HIV infection results in elevated biomarkers for oxidative stress that persist despite viral suppression, and such increases can predict mortality in patients (Hulgan et al., 2003; Masiá et al., 2016; Suresh, Annam, Pratibha, & Prasad, 2009). In the CNS, increased oxidative stress is reported upon post-mortem analyses of HAND individuals, compared to infected yet cognitively sound counterparts, while such augmented levels in the CNS can predict the progression of deterioration in neurocognitive deficits (Boven et al., 1999; Haughey et al., 2004; W. Li et al., 2008; Sacktor et al., 2004; Zhang et al., 2012). In an extensive study that documented increased levels of oxidative stress in the CSF of HAND individuals, the *in vitro* application of such patients' CSF to primary human neurons resulted in increased mitochondrial dysfunction and apoptosis that correlated with the severity of cognitive impairment observed in patients; moreover, *in vitro* application of antioxidants blunted this CSF-induced neurotoxicity (Turchan et al., 2003). Provided that the CNS of HAND individuals is accompanied by ROS dysregulation, while HIV Tat and Nef proteins can compromise neuronal homeostasis, oxidative stress induced by these viral proteins may contribute to the neurobiological underpinnings in HAND.

2.4.1 Tat Effects on Oxidative Stress

Accumulating evidence indeed suggests the increased measures of oxidative stress observed in the CNS of HAND individuals may be caused by HIV viral proteins and their adverse consequences on efficient neuronal functioning (Ivanov et al., 2016; Mollace et al., 2001). In particular, Tat has been shown to reliably induce elevations oxidative stress and compromise viability across neural and glial cell types (Capone et al., 2013; Gorwood et al., 2020; X. Liu et al., 2002; Mastrantonio et al., 2016; Westendorp et al., 1995). Similarly, the intracerebral injection of Tat in rodent models results in neurotoxicity and significantly increased markers of oxidative stress, such as protein carbonylation and reactive gliosis (Aksenov et al., 2001; Bansal et al., 2000; Bruce-Keller et al., 2003). Consistent with detection of Tat in plasma of HIV+ individuals, systemic application of Tat can also induce increased measures of oxidative stress in the CNS (i.e. decreased antioxidant enzymes, lipid peroxidation etc.), suggesting viral proteins emanating from the periphery may contribute to oxidative damage in the brain (Banerjee, Zhang, Manda, Banks, & Ercal, 2010; Henderson et al., 2019; Nicoli et al., 2016). Similar to its effects in the CNS, evidence indicates Tat can induce oxidative stress in multiple peripheral cell types and impair homeostasis through several mechanisms, including DNA damage, disrupted oxidative phosphorylation and overactivation of specific intracellular pathways (e.g. JNK signaling) (El-Amine et al., 2018; Gu, Wu, Xu, Flores, & Terada, 2001).

The consequences of Tat-induced oxidative stress on neuronal homeostasis have further suggested its contributions in HAND development. Initial experiments utilizing primary human neuronal cultures confirmed a significant increase in oxidative stress and ensuing apoptosis in response to Tat (Shi, Raina, Lorenzo, Busciglio, & Gabuzda, 1998). Additional experiments in rodent primary neuronal cultures have supported the causal precedence of Tat-induced oxidative stress, including its significant dysregulation of mitochondrial dynamics and ensuing increases in mitochondrial ROS (Agrawal, Louboutin, Reyes, Van Bockstaele, & Strayer, 2012; Haughey et al., 2004; Kruman, Nath, & Mattson, 1998; Tiede, Cook, Morsey, & Fox, 2011). Such adverse consequences of Tat may be particularly apparent in the BBB, provided that multiple models of CNS endothelial cells reliably demonstrate Tat's induction of oxidative stress and dysregulation of redox-sensitive pathways (Price, Ercal, Nakaoke, & Banks, 2005; Toborek et al., 2003). Moreover, the application of ROS inhibitors (e.g. antioxidants) across neuronal and non-neuronal cell types can inhibit the increased oxidative stress and compromised viability otherwise induced by Tat (Agrawal et al., 2012; Gu et al., 2001; Haughey et al., 2004; Kruman et al., 1998). While the role of Tat-induced oxidative stress has been studied previously, its effects on corresponding mechanisms of neuronal homeostasis (e.g. protein quality control), further suggest its contributions to HAND development.

2.4.2 Nef Effects on Oxidative Stress

Consistent with the effect of Tat, accumulating data suggests elevations in oxidative stress within the CNS can be caused by Nef, while this increase may compromise ensuing neuronal functioning implicated in HAND (Ivanov et al., 2016; Uzasci et al., 2013). Given its diverse polypeptide regions, several investigations have sought to determine which domains are responsible for Nef's dysregulation of ROS. Examinations of microglia cell lines indicate the oxidative stress and associated neurotoxicity of Nef may be attributed to its mitrosylated region, but data employing primary microglia cultures have attributed Nef-induced oxidative stress to its Vav and Pak binding domains (Mangino et al., 2015; Vilhardt et al., 2002). Furthermore, examination of Nef's effects in human neutrophils suggest its interaction with the membrane component of antioxidant enzymes may contribute to elevations in oxidative stress, while in silica binding assays have credited these specific capacities to its FPDW domain (Salmen et al., 2010).

Compared to Tat, evidence for Nef-induced oxidative stress and its consequences for neuronal homeostasis is limited, but nonetheless derived from investigations utilizing both *in vitro* and *in vivo* models. For instance, exposure to Nef in primary human astrocytes results in increased ROS, mitochondria depolarization and apoptosis, while similar findings are observed in the brains of mice injected with Nef in subcortical regions (Acheampong et al., 2009). Such Nef-induced oxidative stress appears to be time-dependent and may be related to its intracellular trafficking, as suggested by data from human macrophages displaying biphasic increases in ROS in response to Nef (Olivetta et al., 2005). In line with these time-dependent effects, Nef induced oxidative stress can result in long term, downstream metabolic regulation (i.e. triglyceride accumulation, insulin resistance) (Gorwood et al., 2020).

Nef dysregulation of ROS may also be related to its direct effects on mitochondria, provided that mitochondrial dysfunction and ensuing oxidative stress following Nef exposure has been replicated across several human stem cell systems (i.e. bone marrow mesenchymal cells, adipose tissue cells) (Beaupere et al., 2015; Gorwood et al., 2020). Moreover, these adverse effects of Nef on mitochondrial dynamics and increased oxidative stress have been documented in neurons (Tiede et al., 2011). Similar to Tat, the application of ROS inhibitors (e.g. antioxidants) appears to inhibit the increased oxidative stress and compromised viability otherwise induced by Nef (Ochoa et al., 2010; T. Wang et al., 2014). Along with its induction of oxidative stress, Nef's effects on corresponding mechanisms of neuronal homeostasis (e.g. protein quality control), further suggest its contributions to HAND development.

2.5 Protein Quality Control (PQC)

Given the abundant diversity in the CNS proteome, efficient protein quality control (PQC) is necessary to maintain neuronal functioning and homeostasis (Hipp, Kasturi, & Hartl, 2019; Höhn, Tramutola, & Cascella, 2020). The specific PQC systems utilized to clear a given protein are dictated by its inherent properties (e.g. quaternary structure), in conjunction with an array of enzymes (e.g. ligases), disposal mechanisms (e.g. lysosomal degradation) and transport complexes (e.g. chaperones) (Dikic, 2017; Schrader, Harstad, & Matouschek, 2009). One facet, the ubiquitin-protease-system (UPS), is responsible for clearing short lived protein species, whereby ubiquitin-bound substrates are shuttled to the 26s proteasome via the Hsc70-BAG1 chaperone complex, and undergo degradation following incorporation into catalytic barrels (Alberti, Esser, & Höhfeld, 2003; Ciechanover, 2005; Ravid & Hochstrasser, 2008). Conversely, peptide assemblies not cleared by the proteasome and its subunits (i.e. 19S, 20S components), can be degraded via autophagy; here, double membrane structures called autophagosomes sequester unwanted proteins and subsequently fuse with lysosomes to ensure catabolism of their contents (Glick, Barth, & Macleod, 2010). While the former degradation pathway is mediated by BAG1, this latter targeting of substrates for autophagic degradation is conversely mediated by BAG3 (Behl, 2016). Given the complementary roles of BAG1 and BAG3 in UPS degradation and autophagy, dynamic levels of these co-chaperones are hypothesized to be crucial for maintaining neuronal PQC and homeostasis (Alberti et al., 2002; S. Zhao et al., 2019)

Through their shared BAG domain, the BCL-2 associated athanogene (BAG) family of co-chaperones are necessary for efficient PQC by functioning as nucleotide exchange factors, thereby modulating the activity of Hsp70 via its ATPase domain (Sondermann et al., 2001; Takayama & Reed, 2001). In its ADP-bound conformation, Hsc70's substrate binding domain can recognize specific hydrophobic amino acid motifs, recruit PQC modulators, and mediate ensuing protein degradation (Hartl, Bracher, & Hayer-Hartl, 2011; Rüdiger, Germeroth, Schneider-Mergener, & Bukau, 1997). BAG family members function as a nuclear exchange factors by hydrolyzing Hsc70-bound-ATP and thus ensuring efficient Hsc70 functioning (Alberti et al., 2003; Rosati, Graziano, De Laurenzi, Pascale, & Turco, 2011). BAG3, in addition to stimulating removal of malformed or aggregated polypeptides via autophagy, is emerging as a crucial regulator of stress granules (SGs), membrane-less compartments that can facilitate aberrant accumulation of aggregated proteins (Ganassi et al., 2016; Mateju et al., 2017). Along with its potent anti-apoptotic properties, growing data also suggest BAG1 levels are inversely proportional to BAG3, potentially due to its compensatory clearance of ubiquitinated substrates via the UPS (Gamerdinger et al., 2009; Minoia et al., 2014; Townsend, Cutress, Sharp, Brimmell, & Packham, 2003). Such a functional transition between BAG1 and BAG3 dependent PQC is postulated to be an adaptive preservation of neuronal homeostasis under conditions that might otherwise facilitate neuropathogenesis, such as viral infection, aging and elevated oxidative stress (Behl, 2016; Gamerdinger, Kaya, Wolfrum, Clement, & Behl, 2011).

In addition to role in autophagy, accumulating data indicate BAG3 is an important regulator of stress granules (SGs) (Ganassi et al., 2016; Kampinga & Craig, 2010; Y.

Kim, Hipp, Bracher, Hayer-Hartl, & Hartl, 2013). SGs are membrane-less cytosolic compartments whose dynamics are increasingly relevant to the underlying mechanisms of neurodegeneration (Benarroch, 2018; Gan, Cookson, Petrucelli, & La Spada, 2018). Under normal physiological conditions, transcripts are bound to RNA binding proteins (RBPs) in ribonucleoprotein (RNP) complexes, which function to translocate mRNA to sites of protein translation and modify protein synthesis (Kiebler & Bassell, 2006). Upon the detection of a stress factor, RNPs are disassembled, while mRNA-bound RBPs are rapidly reformed into SGs in order to sequester transcripts which might otherwise be degraded due to the molecular consequences of the stressor itself or the host cell's non-specific defense mechanisms (Buchan & Parker, 2009; Protter & Parker, 2016). This SG assembly has been observed in response to a variety exogenous (e.g. heat stress, drug treatment) as well as endogenous factors (e.g. oxidative stress), although these applications have been restricted to cell lines (Aulas, Lyons, Fay, Anderson, & Ivanov, 2018; J. Brown et al., 2011; Daigle et al., 2016; M. M. Emara et al., 2012). Subsequently, when stressful conditions are ameliorated, SG disassembly is facilitated by BAG3-dependent machinery (Anderson & Kedersha, 2008; Kedersha & Anderson, 2002; Panas, Ivanov, & Anderson, 2016). However, SG persistence can then hinder disassembly processes by BAG3-depedent mechanisms and contribute to further protein accumulation into aggregates (Daigle et al., 2016; Ganassi et al., 2016; Lechler et al., 2017; A. Patel et al., 2015).

Impaired PQC in neurons can facilitate several adverse consequences, including the aberrant processing of malformed polypeptides as well as subsequent perturbations in homeostasis and impairments in efficient functioning (Currais, Fischer, Maher, &

Schubert, 2017; Invernizzi, Papaleo, Sabate, & Ventura, 2012; Kampinga, Mayer, & Mogk, 2019). In particular, aggregate formation can be initiated following anomalous interactions between polypeptides that are induced by distinct intermolecular properties in said proteins, including aberrant post-translational modifications and variation in amino acid sequences (Fink, 1998). Subsequent interactions amongst proximal counterparts, as well as nascent bonding with distal species, facilitates the polymerization of polypeptides into amorphous assemblies or highly ordered structures (i.e. amyloid) that are not efficiently degraded by PQC (Chiti & Dobson, 2006; Invernizzi et al., 2012). In turn, the persistence of impaired PQC and the associated consequences across a multitude of cellular processes can ultimately result in a net loss of function (Kampinga et al., 2019; Stefani & Dobson, 2003).

2.6 PQC in Aging and Alzheimer's Disease

In addition to oxidative stress, the causal mechanisms underlying neuronal dysfunction in AD and HAND may be related to another common correlate of pathogeneses, namely the failure to maintain intracellular PQC (Aviner & Frydman, 2020; Kurtishi, Rosen, Patil, Alves, & Moller, 2018; Ross & Poirier, 2004). Indeed, aging in general can be characterized by an increased population of aggregate-prone proteins (D. David, 2012; Klaips, Jayaraj, & Hartl, 2018). This elevation in polypeptides that maintain increased aggregation propensities is sometimes referred to as supersaturation (Ciryam, Kundra, Morimoto, Dobson, & Vendruscolo, 2015; Ciryam, Tartaglia, Morimoto, Dobson, & Vendruscolo, 2013). Although the mechanisms that generate such proteins are not fully elucidated, current postulations consider the elevated oxidative stress in aging as a primary causal factor (Hohn, Konig, & Grune, 2013; Santos & Lindner, 2017).

Although the role of an aggregating proteomic profile remains debated in AD, interrogations demonstrating a robust association between AD pathogeneses and specific genomic variations suggest perturbed PQC is a contributing mechanism (J. P. Taylor, Hardy, & Fischbeck, 2002). For instance, numerous mutations within the Aβ sequence in AD patients result in protein conformations that increase its propensity to aggregate (Hatami, Monjazeb, Milton, & Glabe, 2017). Similarly, deleterious mutations amongst those protein quality control mechanisms responsible for maintaining PQC also result in increased propensities for aggregation (Ciechanover & Brundin, 2003; Levine & Kroemer, 2019; Macario, Grippo, & Conway de Macario, 2005). Curiously, as aging can compromise proteostasis and facilitate protein aggregation, while proteome maintenance has been linked to increased fitness across mammalian and non-mammalian species, the consequences of alterations in cellular PQC appear to be particularly relevant to underlying mechanisms in age-related cognitive decline, including AD and HAND (D. C. David et al., 2010; Pérez et al., 2009; R. C. Taylor & Dillin, 2011; Treaster et al., 2014).

In addition to the increased generation of aggregate-prone polypeptides, decreased efficiencies across a variety of PQC processes are observed in aging and AD, particularly those that are otherwise responsible for degrading malformed or aggregated polypeptides (i.e. autophagy) (Klaips et al., 2018; Menzies et al., 2017; Vilchez, Saez, & Dillin, 2014). Interestingly, Hsc7-BAG3 functioning appears to be particularly vulnerable. For instance, mitochondrial functioning and oxidative phosphorylation are hindered with age, which inherently limits the ability of the ATP-dependent chaperones, including Hsc70-BAG3, to fulfill its functions (Beal, 2005; Brehme et al., 2014; Jain et al., 2016). Potentially due to the complementary relationship between the UPS and autophagy, aged cells also maintain mitigated levels of BAG1 and elevated levels of BAG3, indicative of a compensatory yet disproportionate demand for Hsc70-BAG3 functioning in age (Behl, 2016; Gamerdinger et al., 2009). In addition, evidence gathered from preclinical and clinical samples illustrates the aged and AD brain exhibits dysregulated levels of proteins that are necessary for phagophore elongation and ensuing autophagosome formation, including LC3 (Guebel & Torres, 2016; Heckmann et al., 2019; Kaushik et al., 2012; Y. Yu et al., 2017). Similarly, both aged rodents as well as elderly individuals exhibiting AD symptomology maintain decreased levels of Hsc70 in the CNS (Gleixner et al., 2014; Loeffler, Klaver, Coffey, Aasly, & LeWitt, 2016). Interestingly, clinical evidence also suggests the aged brain maintains decreased expression for HDA6, the adapter protein that ensures efficient transport of Hsc70-BAG3 bound substrates (Guebel & Torres, 2016). Therefore, not only does aging and AD induce conditions that are capable of hindering neuronal functioning (i.e. increased PQC demands), but the capacity to mitigate these consequences is concomitantly reduced (i.e. decreased PQC capacity).

In addition to variations in PQC, particularly autophagy, evidence indicates aging conditions may comprise the regulation of SGs (Moujaber et al., 2017). Given the capacity for RNA-Binding Proteins (RBPs) in SGs to recruit aggregate-prone polypeptides through the induction of polypeptide crosslinking, in conjunction with the lack of membrane encapsulation that might otherwise inhibit such contacts, protein-protein interactions in SGs can be strengthened and facilitate the condensation of SGs into insoluble cores that cannot be disassembled (Jain et al., 2016; Mateju et al., 2017).

Thus, as aging is associated with elevation of aggregate-prone proteins and may dysregulate SG formation, these conditions may also induce persistent SGs and contribute to further PQC dysregulation.

2.7 PQC in HAND

Given the importance of maintaining efficient PQC, particularly in response to the demands on autophagy in aging, dysregulation of these homeostatic processes within neurons may contribute to the pathological mechanisms in HAND. Although it can serve as an innate self-defense mechanism to control viral spread, particularly follow initial infection, aberrant cellular autophagy is often correlated of HIV pathogenesis (Chiramel, Brady, & Bartenschlager, 2013; Killian, 2012; Lennemann & Coyne, 2015). In HIV individuals displaying cognitive impairments, modulation of autophagy pathways (i.e. phagophore elongation factors, lysosomal internalization peptides) has been documented across cortical regions of the brain (Dever, Rodriguez, Lapierre, Costin, & El-Hage, 2015; Zhou, Masliah, & Spector, 2011). Rather than severe effects, some data suggest the neuronal modulation of autophagy in HAND may induce subacute intracellular alterations that ultimately decrease the threshold for downstream neuronal dysfunction and apoptosis (Alirezaei, Kiosses, & Fox, 2008). While some evidence suggests aberrant UPS activation also accompanies neurocognitive deficits amongst HIV infected individuals, these data remain limited and encourage further elucidation (Nguyen, Soukup, & Gelman, 2010). Interestingly, an *in vitro* analysis of transcriptional variation in response to HIV viral vectors reported repressed levels of BAG1 (Mitchell, Chiang, Berry, & Bushman, 2003).

In regard to BAG3 regulation following HIV infection, additional examination appears warranted, provided that varying patterns of BAG3 regulation have been reported across differing models of HIV (J. Y. Cheung et al., 2015; Rosati et al., 2009; Rosati et al., 2007). Furthermore, the HIV associated effects on SGs warrant similar examination, as some data indicate viruses can potentiate SG formation, others suggest it may disrupt SG stabilization (Basu, Courtney, & Brinton, 2017; Bonenfant et al., 2019; Mohamed M. Emara & Brinton, 2007; Roth et al., 2017; White & Lloyd, 2012). Indeed, data in cell lines indicates the potential for HIV RNA to accumulate at SG foci, while other studies have noted HIV can increase SG assembly or increase disassembly/inhibit formation (Cinti, Le Sage, Ghanem, & Mouland, 2016; S. Rao et al., 2018; S. Rao et al., 2019; Soto-Rifo et al., 2014). Along with direct investigations of HIV, accumulating data demonstrate dysregulated PQC mechanisms may be caused by HIV proteins themselves, particularly Tat and Nef.

2.7.1 Tat Effects on PQC

The dysregulated PQC mechanisms associated with Tat may contribute to the neurobiological underpinnings of neuronal dysfunction in HAND. Investigations of Tat's effects on UPS are limited, particularly in neuronal models, but indicate its capacity to directly modulate multiple proteasomal mechanisms (e.g. ligases) and induce aberrant downstream consequences, including NMDA receptor malfunctioning (M. V. Green & Thayer, 2016; Lata, Mishra, & Banerjea, 2018). Additionally, accumulating evidence indicates Tat can independently dysregulate autophagy across various cell types, albeit in varying patterns. In primary rodent neuronal cultures as well as cell lines, Tat can induce ubiquitin-independent activation of autophagy, including downstream elevations in autophagosome regulators (J. Li et al., 2018; Sagnier et al., 2015). Conversely, primary rodent hippocampal neurons exposed to Tat also display dose and time dependent decreases in levels of LC3, a protein which is otherwise responsible for initiating autophagosome formation; furthermore, such Tat-induced variation in autophagy is accompanied by elevated neurodegeneration *in vivo*, particularly in the CA3 region of the hippocampus (Fields et al., 2015; Hui, Chen, Haughey, & Geiger, 2012).

While Tat's effects on BAG1 have not yet been investigated, experiments from our laboratory and others have demonstrated similar varying patterns in BAG3 levels in response to Tat. In glioblastoma cell models, Tat induces BAG3-dependent autophagy pathways, while the direct mitigation of BAG3 inhibits such Tat-induced autophagic processes (Bruno et al., 2014; Wu et al., 2018). Conversely, a significant downregulation of BAG3 is characteristic of HIV-transgenic mouse models as well as primary rat neurons expressing the Tat protein (Mohseni Ahooyi et al., 2019). Notably, Tat's effect on SGs remains unknown, although its incorporation into SG foci was recently noted in cell lines (Lai et al., 2013). Although patterns of Tat-induced autophagy dysregulation can vary, disparate cell models and a limited number of investigations behooves further characterization of Tat's effect on neuronal PQC and ensuing neuronal functioning.

2.7.2 Nef Effects on PQC

Similar to Tat, the dysregulated PQC mechanisms associated with Nef may contribute to the neurobiological underpinnings of neuronal dysfunction in HAND. While its effects on UPS are understudied and restricted to cell lines, data indicate Nef can disrupt PQC by triggering the abnormal proteasomal degradation of host cell polypeptides, particularly cell surface proteins (Matheson et al., 2015; Pyeon et al., 2019). While its effects on other UPS modulators require further investigation (i.e. BAG1), Nef's capacity to disrupt cellular autophagy is well documented (Beaupere et al., 2015; Kyei et al., 2009). Here, Nef's binding to BECN1 and ensuing inhibition of autophagosome formation can silence autophagy to levels that are equivalent to uninfected cells (Grant R. Campbell, Pratima Rawat, Rachel S. Bruckman, & Stephen A. Spector, 2015). The sequestration of BECN1 and inhibition of autophagy due to Nef has been replicated in a variety of cell lines and primary cultures, while recent evidence also indicates this effect could be due to Nef's indirect yet further blockade of BECN1 through enhanced binding to Bcl2, as well as Nef's capacity to prevent LC3 lipidation (Castro-Gonzalez et al., 2020). In turn, Nef-dependent inhibition of autophagy can significantly increase cellular susceptibility to apoptosis (Gupta et al., 2017).

The variation in autophagy within the CNS due to Nef may play a crucial role in HAND development, although further investigations employing neuronal cell models are necessary to validate this postulation. In primary human astrocytes transduced with Nef expressing vectors, the increased levels of Nef protein emulated an autophagic blockade induced by bafilomycin treatment (i.e. accumulation of ATG8 and p62). Along with morphological alterations indicative of apoptosis, co-transduction with the tandem LC3 vector in these cells illustrated Nef expression inhibited autophagosome fusion with lysosomes (Saribas, Khalili, & Sariyer, 2015). A more recent study utilizing a similar cell model suggested Nef increases autophagosome degradation but not formation, while its stabilization of p62 levels indicated it effects on autophagy may be dependent on the degradation pathways specific to a given substrate (Cheney et al., 2020). Although its effects on BAG1 and BAG3 remains unknown, the expression of Nef in cell lines can be suppressed via sequestration into SGs, suggesting the translation of Nef protein may perturb SG disassembly processes and contribute to impaired PQC (Henao-Mejia et al., 2009). While further characterization of Nef's effect on neuronal PQC appear warranted, particularly in regard to BAG chaperones and SGs, these alterations in PQC mechanisms may contribute to the neurobiological underpinnings of neuronal dysfunction in HAND.

CHAPTER 3

AIM ONE: EFFECTS OF HIV PROTEINS ON NEURONAL HOMEOSTATIC MECHANISMS

3.1 Rationale

To assess the neurobiological mechanisms of HAND, the current experiments investigated if mechanistic insights from Alzheimer's Disease, such as oxidative stress and protein quality control (i.e. stress granules, BAG chaperones), may similarly contribute to neuronal dysregulation in response to HIV proteins Tat and Nef. Thus, Aim One was designed to assess how the *in vitro* expression of HIV Tat and Nef independently contributed to perturbations in homeostatic mechanisms in primary neuronal cultures. Manipulations employed adenoviral transduction to ensure efficient expression of viral proteins in rat neurons cultured from neonatal embryos, as well as a variety of biochemical techniques to gauge ensuing effects on specific mechanisms. While this aim established a fundamental relationship between viral proteins and SG markers, it also expanded the understanding of how viral proteins, PQC and oxidative stress may interact to perturb neuronal homeostatic mechanisms underlying age-related neurocognitive dysfunction, particularly in HAND.

Given its relevance to age-related cognitive dysfunction, Tat or Nef induced variation in neuronal PQC mechanisms (i.e. SGs, BAG 1&3) was the primary objective for this aim. These targets were assessed by measuring the protein levels (i.e. immunoblot, immunocytochemistry) and transcriptional regulation (i.e. qPCR) of pathway specific polypeptides. In particular, variation in three reliable and valid markers of SGs were quantified, T-cell intracellular antigen 1 (TIA1), Ras GTPase-activating protein-binding protein 1 (G3BP1) and Y-box binding protein 1 (YB1) (Gilks et al., 2004; Somasekharan et al., 2015; Tourriere et al., 2003). Due to their multiple roles in PQC, including the regulation of SGs, levels of BAG1 and BAG3 were also quantified. In addition, variation in oxidative stress induced by viral proteins, as well as the consequences of such oxidative stress on neuronal PQC, were assessed. Specifically, variation in ROS due to Tat or Nef expression were quantified, while ensuing investigations determined if the direct application of ROS via H_2O_2 could recapitulate the effects of viral proteins on neuronal PQC. Along with previous techniques, assessment here employed metabolic and viability assays, as well as live-cell, confocal microscopy.

Provided that the prolonged formation of SGs is associated with dysfunctional PQC, and both Tat as well as Nef can hinder efficient PQC in addition to dysregulating oxidative stress, I hypothesized that viral protein expression will result in increased levels of SG markers, BAG3 and BAG1 chaperones, while these alterations may be mediated by Tat or Nef induced oxidative stress.

3.2 Experimental Procedures

Primary rat neuronal cultures were prepared using dissected E18 prenatal rat embryonic brains and maintained under sterile, controlled conditions until cells obtained sufficient maturity. On Day in Vitro (DIV) 14, cells were be transduced with either Ad-Tat, Ad-Nef or Ad-Null in a manner previously described (Mohseni Ahooyi, Shekarabi, Torkzaban, et al., 2018). Ad-Tat and Ad-Nef refer to adenoviral vectors that induce the expression of the HIV Tat or Nef proteins, whereas Ad-Null contains an empty vector, allowing it to serve as a negative control treatment. Multiplicity of infection (MOI) was maintained at 1 for all vectors throughout procedures (i.e. 1 viral particle/cell).

Transduction durations were maintained in accordance with previous experiments in our laboratory (72 Hr). Two hours prior to transduction, culture media was changed to ensure viability throughout treatment incubation; such two-hour duration was necessary to allow cells to recover from any potential dyshomeostasis induced by media change prior to transduction.

The time at which experiments were conducted (i.e. DIV 12-30) enables the establishment of mature neurons (e.g. synapse formation, action potentials) without compromised neuronal viability or excessive non-neuronal cell proliferation (Basarsky, Parpura, & Haydon, 1994; Gordon, Amini, & White, 2013; Grabrucker, Vaida, Bockmann, & Boeckers, 2009). After transduction treatment, cells were harvested under sterile conditions for subsequent analysis. All experiments were completed in triplicate to confirm the reliability of results. In order to measure protein levels, cells were lysed followed by western immunoblotting utilizing SDS-polyacrylamide gel electrophoresis. To similarly gauge protein levels, as well as assess their spatial disruption within neurons, immunocytochemistry was conducted in conjunction with high-resolution, fluorescent microscopy. In order to examine alterations in the transcription of relevant genes, RNA was extracted, and RT-qPCR was conducted following cDNA synthesis. To directly assess levels of oxidative stress, reactive oxygen species (ROS) were measured using a mitochondrial oxygen free radical indicator coupled with confocal imaging in an open perfusion microincubator. The assessment of neuronal metabolic activity and viability utilized a colorimetric oxidoreductase assay (MTT) and permeability-exclusion assay (Trypan Blue), respectively (for detailed Materials and Methods, please refer to Chapter 6).

3.3 Results

Analyses suggested neither Tat nor Nef influenced the protein levels of the SG marker TIA (Figure 1 A-C). Immunoblotting results demonstrated signal intensities of TIA did not significantly differ as a result of Tat expression compared to control (t (4) = 0.19, p = .851) and Ad-Null conditions (t (4) = 0.59, p = .586). Additionally, signal intensities of TIA did not significantly differ as a result of Nef expression compared to control (t (4) = 0.47, p = .657) and Ad-Null conditions (t (4) = 0.94, p = .399) (Figure 1 A&B). Immunocytochemistry and densiometric analysis indicated that neurons expressing Tat did not maintain significantly different levels of TIA (138.11 ± 15.82 μm^2/cell) compared to those neurons subjected to Ad-Null transduction (159.79 ± 17.39 μm^2/cell; t (4) = 1.57, p = .191). Similarly, neurons expressing Nef (142.41 ± 22.26 μm^2/cell) did not illustrate significant variation in levels of TIA compared to control conditions, (t (4) = 1.09, p = .337) (Figure 1 C). Along with minimal variation protein levels, qPCR indicated TIA expression did not significantly differ in Ad-Tat treated neurons compared to control (t (4) = 2.42, p = .072) and Ad-Null conditions (t (4) = 1.78, p = .149). Additionally, TIA expression did not significantly differ as a result of Nef compared to control (t (4) = 2.44, p = .071) and Ad-Null conditions (t (4) = 2.50, p = .064) (Figure 1 D).

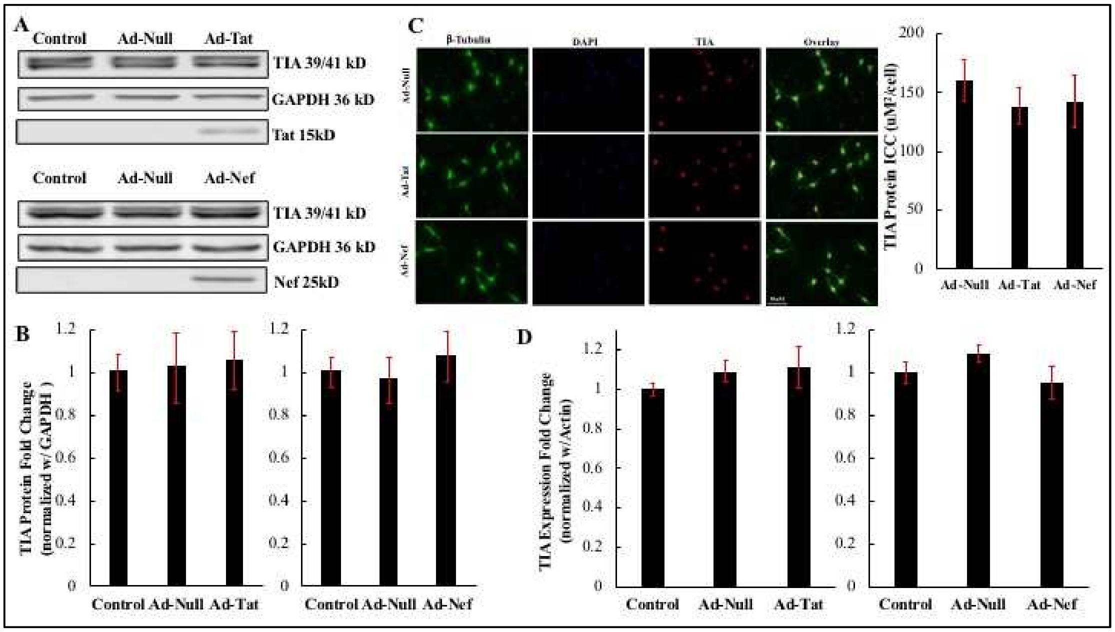

Figure 1. Effects of Tat and Nef on SG-Marker TIA. Immunoblot images (A) and quantifications (B) of TIA protein levels. Immunocytochemistry images and quantifications (C) of TIA protein levels. Quantitative PCR of TIA RNA levels (D).

Similar to results obtained upon analyses of TIA, assessment of G3BP protein levels suggested neither Tat nor Nef substantially perturbed levels of this SG marker (Figure 2 A-C). Immunoblotting results demonstrated G3BP protein levels did not significantly differ as a result of Tat expression compared to control (t (4) = 0.17, p = .869) and Ad-Null conditions (t (4) = 0.09, p = .936). Consistent with the observed effects of Tat, immunoblotting analyses indicated Nef expression did not influence the levels of G3BP protein compared to control (t (4) = 0.06, p = .951) or Ad-Null conditions (t (4) = 0.02, p = .981) (Figure 2 A&B). Immunocytochemistry validated the lack of alterations in G3BP protein levels in Tat (166.89 ± 17.32 μm^2/cell; t (4) = 1.07, p = .343) and Nef expressing neurons (172.23 ± 20.68; t (4) = 0.71, p = .514) compared to control conditions (185.79 ± 23.63) (Figure 2 C). In addition to the benign effects on G3BP protein levels, qPCR indicated its expression did not significantly differ in Ad-Tat treated neurons compared to control (t (4) = 1.51, p = .203) and Ad-Null conditions (t (4) = 0.31, p = .774). Likewise, G3BP expression did not significantly differ as a result of Nef compared to control (t (4) = 1.99, p = .117) and Ad-Null conditions (t (4) = 0.60, p = .579) (Figure 2 D).

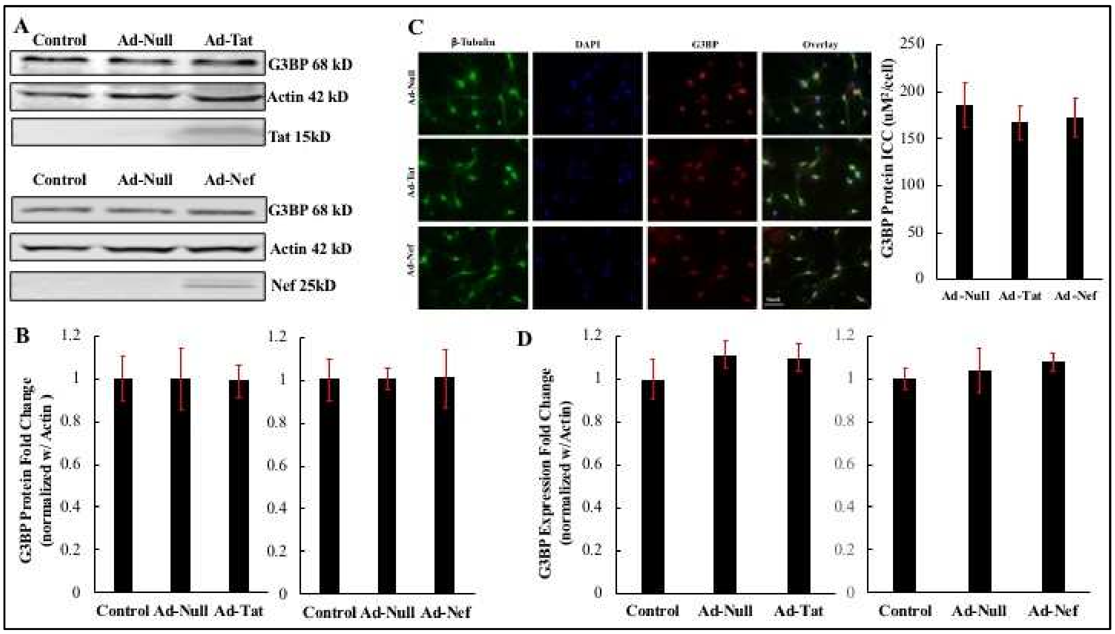

Figure 2. Effects of Tat and Nef on SG-Marker G3BP. Immunoblot images (A) and quantifications (B) of G3BP protein levels. Immunocytochemistry images and quantifications (C) of G3BP protein levels. Quantitative PCR of G3BP RNA levels (D).

While no significant effects of HIV proteins on TIA or G3BP were observed, minor variations were present in YB1 following Tat expression, but not Nef (Figure 3 A-C). Similar to immunoblotting results of other SG markers, signal intensities of YB1 did not significantly differ as a result of Tat expression compared to control (t (4) = 0.18, p = .860) and Ad-Null conditions (t (4) = 0.74, p = .499). Additionally, signal intensities of YB1 did not significantly differ as a result of Nef expression compared to control (t (4) = 0.95, p = .393) and Ad-Null conditions (t (4) = 1.12, p = .324) (Figure 3 A&B). Notably, immunocytochemistry analysis indicated that neurons expressing Tat did maintain significantly elevated levels of YB1 protein (221.89 ± 16.88 μm^2/cell) compared to those neurons subjected to Ad-Null transduction (174.74 ± 11.83 μm^2/cell; t (4) = 4.01, p = .016). This equated to an approximate increase of 27%. However, neurons expressing Nef (179.41 ± 14.36 μm^2/cell) did not illustrate significant variation in protein levels of YB1 compared to Ad-Null conditions, (t (4) = 0.45, p = .674) (Figure 3 C). Along with effects on YB1 protein levels, qPCR indicated its expression was significantly increased in Ad-Tat treated neurons compared to control (t (4) = 4.15, p = .014) and Ad-Null conditions (t (4) = 2.77, p = .050). In Nef expression neurons, YB1 expression did not significantly differ compared to control (t (4) = 0.99, p = .375) and Ad-Null conditions (t (4) = 2.04, p = .110) (Figure 3 D).

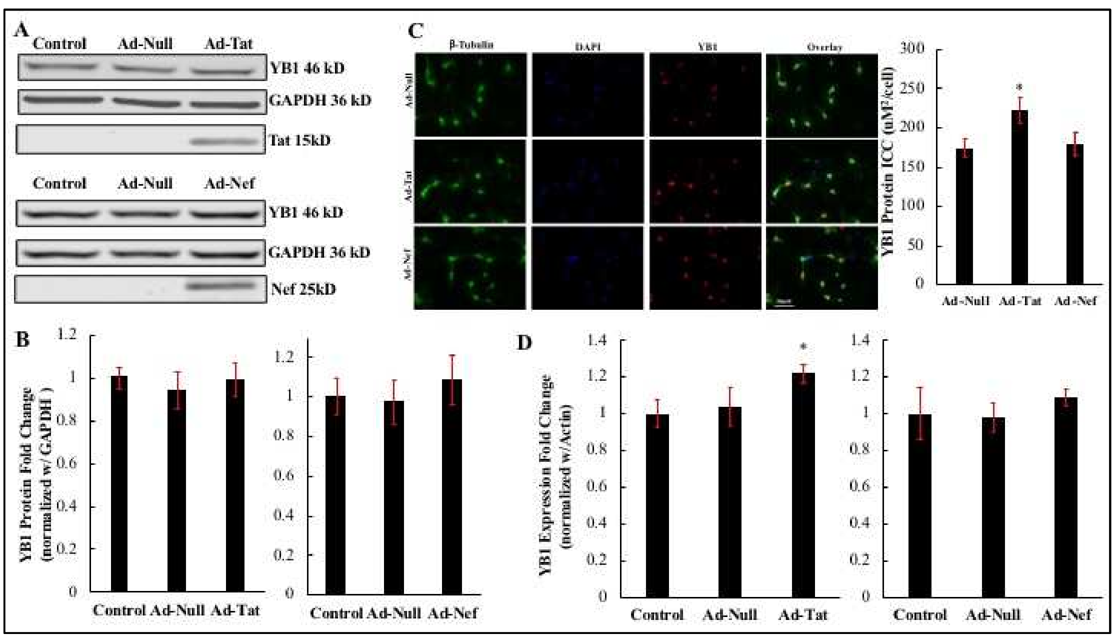

Figure 3. Effects of Tat and Nef on SG-Marker YB1. Immunoblot images (A) and quantifications (B) of YB1 protein levels. Immunocytochemistry images and quantifications (C) of YB1 protein levels. Quantitative PCR of YB1 RNA levels (D). * p < .05 compared to control or Ad-Null conditions.

To explore the regulation of SG markers by Tat or Nef, more advanced biochemical techniques were employed to qualitatively to assess several relevant factors.

First, the subcellular localization of SG markers identified in microscopy (i.e. TIA, YB1 in the nucleus, G3BP in the cytoplasm) suggested the effects of viral proteins may be dependent on location-specific quantities of SGs. However, immunoblotting results following cytoplasmic and nuclear protein extraction revealed the effects of Tat as well as Nef did not appear to be dependent on SG marker localization within neurons (Figure 4 A; cytoplasmic (GAPDH) and nuclear (histones) control proteins are shown to demonstrate sensitivity and specificity). Second, given the predominant cytoplasmic distribution of viral proteins as well as G3BP, confocal microscopy was used to determine if Tat or Nef may physically interact with G3BP. Whereas Ad-Null conditions illustrated diffuse distribution of G3BP throughout the cytoplasm, neurons expressing Tat or Nef illustrated accumulation in cytoplasmic puncta that colocalized with G3BP (Figure 4 B). To further assess if Tat in particular can physically interact with SGs, immunoprecipitation pull-down of Ad-Tat treated neurons was conducted (Figure 4 C). However, immunoblot results suggested G3BP did not bind to Tat proteins in neurons; furthermore, the detection of TIA in both Ad-Tat and Ad-Null conditions suggested TIA may bind directly to immunoglobulin beads during immunoprecipitation procedures, potentially due its unique low complexity domain and increased protein-binding capacities (M. Kato et al., 2012). These results suggest SGs and HIV proteins can localize to similar foci in the cytoplasm (i.e. colocalization of cytoplasmic G3BP with Tat or Nef), but these interactions may be transient or weak (i.e. no evidence of TIA or G3BP immunoprecipitation with Tat).

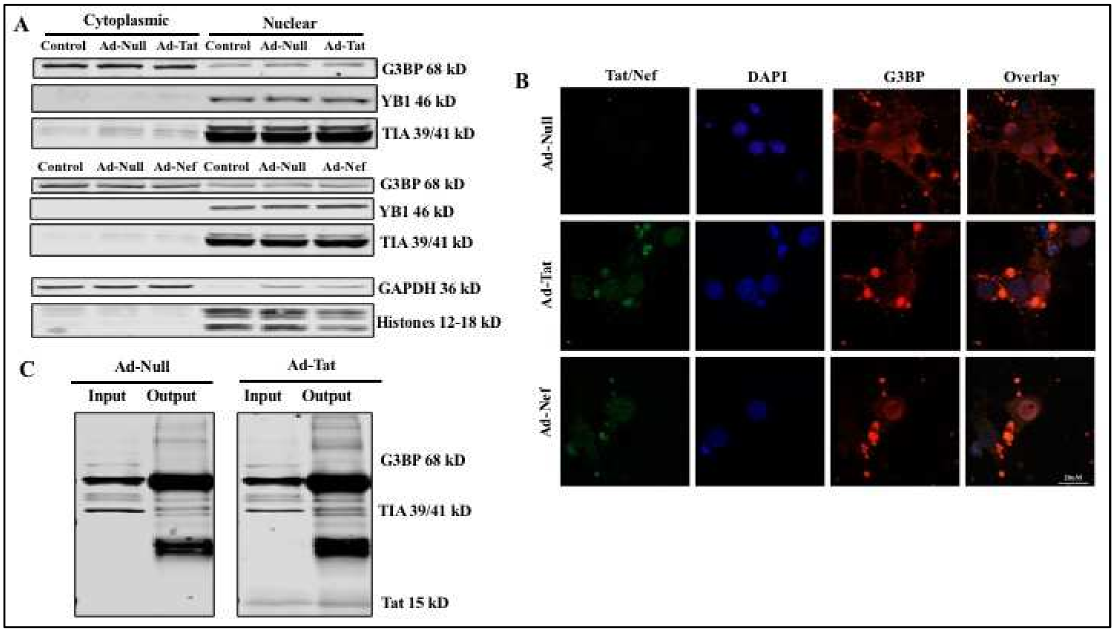

Figure 4. Further Investigation of Tat and Nef Effects on SG-Markers. Immunoblot images of TIA G3BP, YB1 protein levels following cytoplasmic and nuclear fractionation (A). Immunocytochemistry images of G3BP protein colocalization with HIV proteins Tat and Nef (B). Immunoblot images of TIA and G3BP protein levels following immunoprecipitation with HIV Tat (C).

Given their multiple roles in PQC, including the regulation of SGs, variation in levels of BAG1 and BAG3 were also quantified. Notably, variations in BAG1 protein reflected alterations in its two predominant neuronal isoforms (i.e. L&M). Immunoblotting results demonstrated BAG1 protein levels did not significantly differ as a result of Tat expression compared to control (t (4) = 0.70, p = .530) and Ad-Null conditions (t (4) = 0.38, p = .730). Likewise, BAG1 protein levels did not significantly differ as a result of Nef expression compared to control (t (4) = 0.20, p = .853) and Ad-Null conditions (t (4) = 0.94, p = .400) (Figure 5 A&B). Immunocytochemistry validated the lack of alterations in BAG1 protein levels in Tat (176.66 ± 31.36 µm^2/cell; t (4) = 1.31, p = .260) and Nef expressing neurons (167.38 ± 26.04 µm^2/cell; t (4) = 0.97, p =.386) compared to control conditions (151.62 ± 10.57 µm^2/cell) (Figure 5 C). In contrast, significant reductions in BAG1 RNA were noted in Ad-Tat treated neurons compared to control (t (4) = 7.06, p = .002) and Ad-Null neurons (t (4) =5.85, p = .004). Similar reductions in BAG1 transcription were observed in Nef expressing neurons compared to control (t (4) = 5.67, p = .005) and Ad-Null neurons (t (4) =8.06, p = .001) (Figure 5 D).

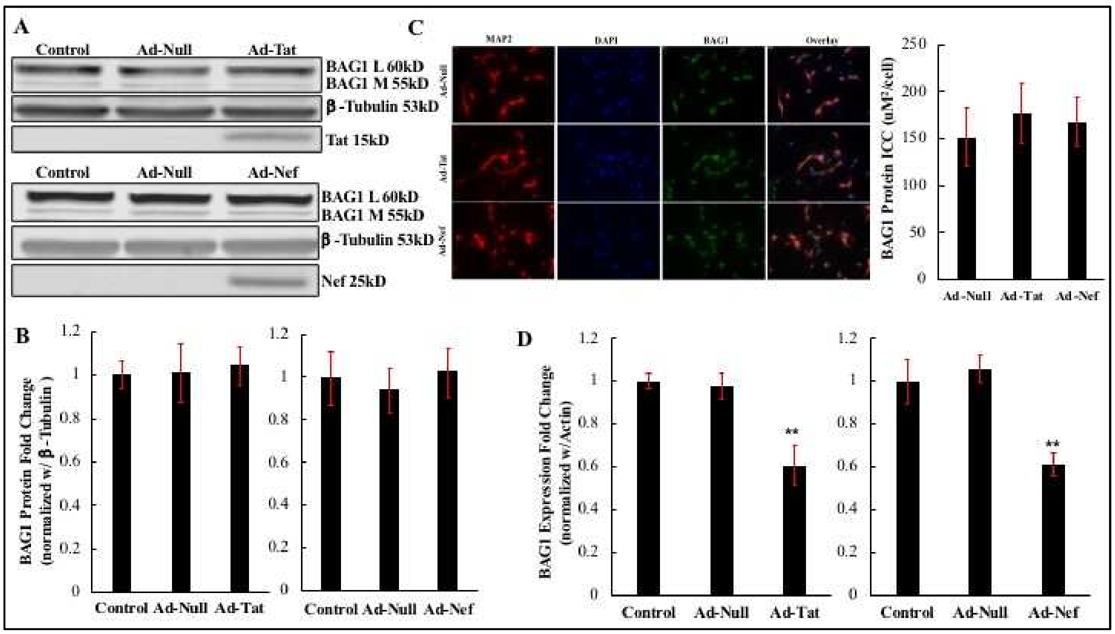

Figure 5. Effects of Tat and Nef on BAG1. Immunoblot images (A) and quantifications (B) of BAG1 protein levels. Immunocytochemistry images and quantifications (C) of BAG1 protein levels. Quantitative PCR of BAG1 RNA levels (D). ** p < .01 compared to control or Ad-Null conditions.

In contrast to the limited variations in BAG1, primary neurons expressing HIV Tat or Nef displayed significant and consistent reductions in levels of BAG3. Immunoblotting data demonstrated a significant decrease in BAG3 protein in response to Tat compared to control (t (4) = 10.79, p = .001) and Ad-Null conditions (t (4) = 7.74, p = .006). Similar immunoblotting results were found in Ad-Nef treated neurons compared to control (t (4) = 7.86, p = .006) and Ad-Null conditions (t (4) = 9.96, p = .001) (Figure 6 A&B). Immunocytochemistry validated decreases in BAG3 protein levels found in Tat

(54.63 ± 8.19 μm^2/cell; t (4) = 12.54, $p < .001$) and Nef expressing neurons (83.33 ± 6.76 μm^2/cell; t (4) = 10.73, $p < .001$) compared to control conditions (233.92 ± 6.76 μm^2/cell) (Figure 6 C). Along with decreases in protein levels, significant reductions in BAG3 transcription were observed in response to Tat compared to control (t (4) = 14.63, $p < .001$) and Ad-Null neurons (t (4) =11.85, $p < .001$). Similarly, neurons treated with Ad-Nef illustrated robust decreases in BAG3 transcription compared to control (t (4) = 14.40, $p < .001$) and Ad-Null neurons (t (4) = 13.58, $p < .001$) (Figure 6 D). While both viral proteins lead to significant downregulation of BAG3, these effects were particularly apparent in Tat expressing neurons.

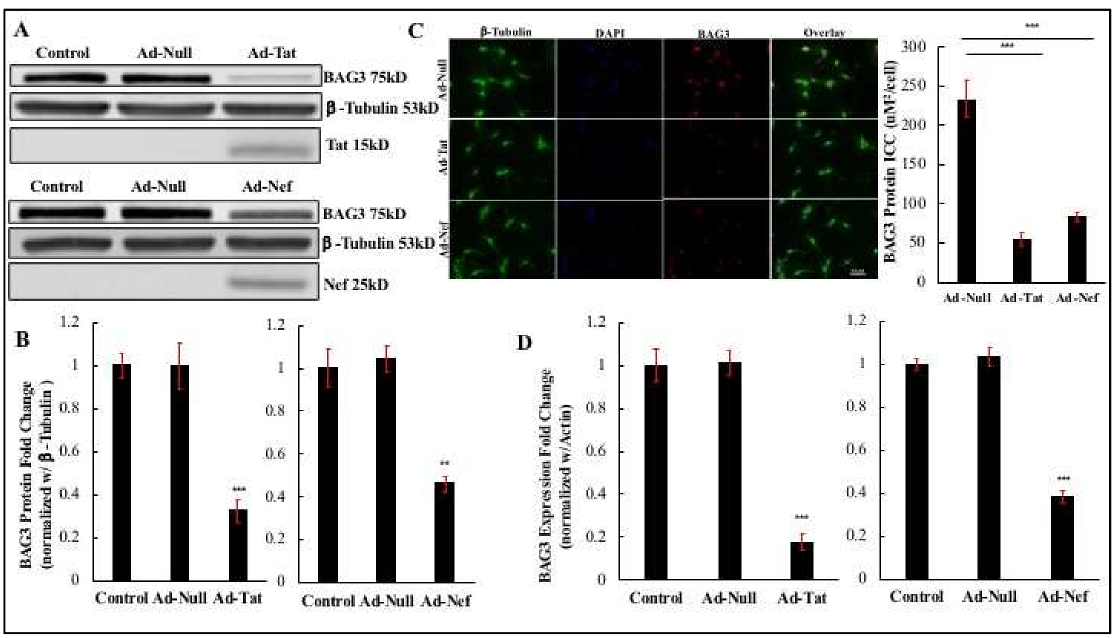

Figure 6. Effects of Tat and Nef on BAG3. Immunoblot images (A) and quantifications (B) of BAG3 protein levels. Immunocytochemistry images and quantifications (C) of BAG3 protein levels. Quantitative PCR of BAG3 RNA levels (D). ** $p < .01$, *** $p < .001$ compared to control or Ad-Null conditions.

Provided its potential role in HAND, variation in oxidative stress induced by HIV proteins was assessed, as well as the consequences of such ROS imbalances on neuronal PQC, particularly H_2O_2. In assessing variations in oxidative stress, live-cell confocal microscopy illustrated significant elevations ROS following the expression of Tat (317.10 ± 21.57 μm^2/cell; t (4) = 6.94, p = .002) or Nef (327.19 ± 28.96 μm^2/cell; t (4) = 6.58, p = .003) compared to Ad-Null conditions (166.64 ± 30.72 μm^2/cell) (Figure 7).

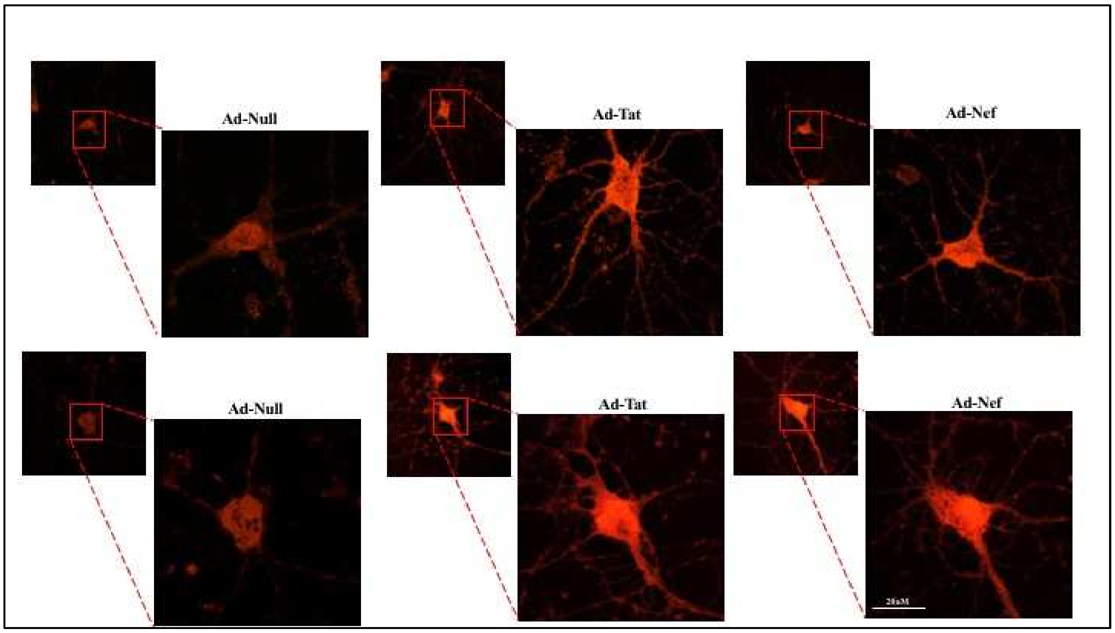

Figure 7. Tat and Nef Effects on Reactive Oxygen Species (ROS). Confocal microscopy images of ROS levels in neurons.

Subsequent experiments examined if oxidative stress, particularly H_2O_2 due to its unique characteristics (see Introduction; Oxidative Stress), was capable of regulating neuronal BAG family members independent of HIV protein expression. However, optimal treatment parameters for H_2O_2 in primary neuronal culture are not well-established, due to the lack of studies employing H_2O_2 in primary neuronal cultures as well as the reliability of techniques used to assess its kinetics (Bilan & Belousov, 2018; O'Riordan & Lowry, 2017; Ricart & Fiszman, 2001; Whittemore, Loo, Watt, & Cotman, 1995). Based on the conditions and techniques available in the limited literature, several experiments were initially conducted to determine the parameters of H_2O_2 that alter general metabolic activity without compromising viability. At a fixed duration of 6 hours,

H_2O_2 significantly decreased metabolic activity at dosages above 150uM as indicative by MTT assay compared to control conditions (t (4) = 3.99, p = .016) (Figure 8 A), while trypan blue staining suggested cell viability was significantly mitigated at concentrations above 250uM compared to control (t (4) = 3.25, p = .031) (Figure 8 B). Utilizing a 250uM concentration, time course experiments illustrated significantly decreased metabolism beginning at 2 hr post-treatment compared to untreated samples (t (4) = 4.61, p = .010) (Figure 8 C), while this decrease stabilized over the course of 6 hr post-treatment (t (4) = 0.30, p = .977); significant increased cell death was not observed until after 8 hours of treatment compared to control conditions (t (4) = 6.30, p = .003) (Figure 8 D). Therefore, such fixed parameters (i.e. 250uM, 6 hr) were selected as the optimal treatment conditions in succeeding experiments.

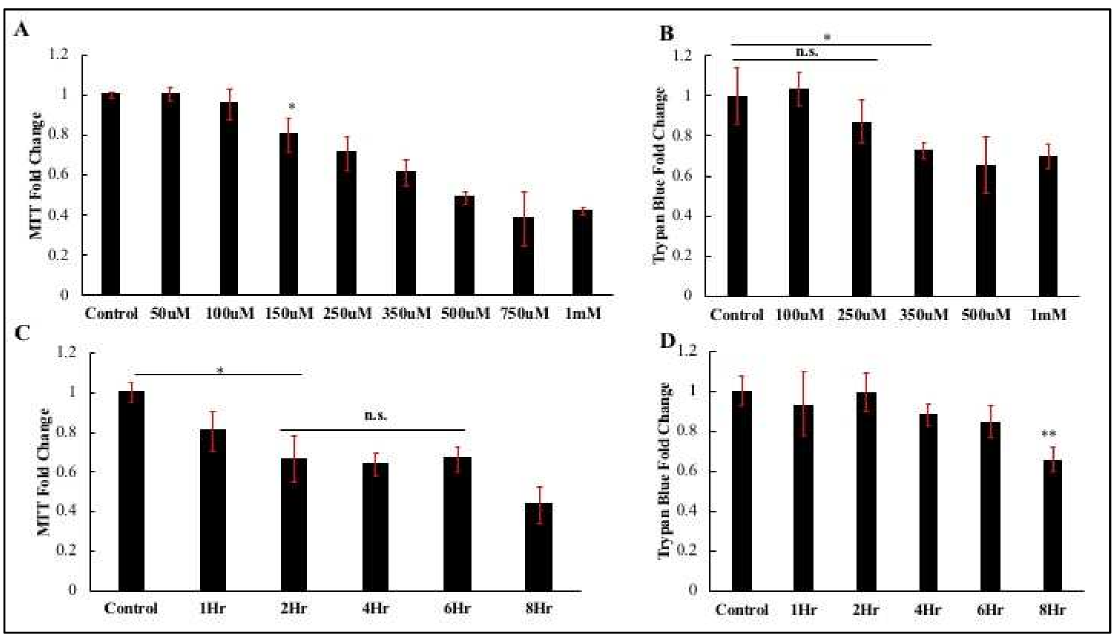

Figure 8. Metabolic and Viability Assays for H_2O_2 Treatment Optimization. Dosage responses to H_2O_2 in metabolic activity (A) and viability (B). Time course responses to H_2O_2 in metabolic activity (C) and viability (D). * $p < .05$,** $p < .01$ compared to control conditions.

While Tat and Nef induced oxidative stress was not associated with variation in BAG1, direct application of oxidative stress in the form of H_2O_2 triggered alterations in BAG1 transcription and translation. Preliminary studies and immunoblot results indicated dose and time dependent effects of H_2O_2 on BAG1 protein levels (Figure 9 A). Consistent with these time dependent effect on protein levels, neurons exposed to H_2O_2 initially displayed significant decreases in BAG1 RNA, most notably following 2 hours of treatment (t (4) = 7.78, p = .002); however, H_2O_2 also triggered a significant increase in BAG1 RNA after this initial decrease (t (4) = 4.66, p = .010), although levels did not completely return to baseline (Figure 9 B). Subsequent immunoblot results indicated the up regulation of BAG1 protein by H_2O_2 (i.e. 6 hours, 250uM) was not dependent on Tat or Nef expression and was significant compared to Ad-Null transduced neurons (t (4) = 5.38, p = .006) (Figure 9 C&D). Immunocytochemistry validated the H_2O_2-specific increases in BAG1 protein (254.51 ± 29.80 μm^2/cell) relative to control conditions (142.83 ± 18.24 μm^2/cell; t (4) = 5.54, p = .005) (Figure 9 E; high magnification images are shown; low magnification images are included in raw data).

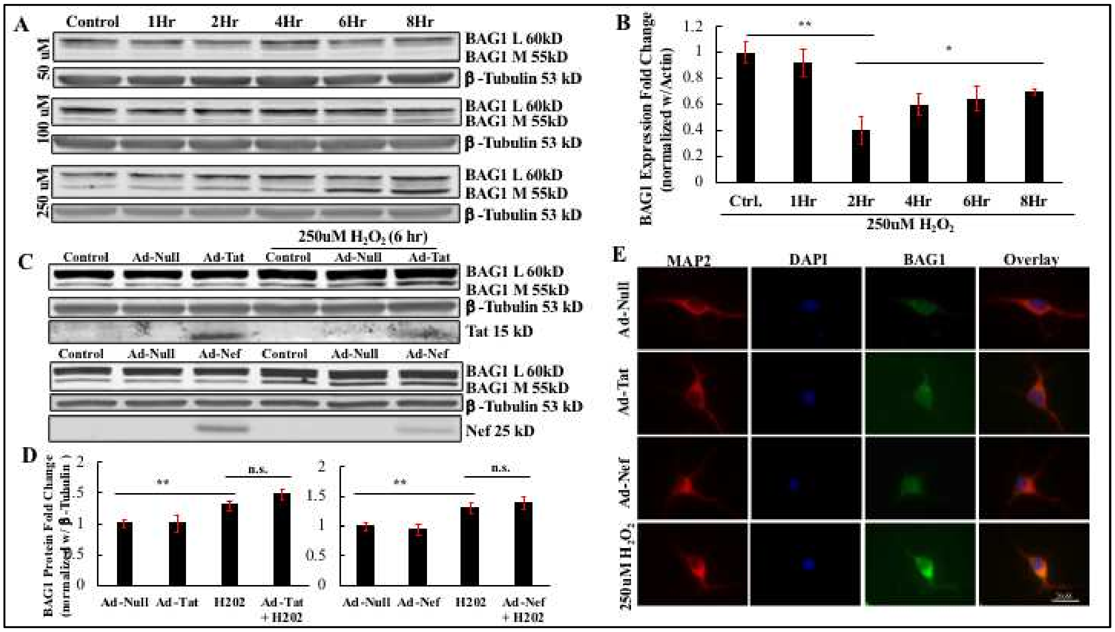

Figure 9. Effect of Oxidative Stress (H_2O_2) on BAG1. Immunoblots of dosage and time course responses to H_2O_2 in BAG1 protein levels (A). Time course responses to H_2O_2 in BAG1 RNA levels (B). Immunoblot (C) and quantifications (D) of BAG1 protein levels in response to specified conditions. Immunocytochemistry of BAG1 protein levels (E) in response to specified conditions. ** $p < .01$ compared to control conditions. * $p < .05$ compared to 2Hr conditions.

In primary neurons exposed to H_2O_2, significant reductions in BAG3 transcription and translation were observed. Preliminary studies and immunoblot results indicated dose and time dependent effects of H_2O_2 on BAG3 protein levels (Figure 10 A&D). Subsequent immunoblot results confirmed significant reductions in BAG3 protein levels due to oxidative stress at the levels optimized in preceding experiments (i.e. 6 hours, 250uM) ($t(4) = 8.05$, $p = .001$). In addition to reductions in protein levels, BAG3 transcription was significantly mitigated at these optimized conditions ($t(4) = 6.88$, $p = .002$). Interestingly, reduced transcription by H_2O_2 began at 2 hrs post-treatment ($t(4) = 6.20$, $p = .003$), while this reduction did not extend further over the course of the experiment ($t(4) = 0.16$, $p = .876$) (Figure 10 B). Immunocytochemistry confirmed reductions in BAG3 due to oxidative stress (60.02 ± 14.74 μm^2/cell) compared to control conditions (185.47 ± 17.17 μm^2/cell; t (4) = 0.16, $p < .001$). Furthermore, microscopy illuminated morphological alterations in neurons exposed to H_2O_2, with some of these cells developing dystrophy in their processes compared to control conditions (Figure 10C; high magnification images are shown; low magnification images are included in raw data). Interestingly, the co-expression of both viral proteins did not exacerbate neuronal reductions in BAG3 protein levels (Figure 10 E), while such decreases appeared to be neuronal-specific, provided that primary rat astrocytes failed to show similar sensitivity to H_2O_2 treatment (Figure 10 F).

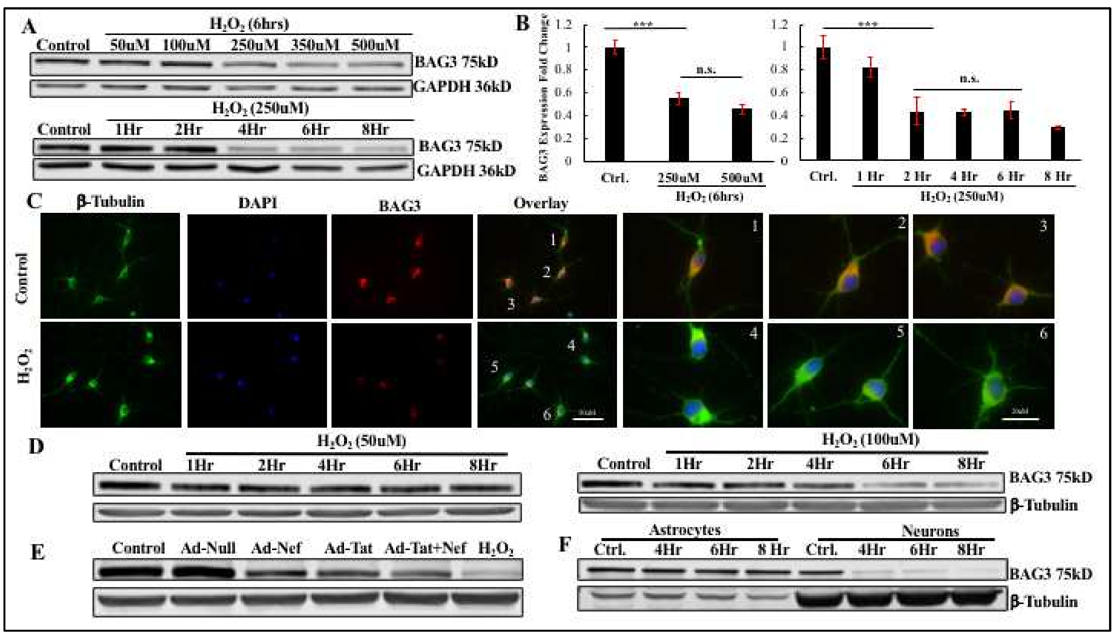

Figure 10. Effect of Oxidative Stress (H_2O_2) on BAG3. Immunoblots of dosage and time course responses to H_2O_2 in BAG3 protein levels (A). Responses to H_2O_2 in BAG3 RNA levels (B). Immunocytochemistry of BAG3 protein levels (C) in response to H_2O_2. Immunoblots of dosage and time course responses to H_2O_2 in BAG3 protein levels (D). Immunoblots of BAG3 protein levels in response to specified conditions (E). Immunoblots of neuronal and astrocytic BAG3 protein levels in response to H_2O_2 (F). *** $p < .001$ compared to control conditions.

As indicated by additional immunoblot data, H_2O_2 replicated and exacerbated a reduction in neuronal BAG3 induced by the expression of HIV viral proteins (i.e. Tat and

Nef). The combination of H_2O_2 and Tat resulted in an additive effect, enhancing the decrease in BAG3 compared to control conditions (t (4) = 8.43, p = .001) and neurons expressing Tat alone (t (4) = 5.01, p = .008) (Figure 11 A). Similar results were noted in Nef expressing neurons, where the addition of H_2O_2 exacerbated the reduction in BAG3 compared to control conditions (t (4) = 7.21, p = .008) and neurons expressing Nef alone (t (4) = 4.18, p = .014) (Figure 11 B). When normalized ratios of BAG1 to BAG3 immunoblot levels were assessed, it suggested a clear upregulation of BAG1 relative to BAG3 in primary neurons (Figure 11 C).

Subsequent gain and loss of function experiments (i.e. BAG3 overexpression with Ad-BAG3 and BAG3 knockdown with Ad-SiBAG3) suggested oxidative stress was responsible for triggering compensatory BAG1 upregulation. Neurons treated with H_2O_2 following BAG3 knockdown displayed a substantial BAG1 upregulation compared to knockdown of BAG3 alone (t (4) = 9.21, p =.001). (Figure 11 D). These decreases in BAG3 were associated with increased dysregulation of proteins responsible for maintaining mitochondrial membrane permeability. Specifically, decreases in ANT and increases in VDAC following BAG3 knockdown alone were significantly exacerbated by H_2O_2 (ANT, t (4) = 6.09, p =.004 ; VDAC1, t (4) = 5.36, p =.006) (Figure 11 D). However, largely consistent with the effects of Tat and Nef, oxidative stress in the form of H_2O_2 did not perturb protein levels of SG markers (i.e. G3BP, YB1, TIA) (Figure 11 D).

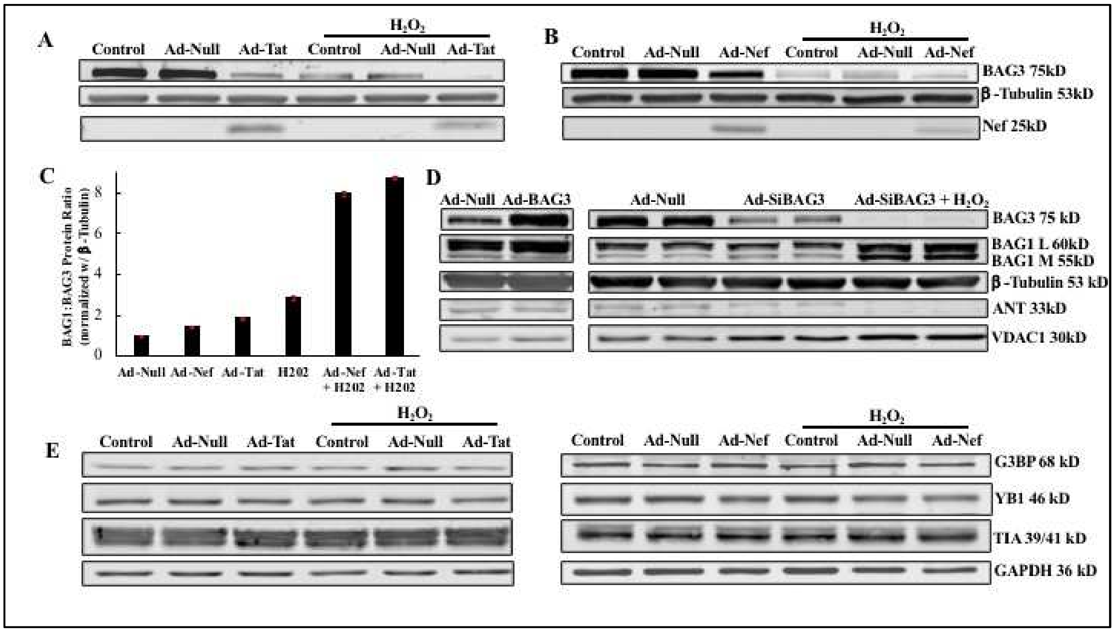

Figure 11. Further investigation of Oxidative Stress Effects (H_2O_2) on BAG Chaperones. Immunoblot images of BAG3 protein levels in response to Tat and oxidative stress (A) as well as Nef and oxidative stress (B). Ratio of BAG1 to BAG3 protein levels, assessed via immunoblot, in response to specified conditions (C). Immunoblot images of target proteins in response to gain and loss of function manipulations (D). Immunoblot images of TIA, G3BP and YB1 protein levels in response to viral proteins and oxidative stress (E).

3.4 Discussion

The current findings address the potential for HIV viral proteins to mediate the neuronal mechanisms implicated in HAND, particularly PQC. Although neither viral protein reliably perturbed the regulation of SGs, Tat and Nef facilitated elevations in intraneuronal ROS, decreased BAG1 transcription and suppressed BAG3 levels (i.e. protein and RNA). While Tat expression was associated with increased YB1 protein (i.e. immunocytochemistry) and RNA, these effects were not observed in other SG markers. Oxidative stress, in the form of H_2O_2, exacerbated the reduction in BAG3 caused by Tat or Nef, induced an upregulation of BAG1 and dysregulated proteins responsible for maintaining mitochondrial membrane permeability (i.e. ANT, VDAC). While data indicate limited impacts on SGs, these results suggest oxidative stress induced by HIV proteins or other factors (i.e. aging) may dysregulate neuronal PQC, particularly BAG chaperones, and contribute to the mechanisms underlying HAND.

The pattern of BAG3 and BAG1 regulation reported herein warrants consideration and emphasizes the implications of cell-specific experimental paradigms. As oxidative stress and aging can mitigate PQC while also increasing concentrations of aggregate-prone polypeptides, a compensatory yet disproportionate demand for BAG3 over BAG1 has been hypothesized in response to perturbation in neuronal homeostasis (Behl, 2016; Klaips et al., 2018). Indeed, in a cell line model of aging as well as neural cell lines clonally selected for resistance to oxidative stress, a group of researchers recently reported increasing BAG3 and decreasing BAG1 in response to H_2O_2 (Chakraborty et al., 2019; Gamerdinger et al., 2009; Gamerdinger et al., 2011). This group reported similar results in aged rodent brain samples containing both neurons and glia (i.e. tissue homogenates, *ex vivo* hippocampal slices) (Gamerdinger et al., 2009). However, researchers indicated such BAG regulation was specific to neurons (see Figure 2F in Gamerdinger at al., 2009), consistent with results obtained here. Therefore, the direction of BAG3 and BAG1 regulation by H_2O_2 in the current experiments may be reflective of cell-specific responses obtained from primary neurons, whereas prior experimental procedures may reflect general cellular adaptations in the neural milieu. In light of accumulating evidence distinguishing the molecular biology of neurons from other cell types in the brain, particularly in response to aging and neuropathogenesis, such cell-specific responses are plausible and increasingly relevant (Grubman et al., 2019; Mattson & Magnus, 2006).

Although HIV viral proteins influenced several homeostatic processes in neurons, results suggested neither Tat nor Nef reliably influenced the regulation of RNA-Binding Proteins (RBPs), which are involved in stress granule (SG) formation (Gilks et al., 2004; Somasekharan et al., 2015; Tourriere et al., 2003). This finding may be due to the physical characteristics of SGs. Specifically, SGs exist in the cytoplasm as liquid-liquid phase separated (LLPS), a dynamic, fluid-like structure that, although initially homogenous, separates into two liquid phases that coexist: a slightly denser core encased by an outer shell that is in direct contact with surrounding cytosolic elements (Jain et al., 2016; M. Kato et al., 2012; Molliex et al., 2015; Shin & Brangwynne, 2017). Given this unique physical state, specialized equipment, reagents and protocols may be required to determine reliable SG variation (Aulas et al., 2017; Wheeler, Jain, Khong, & Parker, 2017). Conversely, measuring RBPs by means of traditional biochemical techniques (e.g. immunoblotting, immunocytochemistry, qPCR) may fail to reliably assess this recently identified biological process (Kedersha, Gupta, Li, Miller, & Anderson, 1999; Nover, Scharf, & Neumann, 1989).

The observed Tat and Nef effects on SGs may also be dependent on the traditional RBP markers of SGs, coupled with the inherent biological characteristics of neurons. Indeed, the post-mitotic nature of neurons is associated with a distinctive proteomic profile, composed of cell-specific adaptations that may otherwise inhibit the LLPS of SGs, including protein chaperones and co-chaperones, post-translational modifications, and specialized cytoskeletal proteins (Sjöstedt et al., 2015). In addition, the majority of investigations have measured RBPs as markers of SGs in proliferative cell types under acute, severe stress conditions (e.g. thermal stress) (Kedersha et al., 2000). Conversely, some RBPs colocalize with SGs in neurons but not peripheral cells, while others inhibit formation of SGs despite their neuronal abundance (Bounedjah et al., 2014; Figley, Bieri, Kolaitis, Taylor, & Gitler, 2014). Moreover, while RBP accumulation in distinct granular foci has been documented in the brain, including colocalization with pTau in AD mice, RBPs can also remain dispersed throughout the cytoplasm in neuropathological conditions (Armstrong, Carter, & Cairns, 2012; Ash, Vanderweyde, Youmans, Apicco, & Wolozin, 2014). Thus, the RBPs utilized in proliferative cells to characterize SGs may not be suitable to precisely measure their dynamic variation in neurons. Nonetheless, current findings add to the limited existing literature of SGs in primary neurons and appear to be the first investigation of SGs in primary rat neurons (Khalfallah et al., 2018; Shelkovnikova et al., 2017).

CHAPTER 4

AIM TWO: IMPLICATIONS OF VIRAL PROTEIN-INDUCED ALTERATIONS FOR COGNITIVE DYSFUNCTION

4.1 *In vivo* Effects Associated with HIV Proteins

4.1.1 Rationale

To further assess the neurobiological mechanisms of HAND, the current experiments investigated if the neuronal variation observed in response to HIV proteins and their associated oxidative stress (e.g. levels of SG markers, BAG proteins) is translatable across experimental paradigms. Thus, Aim Two A was designed to assess if the *in vitro* effects of HIV Tat and Nef on homeostatic mechanisms in neurons were maintained in the brains of transgenic animal models that expresses HIV viral proteins (Tg26 & iTat mice). While this aim established a fundamental association between HIV proteins and SG markers *in vivo*, it also examined if the Tat and Nef induced effects on neuronal PQC (e.g. BAG1, BAG3) were translatable across experimental models.

Similar to *in vitro* experiments, variation in neuronal PQC processes (i.e. SGs, BAG 1&3) associated with the expression of HIV viral proteins was the primary objective for this aim. These targets were assessed in Tg26 mice by measuring the protein levels of three SG markers (i.e. TIA, G3BP1 and YB1) (Gilks et al., 2004; Somasekharan et al., 2015; Tourriere et al., 2003). Immunohistochemistry data reflected alterations in SG markers in the frontal cortex and subregions of the hippocampus (CA1, CA3) while immunoblot results reflected variations in the frontal cortex and the whole hippocampus. In addition, levels of BAG1 and BAG3 were quantified in Tg26 animals, while these findings were extended to a separate animal model of HIV (iTat mice). Here, variation in

BAG3 protein levels (i.e. immunoblot; frontal cortex and the whole hippocampus) prompted further analysis of BAG1&3 transcriptional regulation (i.e. qPCR).

Provided that the prolonged formation of SGs is associated with dysfunctional PQC, and HIV proteins can hinder efficient PQC, I hypothesized that HIV animal models would maintain increased levels of SG markers, as well as BAG3 and BAG1 chaperones.

4.1.2 Experimental Procedures

Adult male Tg26 (n = 4) and C57Bl/6J mice (n = 4) were maintained until approximately 6 months of age, at which point brain tissue was harvested for subsequent analyses. Here, the right and left hemispheres of each animal were processed for immunoblotting/qPCR and immunohistochemistry, respectively. After the right hemisphere was dissected (i.e. frontal cortex, whole hippocampus) and homogenized, the total volume from each region was split in equal parts for protein and RNA analysis. For protein quantification, tissue was then lysed followed by western immunoblotting utilizing SDS-polyacrylamide gel electrophoresis. To similarly gauge protein levels, immunohistochemistry of formalin fixed and paraffin embedded tissue was conducted in conjunction with brightfield microscopy. In order to examine alterations in the transcription of relevant genes, RNA was extracted, and RT-qPCR was conducted following cDNA synthesis (for detailed Materials and Methods, please refer to Chapter 6).

In addition, the analyses of BAG1 and BAG3 were extended to the iTat model of HIV, which conditionally-expresses the HIV Tat protein in the presence of doxycycline (DOX). Beginning at 4.5 months of age, both iTat (n = 4) and their control littermates (n = 4) received doxycycline hyclate orally over the course of 6 weeks (200mg/kg; rodent chow), at which point brain tissue was harvested for subsequent analyses. iTat analyses were restricted to immunoblotting and qPCR from right hemisphere homogenates (left hemisphere tissue were used for procedural validation of Tat expression).

4.1.3 Results

Similar to results in Aim One, *in vivo* analyses suggested minimal variation in SG markers in the brains of HIV animals. Immunohistochemistry of TIA protein levels indicated no significant variation across the frontal cortex or hippocampus (CA1, CA3) of Tg26 animals compared to controls (Figure 12 A&B); however, within control animals, there was a non-significant trend of TIA downregulation of in the CA3 region (203.27 ± 26.25 μm^2/cell) compared to CA1 (240.16 ± 20.03 μm^2/cell; t (6) = 2.23, p = .066). Moreover, immunoblot data confirmed similar levels of TIA protein between HIV animals and controls in both the frontal cortex (t (6) = 0.56, p = .600) as well as hippocampus (t (6) = 2.27, p = .064) (Figure 12 C&D).

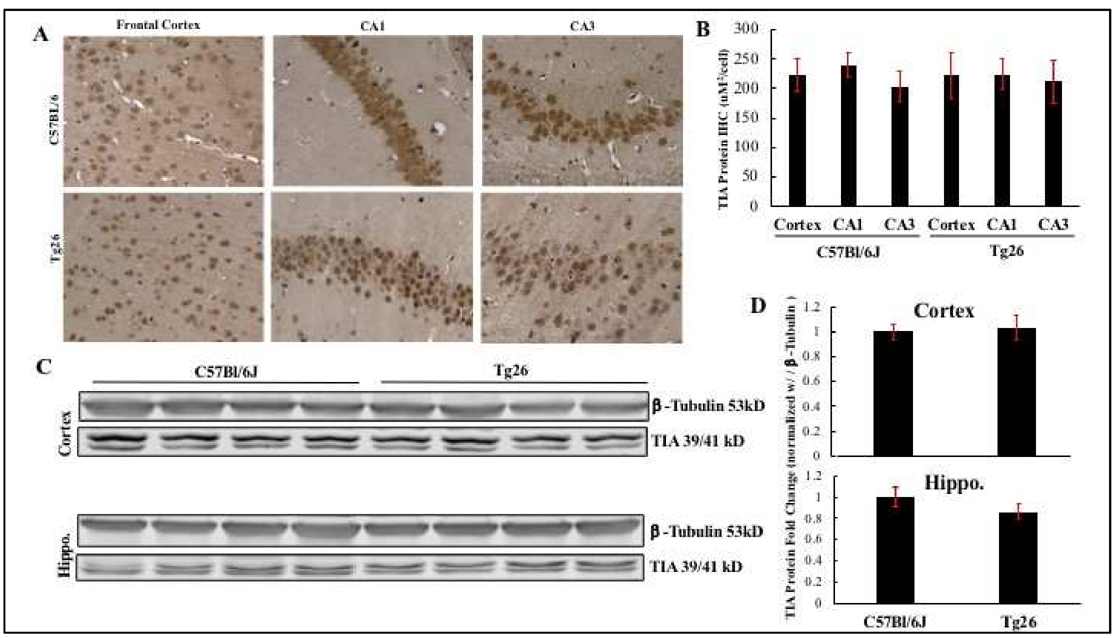

Figure 12. *In Vivo* Effects Associated with HIV Proteins on SG-Marker TIA. Immunohistochemistry images (A) and quantifications (B) of TIA protein levels. Immunoblot images (C) and quantifications (D) of TIA protein levels.

In line with TIA data, results illustrated limited variation in G3BP levels in the brains of HIV animals. Immunohistochemistry indicated no significant differences between groups in the frontal cortical (t (6) = 1.47, p = .192) or CA3 regions (t (6) = 1.47, p = .344), although a significant decrease was found in the CA1 region of Tg26 animals (203.33 ± 19.16 μm^2/cell) compared to their control counterparts (155.55 ± 24.78 μm^2/cell; t (6) = 2.71, p = .035) (Figure 13 A&B). Immunoblot results suggested such observed group differences in G3BP within CA1 were not extended across the entirety of hippocampal tissue (t (6) = 0.87, p = .416), while levels of this SG marker did not differ in the frontal cortex across groups (t (6) = 1.41, p = .208) (Figure 13 C&D).

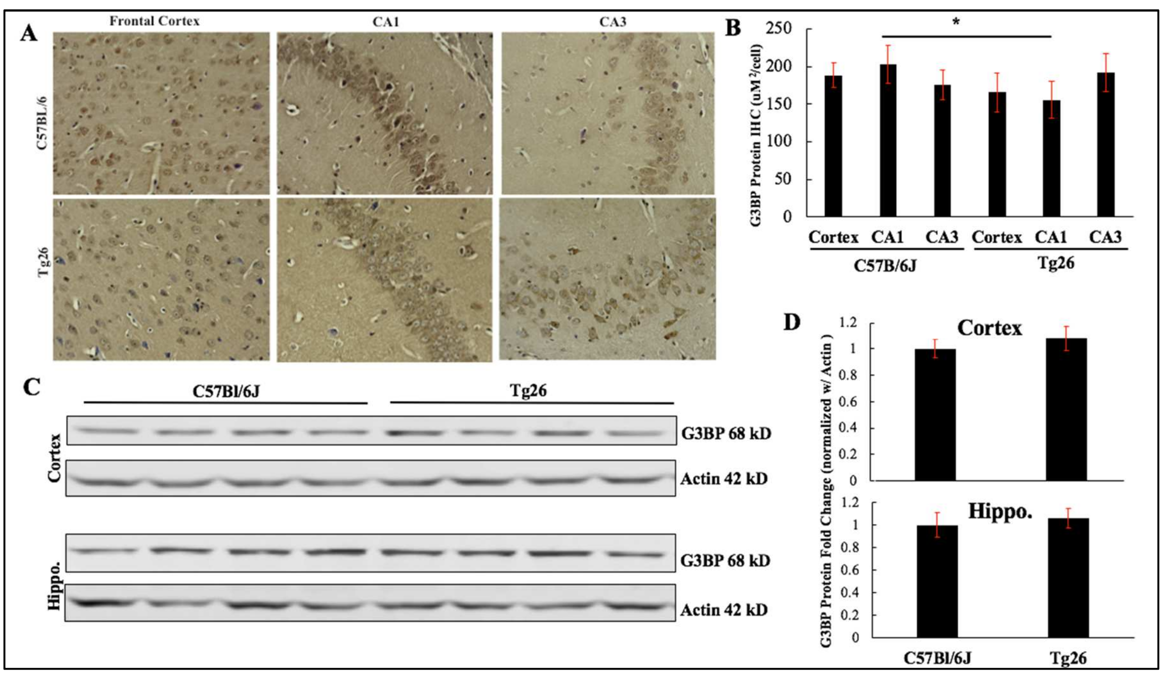

Figure 13. *In Vivo* Effects Associated with HIV Proteins on SG-Marker G3BP. Immunohistochemistry images (A) and quantifications (B) of G3BP protein levels. Immunoblot images (C) and quantifications (D) of G3BP protein levels. * $p < .05$ compared to control conditions.

In addition to TIA and G3BP, protein levels of YB1 remained consistent in the brains of Tg26 animals compared to control subjects. Although immunohistochemistry revealed a trend of increasing YB1 in the CA1 region of HIV animals (188.91 ± 29.74 μm^2/cell) compared to CA1 regions in controls (222.25 ± 25.36 μm^2/cell), this pattern was not statistically significant (t (6) = 2.06, p = .085). Furthermore, no significant variation between groups was observed across cortical (t (6) = 1.03, p = .343) and CA3 regions (t (6) = 0.14, p = .897) (Figure 14 A&B). Immunoblot data supported these findings and illustrated similar levels of YB1 protein in the frontal cortex (t (6) = 0.67, p = .531) and hippocampus (t (6) = 2.03, p = .089) across groups (Figure 14 C&D).

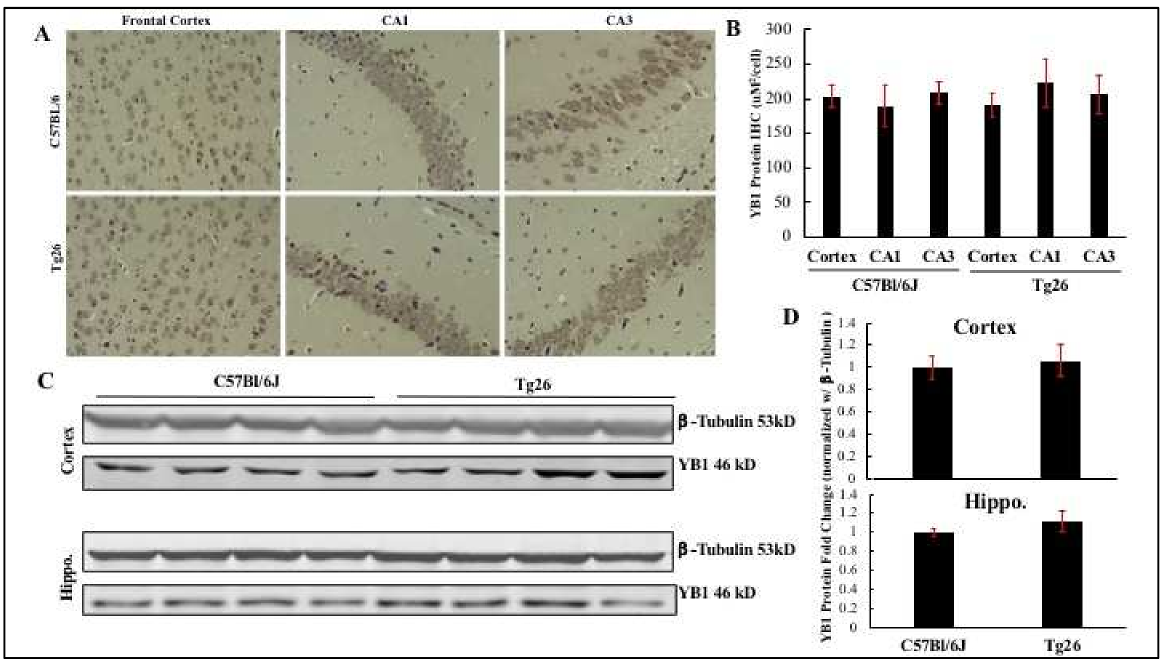

Figure 14. *In Vivo* Effects Associated with HIV Proteins on SG-Marker YB1. Immunohistochemistry images (A) and quantifications (B) of YB1 protein levels. Immunoblot images (C) and quantifications (D) of YB1 protein levels.

To assess if the *in vitro* effects of HIV Tat and Nef on BAG chaperones may be maintained *in vivo*, subsequent analyses interrogated variation in BAG3 as well as BAG1. Initial immunoblot results suggested a significant reduction in BAG3 protein levels in the frontal cortex (t (6) = 3.91, p = .008) and hippocampal tissue (t (6) = 6.12, p = .001) of Tg26 mice (Figure 15 A&B). To examine if BAG regulation is maintained in other animal models of HIV, brain tissue from the iTat mouse model was analyzed. Here, decreases in BAG3 protein were observed in frontal cortex compared to control subjects (t (6) = 3.34, p = .016), although this pattern of regulation did not extend to the hippocampus (t (6) = 0.424, p = .687) (Figure 15 A&B). Variation in BAG3 protein levels (i.e. immunoblot) prompted further analysis of BAG1&3 transcriptional regulation. Compared to control counterparts, Tg26 animals displayed significantly decreased transcription of BAG3 (t (6) = 4.80, p = .003) and increased transcription of BAG1 in cortical tissue (t (6) = 3.19, p = .019). Similarly, iTat mice displayed significantly decreased levels of BAG3 RNA (t (6) = 3.54, p = .012) and increased levels of BAG1 RNA in the frontal cortex compared to control animals (t (6) = 3.75, p = .010) (Figure 15 C). Consistent with cortical tissue, significantly reduced transcription of BAG3 (t (6) = 4.68, p = .003) and increased transcription of BAG1 (t (6) = 4.98, p = .002) was observed in the hippocampus of Tg26 animals. Likewise, iTat mice displayed an inverse pattern of BAG3 (t (6) = 3.23, p = .018) and BAG1 (t (6) = 3.55, p = .012) transcription in hippocampal tissue compared to control animals (Figure 15 D).

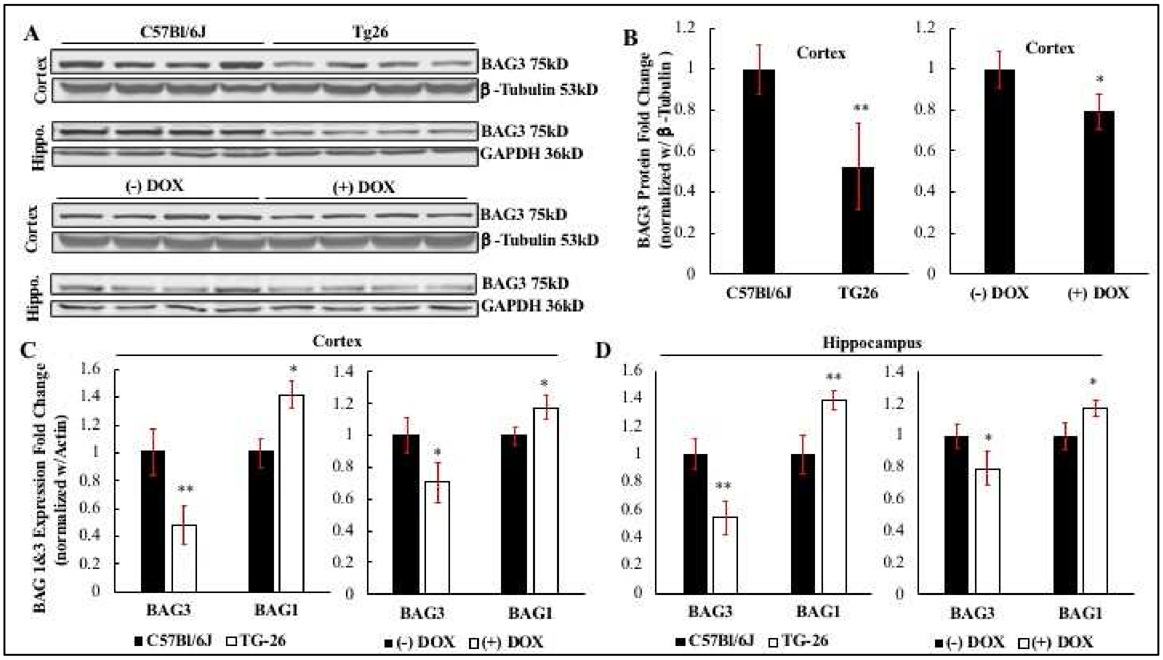

Figure 15. *In Vivo* Effects Associated with HIV Proteins on BAG1 & BAG3. Immunoblot images (A) and quantifications (B) of BAG3 protein levels. Quantitative PCR of BAG3 and BAG1 RNA levels in the cortex (C) and hippocampus (D). * $p < .05$, ** $p < .01$ compared to control conditions.

4.1.4 Discussion

The objective of Aim Two A was to determine if the *in vitro* effects of Tat and Nef on PQC mechanisms in neurons might be maintained in the brains of transgenic animal models that expresses HIV viral proteins (Tg26 & iTat mice). Compared to control animals, analyses of Tg26 tissue across multiple brain regions indicated limited variation in SGs, although increased levels of G3BP were noted in the CA1 region of the hippocampus. Subsequent assessments revealed decreased levels of BAG3 protein and RNA in both cortical and hippocampal tissue, which was accompanied by increased transcription of BAG1. A similar inverse pattern of BAG1 and BAG3 transcription was observed in the iTat mouse model, while decreases in BAG3 protein were limited to the frontal cortex only. These results suggest HIV proteins may induce alterations in PQC within the brain that could contribute to the aberrant neuronal functioning underlying age-related cognitive dysfunction amongst HIV infected individuals.

As discussed in Aim One, the minimal variation in SG markers in Tg26 mice may be due to neuron-specific regulatory mechanisms of SGs (i.e. differential regulation of RBPs, proteome specific inhibition of LLPS etc.), or the unique physical state of SGs combined with assessment using traditional biochemical techniques. In addition, while we reported increased BAG1 and decreased BAG3 RNA in brain tissues of two different HIV animal models, an opposite pattern of regulation has been reported in the aged rodent brain samples (i.e. tissue homogenates, *ex vivo* hippocampal slices) (Gamerdinger et al., 2009). Thus, the pattern of regulation reported here may reflect a compensatory adaptation between neuronal and non-neuronal cells to confer neuroprotection against HIV proteins during adulthood (Malva, Cunha, Oliveira, & Rego, 2007; Valles et al., 2019). Indeed, this regulation of neuronal BAG proteins in the brains of HIV animals may be age dependent, given that cell-specific population densities and functioning can vary in aging CNS, compared to adolescence or adulthood (Salas, Burgado, & Allen, 2020).

While this aim examined if the effects of HIV proteins on neuronal PQC pathways were translatable across experimental models, the dysregulation of BAG chaperones in the brains of HIV animals suggests potential contributions to the cognitive impairments previously reported in these models. In a recent study that utilized Tg26 mice from the same colony as current animals, significant impairments in Barnes Maze performance were discerned. Specifically, female mice illustrated deficits in maze learning (i.e. trials to acquisition) and short-term (Day 5) memory, while male mice displayed deficits in short as well as long term (Day 14) memory (Putatunda et al., 2019). Furthermore, such Barnes Maze impairments have been documented in iTat mice, and rat models which express the same transgene as Tg26 mice maintain significantly impaired performance in a species-comparable spatial memory task (i.e. Morris water maze) (Carey et al., 2012; Vigorito et al., 2007). Therefore, the altered regulation of BAG1 and BAG3 in the brains of HIV animal models that otherwise display cognitive impairments suggests this PQC dysregulation may contribute to the neurobiological underpinnings of cognitive dysfunction observed in HAND.

4.2 Electrophysiological Functioning Associated with HIV Proteins

4.2.1 Rationale

To assess the neurobiological mechanisms of HAND, the current experiments investigated if mechanistic insights from Alzheimer's Disease, such as oxidative stress and protein quality control (i.e. stress granules, BAG chaperones), may similarly contribute to functional electrophysiological impairments amongst neurons in response to HIV proteins Tat and Nef. Thus, Aim Two B was designed to investigate the electrophysiological functioning associated with Tat and Nef-induced perturbations in neuronal homeostatic mechanisms. While these experiments provided evidence for the functional underpinnings of neuronal mechanisms implicated in HAND, they also explored how the adverse consequences of Tat and Nef-induced oxidative stress may be targeted via antioxidants (AOX) to preserve neuronal homeostasis.

Given its relevance to cognitive dysfunction in HAND, Tat or Nef induced variation in neuronal electrophysiology was the primary objective for this aim. Neuronal firing patterns were measured from primary rat neurons using MicroElectrode Arrays

(MEAs), which gauge electrical variation from 60 independent electrodes that are located underneath the surface on which neurons are cultured. Here, activity from a single electrode is indicative of firing capacities from populations of neurons proximal to the electrode's location, also known as a local field potentials (LFP). Neuronal firing was assessed pre-treatment (Day 0) to confirm synchronous activity and establish baseline measurements. After neurons were transduced in a manner previously described (i.e. 72 hrs), Ad-Null, Ad-Nef and Ad-Tat conditions were maintained with control media or media containing AOX. Effects were then assessed 24 hours (Day 4) and 96 hours (Day 7) after AOX treatment. Together, these experiments quantified Tat and Nef consequences on neuronal firing, and established if the mitigation of oxidative stress could preserve functional capacities otherwise hindered by these viral proteins.

In parallel to electrophysiology, separate experiments determined the direct effects of AOX on homeostatic processes in neurons, particularly oxidative stress and PQC mechanisms. Specifically, experiments assessed if AOX treatment directly ameliorated the Tat or Nef induced ROS dysregulation. Additionally, these experiments determined if the mitigation of viral protein-induced oxidative stress could partially preserve BAG chaperone regulation. Similar to prior experiments (Aim One), assessment here employed live-cell, confocal microscopy, as well as the measurement of protein levels (i.e. immunoblot, immunocytochemistry).

As HIV proteins can increase oxidative stress and modulate PQC, I hypothesized the adverse consequences on neuronal functioning and PQC mechanisms induced by Tat and Nef may be mitigated through the application of antioxidants.

4.2.2 Experimental Procedures

To gauge variation in neuronal firing activity, primary neuronal cultures were isolated as previously described (see Aim One) and cultured in MicroElectrode Arrays (MEAs). After reaching maturity, neurons expressing Tat or Nef were maintained with or without AOX to determine if the mitigation of oxidative stress induced by these viral proteins may preserve functional firing capacities. Parallel experiments utilizing live-cell, confocal microscopy validated the inhibition of oxidative stress by AOX treatment that was otherwise induced by Tat or Nef expression. In addition, protein levels were assessed (immunoblot, immunocytochemistry) to determine if antioxidants could inhibit the dysregulation of homeostatic mechanisms by viral proteins (i.e. BAG chaperones).

Neuronal activities were recorded pre-treatment (Day 0) and 96hrs (Day 4) as well as 168hrs (Day 7) after transduction. To ensure the assessment of AOX effects were temporally consistent, recordings were conducted 24 hours after AOX was added to half of the cultures post-transduction (i.e. 96 hours). Media with or without AOX was then maintained until the final recording session on Day 7 to assess potential chronic effects of AOX treatment on neuronal functioning. In each recording session, five minutes of recordings were performed to acquire local field potential (LFP) of neuronal extracellular action potential at 2000 Hz (2kHz) from 60 electrodes simultaneously. To avoid confounding measures with electrical interference in the beginning or conclusion of experiments, stabilized signals from the middle of the recording session (i.e. three, 20-second averages) were used for statistical analyses.

Measures of firing frequency reflected total quantity of action potentials and subthreshold oscillations, while measures of amplitude reflected total area under the curve upon detection of action potential spike. Measures were then normalized to baseline conditions to maintain reliability across groups. Along with variations in firing frequency and amplitude, experiments assessed group differences in conduction velocities, which were computed using the differences in time at which signals were detected amongst proximal electrodes as well as the distance between such electrodes (for detailed Materials and Methods, please refer to Chapter 6).

4.2.3 Results

Initial experiments employing live-cell microscopy confirmed the application of AOX was capable of inhibiting the increase in ROS otherwise induced by Tat (t (4) = 5.11, p = .007) or Nef expression (t (4) = 6.41, p = .003) (Figure 16 A). In turn, immunocytochemistry results indicated the direct inhibition of oxidative stress in neurons was capable of partially preserving BAG3 levels (Figure 16 B). Here, neurons expressing Tat (215.16 ± 23.64 μm^2/cell) or Nef (196.90 ± 9.33 μm^2/cell) in the presence of AOX maintained significantly increased BAG3 protein levels compared to Tat (124.09 ± 10.17 μm^2/cell; t (4) = 5.08, p = .007) or Nef (137.10 ± 15.08 μm^2/cell; t (4) = 5.84, p = .004) expressing neurons without AOX. While immunoblotting confirmed AOX partially preserved BAG3 protein levels otherwise reduced by Tat (t (4) = 5.27, p = .006) or Nef (t (4) = 3.35, p = .029) proteins, results indicated AOX treatment was not capable of mitigating the dysregulation induced by H_2O_2 (i.e. alterations in BAG3, BAG1 and mitochondrial proteins) (Figure 16 C). Due to cross-reactivity between MitoSOX reagents with H_2O_2 in solution, it was not possible to reliably discern if direct application of H_2O_2 was ameliorated by AOX.

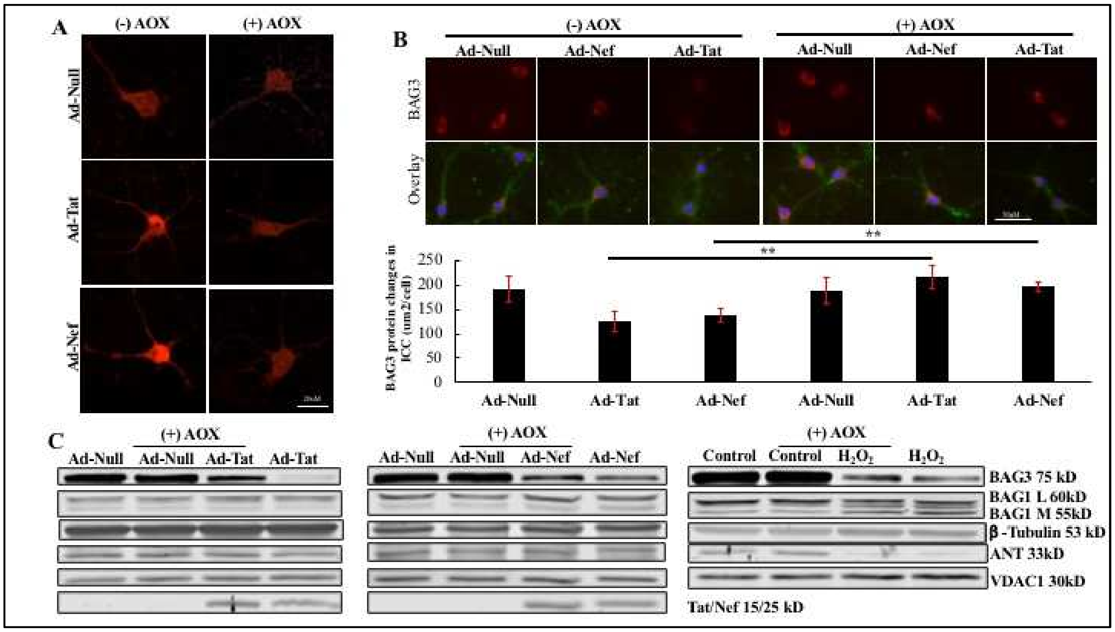

Figure 16. Effects of Reducing Oxidative Stress. Confocal microscopy images of ROS levels in neurons (A). Immunocytochemistry images and quantifications (B) of BAG3 protein levels in response antioxidant treatment under specified conditions. Immunoblot images of target protein levels in response antioxidant treatments under specified conditions (C). ** $p < .01$ compared to control conditions.

Assessment of electrophysiological measurements indicated AOX was capable of partially preserving neuronal functioning otherwise dysregulated by HIV viral proteins, particularly Tat (Figure 17 A&B). As shown by LFP signals and quantifications, suppression of peak amplitudes in Tat expressing neurons continued to progress between Days 4 and 7 (t (4) = 3.99, p = .016), while Tat expressing neurons maintained in AOX did not illustrate further attenuation in amplitudes (t (4) = 1.46, p = .219) (Figure 17 C). Similarly, suppression of firing frequencies in Tat expressing neurons continued to progress between Days 4 and 7 (t (4) = 5.25, p = .006), while Tat expressing neurons maintained in AOX did not illustrate further attenuation in firing frequencies (t (4) = 0.31, p = .775) (Figure 17 D). Interestingly, while AOX treatment in Nef expressing neurons partially preserved firing amplitudes under acute conditions (t (4) = 1.51, p =

.207), it was incapable of inhibiting long term attenuation in amplitudes or preserving firing frequencies.

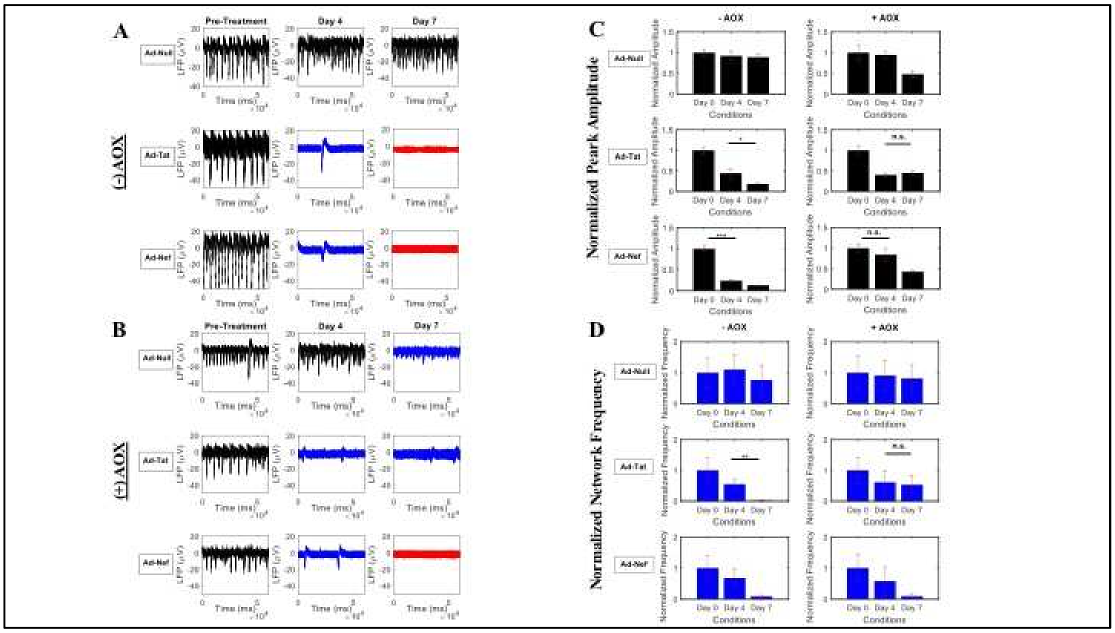

Figure 17. Effects of Reducing Viral Protein-Induced Oxidative Stress on Neuronal Firing Frequencies and Amplitudes. Raw signal traces of specified conditions in the absence (A) and presence (B) of antioxidants. Quantifications of signal amplitudes (C) and firing frequencies (D) in response to specified conditions. * $p < .05$, ** $p < .01$, *** $p < .001$ in comparison to baseline conditions specified in figure (i.e. Day 0 or Day 4).

Upon visual presentation of spontaneous frequency activity across neuronal populations, evaluation of baseline activity confirmed regular bursting activity in all MEAs (Figure 18 A). Here, dots represent time-dependent activity of individual electrodes. In the absence of AOX treatment, neurons transduced with Ad-Tat or Ad-Nef consistently displayed disrupted firing patterns across electrodes. Treating neurons with AOX resulted in a partial restoration of synchronous activity in the Tat expressing group, but not Nef. Analyses of conduction velocities quantified the speed at which similar patterns of activity propagated across proximal electrodes, whether in the form of spikes or subthreshold oscillations (Figure 18 B). In agreement with the measures above, data indicated deterioration in conduction velocities in neuronal cultures expressing Tat (t (4) = 3.01, p = .040) or Nef (t (4) = 3.07, p = .037) between Day 4 and 7. However, the application of AOX prevented further attenuation in activity patterns amongst Tat expressing neurons (t (4) = 0.58, p = .591), and improved activity in the Nef expressing group (t (4) = 3.65, p = .022). Notably, the effects of AOX under H_2O_2 conditions could not be reliably examined due to peroxide-induced electrical interference during recording sessions.

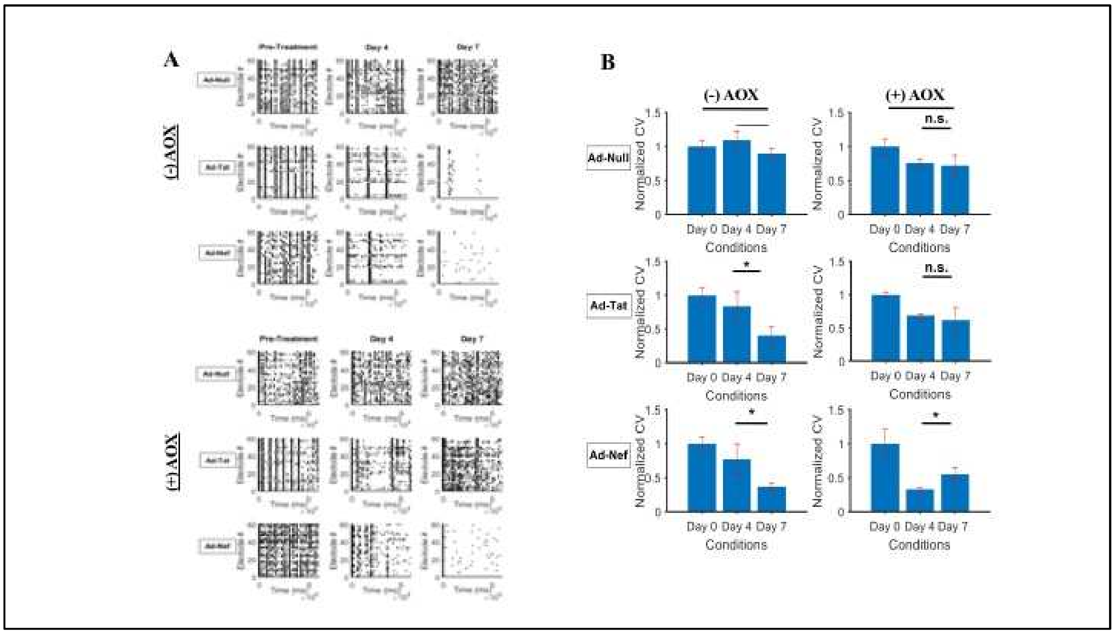

Figure 18. Effects of Reducing Viral Protein-Induced Oxidative Stress on Neuronal Conduction Velocities. Raw depictions of spontaneous frequency activity across neuronal populations in the absence and presence of antioxidants (A). Quantifications of signal conduction velocities in response to specified conditions (B). * $p < .05$ in comparison to baseline conditions specified in figure (i.e. Day 4).

4.2.4 Discussion

Aim Two B was designed to assess the functional variation in neuronal firing patterns associated with HIV proteins, thus providing insights for the underpinnings of neuronal malfunctioning observed in HAND. The current findings indicate the inhibition of oxidative stress in primary neurons was capable of partially preserving electrophysiological functioning and BAG3 levels otherwise altered by HIV viral proteins. Taken together, results support conclusions in Aim One, which suggested that oxidative stress serves a modulator of neuronal functioning and homeostasis by regulating BAG family members in response to HIV proteins or other potential sources of oxidative stress, such as aging. In turn, Tat and Nef induced alterations in PQC via oxidative stress may facilitate aberrant neuronal functioning underlying age-related cognitive dysfunction among HIV infected individuals.

Electrophysiological results presented here support previous investigations of altered neuronal functioning in response to HIV viral proteins. Along with the replication of Tat-induced variation in neuronal firing capacitates, current data expanded these findings to document the adverse effects of Nef (Krogh, Green, & Thayer, 2014; Mohseni Ahooyi, Shekarabi, Decoppet, et al., 2018; Mohseni Ahooyi et al., 2019; Saribas et al., 2018). Current results also complement *ex vivo* electrophysiological experiments, which illustrate disrupted subthreshold oscillations and altered action potential kinetics in neurons following *in vivo* Tat expression across the cortex and hippocampus (Cirino, Harden, McLaughlin, & Frazier, 2020). Furthermore, the disruptions in neuronal functioning observed here emulate the variation observed in HIV patients with decreased cognitive capacities, which include blunted ERP amplitudes, dyssynchronous activation patterns and abnormal contributions of specific frequency bands (Babiloni et al., 2014; Becker et al., 2012; Fletcher, Raz, & Fein, 1997; Nielsen-Bohlman, Fein, Boyle, & Ezekiel, 2002; T. Wilson et al., 2013).

Interestingly, the observed adaptation of neuronal functioning in response to antioxidants suggests the possibility of compensatory functional mechanisms in neurons that confer resiliency following exposure to viral proteins. This postulation aligns with theories developed among clinical investigators, who suggest the elevated recruitment of certain cortical networks in aging could reflect an adaptive reorganization process, enabling an individual to compensate for the declining sensory and information processing that would otherwise constrain attentional capacities. For instance, aged individuals faced with attention demanding tasks display elevated bilateral recruitment of pre-frontal cortex, whereas adults and youth asymmetrically engage this circuitry (Cabeza, 2002; Reuter-Lorenz, Grady, Cabeza, & Dennis, 2013). Furthermore, such overactivation is proportional to task difficulty itself, with aged individuals requiring more activation as attention demands increase (Reuter-Lorenz & Cappell, 2008; Reuter-Lorenz & Lustig, 2005). Similar compensatory mechanisms are observed in HIV infected individuals, where recruitment of previously under-utilized brain networks or more efficient use of existing networks can facilitate individual variation in cognitive performance (Chang, Holt, Yakupov, Jiang, & Ernst, 2013; Chang et al., 2004; Chang, Yakupov, Nakama, Stokes, & Ernst, 2008; Ernst et al., 2009). Thus, the data reported here provide mechanistic evidence suggesting the compensatory processes observed in age-related cognitive decline may be mediated by adaptations in neuronal functioning following exposure to HIV proteins or other sources of oxidative stress, including aging.

CHAPTER 5

CONCLUSIONS, LIMITATIONS AND IMPLICATIONS

5.1 Conclusions

The current findings address the potential for HIV proteins to mediate the neuronal mechanisms implicated in age-related cognitive dysfunction, particularly HAND. Although data indicate limited effects on SGs, Tat and Nef facilitated elevations in intraneuronal ROS, decreased BAG1 transcription and suppressed BAG3 levels (i.e. protein and RNA), while these disrupted neuronal mechanisms coincided with impaired neuronal firing capacities. Oxidative stress, in the form of H_2O_2, exacerbated the reduction in BAG3 and induced an upregulation of BAG1. Such inverse ratios were maintained in two animal models which express viral proteins in the CNS, the doxycycline-inducible Tat (iTat) and Tg26 mouse. Interestingly, the inhibition of oxidative stress in primary neurons was capable of partially preserving electrophysiological functioning and BAG3 levels otherwise altered by HIV viral proteins. Results suggest oxidative stress induced by HIV proteins or other factors (i.e. aging) may dysregulate neuronal PQC, particularly BAG chaperones, and contribute to the neurobiological mechanisms underlying HAND.

In addition to renewed enthusiasm for HAND, contemporary findings highlight important considerations in assessing the etiology of cognitive dysfunction in our aging HIV population, including the role of oxidative stress and altered PQC. These studies also underscore the value of advanced analytic techniques in aging neuroscience and neurovirology, as well as the growing necessity for translational, interdisciplinary approaches in determining the underlying mechanisms of age-related neurological dysfunction. Overall, current results illuminate novel means of enhancing neuronal homeostatic functioning to ameliorate the adverse consequences of HIV viral proteins in the CNS and to mitigate neuropathological manifestations associated with HAND.

5.2 Limitations

Current findings provided further mechanistic insights for the biomolecular underpinnings of HIV neuropathogenesis, particularly regarding the consequences of viral proteins Tat and Nef on homeostatic functioning in neurons (Gurwitz et al., 2017; M. H. Lehmann, J. M. Lehmann, & V. Erfle, 2019). However, several limitations in existing literature as well as the current experiments warrant consideration.

Although there are investigations indicating an association between HIV infection and cognitive deficits, some studies do not support this finding, while others have documented seropositive individuals who do not illustrate cognitive decline (Gisslén et al., 2011; McDonnell et al., 2014; Winston & Spudich, 2020). To address this discrepancy, future studies are encouraged to assess the role of specific HIV strains and cognitive impairments, provided that non-pathogenic viral strains can obscure otherwise reliable associations between infection and disease progression (T. Kato et al., 2008; Mandell et al., 2014). Furthermore, as assessment of viral levels can vary across studies (i.e. host immunoglobulins, integrated DNA, viral transcripts etc.), and these inconsistent measurements may fail to precisely distinguish states of viremia between studies (i.e. acute infection, reactivation, latency and/or multiple reactivations), it behooves future investigations to employ parallel techniques in viral assessment across analyses in order to enhance the reliability of conclusions.

In addition, the cross-sectional designs employed by some investigations may confound the assessment for neurocognitive deficits following HIV infection, provided that neurotropic reactivation(s) over time may facilitate neuropathological progressions. Consistent with this postulation, some data indicate elevated neurotropic infections amongst MCI patients compared to those diagnosed with AD, while significant associations between neurotropic infection and AD risk may only become apparent after extended durations (Kobayashi et al., 2013; Lövheim et al., 2015; Mancuso, Cabinio, Agostini, Baglio, & Clerici, 2020). Thus, the cross-sectional and factorial experimental designs utilized in some studies may fail to reflect time-dependent variation in vector-host interactions that could overlap with physiological aging processes. In turn, the adoption of longitudinal study designs is encouraged to more accurately capture the heterogeneous trajectories and potentially time-dependent risks of cognitive impairments due to HIV.

Although current evidence has elucidated a number of crucial mechanisms, several methodological confounds remain, including the limited external validity with respect to *in vitro* models. Whereas many current studies have utilized *in vitro* techniques and cell line models to establish causal precedence amongst HIV associated mechanisms and ensuring cellular dysfunction, further examinations are necessary to determine if such causal relationships are relevant to behavioral performance in animal models as well as clinical samples, provided that results on cognitive tasks can fail to translate across models (Sarter, 2004; Sarter et al., 1992a, 1992b). While contemporary extraneous circumstances inhibited *in vivo* assessment in cognitive tasks, it should be noted the HIV animal models employed herein otherwise maintain cognitive deficits and display increased levels of oxidative stress (Carey et al., 2012; Putatunda et al., 2019; Rai et al., 2013; Sivalingam, Cirino, McLaughlin, & Thangavel, 2020).

Consistent with such methodological concerns, sample sizes in the current statistical analyses limits the reliability of findings. Such comparison of means in restricted sample sizes is characteristic of biomolecular experiments and limits the power of analyses; however, it nonetheless enables the interpretation of experimental findings for biological relevance that would otherwise be obscured by the use of non-parametric (e.g. Mann-Whitney-U) or discrete (e.g. chi-square) alternatives (i.e. due to the inherent limited variation in rankings/levels between groups) (Pollard, Pollard, & Pollard, 2019). Despite limited sample sizes, it should be noted the results reported herein were replicated across three independent primary neuronal cultures, while the well-controlled laboratory conditions reduced the variation between samples sometimes reported in observational studies.

Along with the lack of behavioral data and limited statistical power, the *in vitro* culture system employed herein deserves consideration. Consistent with techniques in the field, the efficient culturing of primary rat neurons in the current experiments renders minimal proliferation non-neuronal cell populations (<5% glia); moreover, the time at which experiments were conducted (i.e. DIV 12-30) enables the establishment of mature neurons (e.g. synapse formation, action potentials) without compromised neuronal viability or excessive non-neuronal cell proliferation (Basarsky et al., 1994; Gordon et al., 2013; Grabrucker et al., 2009). Thus, findings presented here reflect of neuron-specific effects, and suggest caution when translating findings to other models without further validation. In line with this limitation, while we reported increased BAG1 and decreased BAG3 RNA in brain tissues of two different HIV animal models, this pattern of regulation may reflect a compensatory adaptation between neuronal and non-neuronal cells to confer neuroprotection during adulthood (Malva et al., 2007; Valles et al., 2019). Therefore, future studies should determine if this regulation of neuronal BAG proteins in animal models of HIV is age dependent, given that cell-specific population densities and functioning can vary in aging CNS, compared to adolescence or adulthood (Salas et al., 2020).

In line with potential limitations in an *in vitro* model, current experiments utilized adenoviral transduction to provide efficient delivery of HIV DNA necessary for Tat and Nef transcription and translation (Wold & Toth, 2013). While such a technique enables the determination of neuron-specific effects and the recapitulation of persistent viral protein expression in the HIV CNS, this model has two primary drawbacks. First, it remains unclear is neurons can support constitutive viral replication; thus, the intracellular expression of Tat and Nef in the current neuronal system may itself not be representative of additional extracellular effects these proteins could have on neurons (Bissel & Wiley, 2004). Nevertheless, it should be noted that Tat and Nef are rapidly internalized in neurons and compromise firing capacitates following section by actively infected CNS cells (Y. Liu et al., 2000; Saribas et al., 2018). Second, although adenoviral genetic materials are not integrated into the host genome, it is plausible that their interaction with host cell processes (e.g. gene regulatory machinery) could confound the subsequent assessment on neuronal homeostatic mechanisms (Miciak, Hirshberg, &

Bunz, 2018; Váňová, Hejtmánková, Kalbáčová, & Španielová, 2019). Current experimental designs addressed this potential discrepancy and illustrated that Ad-Null transduced neurons (i.e. viral vector without HIV DNA) do not display adverse effects. To enhance the reliability of present results, further investigations are encouraged to validate the physiological relevance of these data in the context of recombinant Tat and Nef proteins.

Another limitation of the current experiments is due to the specificity of HIV proteins employed. Indeed, additional data is necessary to determine if the variation in neuronal mechanisms induced by Tat and Nef is similar to other viral proteins, such as gp120 and VpR. More specifically, the strain of HIV DNA (89.6, Group M, subtype B) used for construct design of Tat and Nef adenoviral vectors in current experiments represents HIV populations in USA, Western Europe and Australia (i.e. ~12% of all infections), whereas different HIV strains persist in other populations (e.g. subtype C, ~50% of all infections; predominates in Southern Africa and India) (Geretti, 2006; B. Taylor, Sobieszczyk, McCutchan, & Hammer, 2008). Furthermore, Tat and Nef protein structures can vary between and within strains, while these variations confer distinct effects in host cells (Arya, 1993; J. Foster et al., 2001; Hirao et al., 2020; Laguette, Brégnard, Benichou, & Basmaciogullari, 2010; Mele, Marino, Dampier, Wigdahl, & Nonnemacher, 2020; M. E. Williams, Zulu, Stein, Joska, & Naudé, 2020). In order to further elucidate the role of HIV proteins in facilitating mechanisms in HAND, future studies should assess if the adverse effects on neuronal homeostasis are dependent on strain- and subtype-specific HIV polypeptides.

5.3 Alternative Mechanisms

5.3.1 Neuroinflammation

While the current experiments primarily focused on the adverse effects of HIV proteins on neuronal PQC and oxidative stress, several other mechanisms may contribute to the underpinnings of age-related neuronal dysfunction and warrant consideration, including chronic neuroinflammation as well as disrupted lipid homeostasis. Indeed, dysregulated neuroinflammation increases with advancing age, and poses a risk for developing AD as well as HAND (Cribbs et al., 2012; Gabuzda & Yankner, 2013; Hong & Banks, 2015; Lynch, 2010). Here, neuroinflammation is broadly associated with increased production of proinflammatory mediators, such as TNF-α, IL-1β, and IL-6, and reduced levels of anti-inflammatory cytokines, such as IL-10 (Baron, Babcock, Nemirovsky, Finsen, & Monsonego, 2014; Chung et al., 2009; Lucin & Wyss-Coray, 2009; Von Bernhardi, Tichauer, & Eugenin, 2010). Age-related neuroinflammation, if left controlled, can result in an exaggerated production of pro-inflammatory cytokines, as well as other cytotoxic mediators, that can exert detrimental effects on neurons and contribute to cognitive dysfunction in AD and HAND (Denver & McClean, 2018; Hong & Banks, 2015; Rea et al., 2018; Simen, Bordner, Martin, Moy, & Barry, 2011).

Consistent with these adverse consequences of aberrant neuroinflammation, evidence from postmortem human studies demonstrate significantly increased expression of pro-inflammatory cytokines and immune dysregulation across numerous brain regions of elderly subjects displaying cognitive impairments, including those diagnosed with either mild cognitive impairment (MCI) or AD (Minett et al., 2016; Perez-Nievas et al., 2013; Styren, Civin, & Rogers, 1990; Xiang, Haroutunian, Ho, Purohit, & Pasinetti, 2006). Furthermore, in transgenic AD mouse models, ablation of genes associated with proinflammatory signaling (e.g. TNF or IL12/23 receptor) can reduce Aβ load, maintain neuronal viability, and preserve hippocampus-dependent memory capacities across multiple behavioral paradigms, including the hole-board memory test, object recognition task, contextual fear conditioning and the Barnes maze (He et al., 2007; Vom Berg et al., 2012). Similarly, treatment with anti-inflammatory cytokines in rat models can reduce inflammation triggered by intra-hippocampal Aβ injections and reverse memory impairments (Jacobsen et al., 2006; Mudò et al., 2019).

In addition to a tonic neuroinflammatory profile, variation in the primary mediators of the brain's immune responses, (i.e. microglia), may contribute to neuronal dysfunction observed in AD and HAND (Rivest, 2009; Sousa, Biber, & Michelucci, 2017). Recent evidence indicates that during the course of aging and AD, these cells lose their functionality and contribute to the previously characterized chronic state of inflammation (Hickman, Izzy, Sen, Morsett, & El Khoury, 2018; Spittau, 2017). Indeed, numerous genome wide association studies (GWAS) and other genomic interrogations have discerned a variety of microglia-specific genetic associations with AD, including the triggering receptor expressed on myeloid cells 2 (TREM2) (Hollingworth et al., 2011; Jonsson et al., 2012; Lambert et al., 2013; Villegas-Llerena, Phillips, Garcia-Reitboeck, Hardy, & Pocock, 2016). Studies in rodents and humans have shown that during aging, an increasing proportion of microglia across different brain regions transition to a dystrophic (senescent) phenotype, characterized by deramification (loss of branching), cytoplasmic deterioration/edema, as well as the thinning, twisting and fragmentation of protrusions (Sierra, Gottfried-Blackmore, McEwen, & Bulloch, 2007; Streit, Braak, Xue, & Bechmann, 2009; Streit, Sammons, Kuhns, & Sparks, 2004; Wong, 2013). In support of their role in contributing to age-related neuronal dysfunction, the mitigation of reactive microglia across various rodent models can preserve or rescue cognitive detriments (Elmore et al., 2018; Jin et al., 2019; Kiyota et al., 2010).

Several lines of research support the postulation that variation in neuroinflammatory mechanisms in response to HIV infection can increase risk for HAND, including cell surface expression of receptors, cytokine signaling and the regulation of peripheral immunity. For example, as one of the most abundant and versatile classes of immune receptors, disruptions in MHC/HLA signaling is associated with HAND susceptibility as well as AD pathogenesis (Cifuentes & Murillo-Rojas, 2014; Kløverpris, Leslie, & Goulder, 2015; Lu et al., 2017). Additionally, evidence indicates HIV elevates levels of various pro-inflammatory cytokines and chemokines in the CNS, including I-1β, IL-6 and IL-8 (Kaul, Garden, & Lipton, 2001; Tyor et al., 1992). Indeed, compared to uninfected controls and non-demented counterparts, HIV infected individuals suffering from cognitive dysfunction display significantly elevated levels of IFNα and MCP-1 in CSF (Conant et al., 1998; Rho et al., 1995). Along with variation in immune receptors and cytokine signaling, elevated inflammatory processes originating in the periphery (e.g. pro-inflammatory chemokines and cytokines, infiltrating T lymphocytes and monocytes) are thought to exacerbate underlying inflammation within the CNS, thus furthering neuropathology in AD as well as HANDs (Clifford & Ances, 2013; Hong & Banks, 2015; Niraula, Sheridan, & Godbout, 2017; Norden, Muccigrosso, & Godbout, 2015). Thus, given its trophic capacities for immune cells outside the CNS, HIV may also augment systemic inflammation that subsequently contributes to infiltrating immune processes in HAND (D. Williams et al., 2014).

Modified microglial functioning may also contribute to age-related cognitive dysfunction HAND; post-mortem analyses indicate microglia densities are positively correlated with the degree of cognitive dysfunction in infected patients, while these cells also adopt distinct immune responses and may contribute to an observed disruption in glutamate synthesizing enzymes (Anthony, Ramage, Carnie, Simmonds, & Bell, 2005; Ghorpade et al., 2005; Glass, Fedor, Wesselingh, & McArthur, 1995; Y. Huang et al., 2011). Moreover, amongst virally suppressed individuals who remain cognitively sound, impaired executive performance is nonetheless associated with greater microglia activation across multiple cortical regions, whereas such an association is absent among uninfected controls (Garvey et al., 2014).

5.3.2 Lipid Homeostasis

As another factor that may contribute to the neurobiological underpinnings of cognitive decline in HAND and AD, variation in lipid processing has been subject to increasing scrutiny. Initial reports have long suggested global changes in lipid concentrations accompany aging in the brain (Rouser & Yamamoto, 1968; Seidel, 1958). Indeed, alterations in specific lipid species, such as phospholipids, cholesterol, and polyunsaturated fatty acids, have been documented in the CNS of elderly subjects (Farooqui, Liss, & Horrocks, 1988; McNamara, Liu, Jandacek, Rider, & Tso, 2008; Söderberg, Edlund, Kristensson, & Dallner, 1990). Moreover, due to dietary interventions, alterations in lipid composition are suggested to protect against age related cognitive impairments across animal and clinical models (Roberts et al., 2017; Yurko-Mauro et al., 2010). Along with differing composition, alterations in lipid metabolism may contribute to age-related neuronal malfunctioning implicated in HAND. For instance, peroxidized lipids can generate reactive and unstable byproducts that are known to compromise neuronal viability and functioning (Pamplona, 2008). Interestingly, such increased levels of lipid peroxidation are associated with significantly shorter life expectancies across a variety of mammalian species and correlate with the onset of age-associated neuropathologies (de Diego, Peleg, & Fuchs, 2019; Jové et al., 2013). Together, these data indicate modifications in lipid composition and metabolism accompany aging, while these effects may contribute to neuronal malfunctioning in aged HIV individuals.

Several lines of evidence have focused on alterations in cholesterol in particular as an important modulator of age-related cognitive decline (Puglielli, Tanzi, & Kovacs, 2003). Indeed, observations from the AD clinical population have indicated excessive Aβ deposition accompanies the accumulation of cholesterol within the CNS (Cutler et al., 2004). Additionally, decreased levels of cholesterol in neuronal cultures can promote the nonamyloidogenic processing of amyloid precursor protein (APP), while complete cessation of cholesterol can block its amyloidogenic pathway, and deplete its processing entirely by inhibiting gamma secretase activity (Kojro, Gimpl, Lammich, Marz, & Fahrenholz, 2001; Simons et al., 1998; Wahrle et al., 2002). Conversely, elevated neuronal cholesterol can promote APP's toxic amyloidgenic pathway *in vivo*, potentially due to its positive modulation of BACE1 within intracellular lipid rafts (Ghribi, Larsen, Schrag, & Herman, 2006; Marquer et al., 2011; von Arnim et al., 2008).

Further consideration for cholesterol's role in AD arises from the mechanism which allows its transport between neuronal and glial cell bodies, specifically apolipoproteins (APOE) (De Chaves & Narayanaswami, 2008). Here, a robust association is observed throughout the clinical population between the APOE-4 isoform and the diagnosis of AD (Corder et al., 1993; Lambert et al., 2013). Moreover, APOE-4 status can dictate the efficacy of both pharmacological and non-pharmacological AD therapeutics (Solomon et al., 2018; L. Wang et al., 2014). Consistent with its proposed physiological function, some evidence suggests the association between APOE and AD is due to the dysregulation of homeostatic cholesterol transport (Y. Lin et al., 2018; J. Zhao et al., 2017). However, other evidence suggests such a correlation could be due to APOE's capacity to directly bind and facilitate the clearance of Aβ; indeed, APOE-4 maintains the lowest binding capability for Aβ compared to its other APOE isoforms (Jiang et al., 2008; Tokuda et al., 2000). Consistent with this hypothesis, transgenic models harboring APOE-4 routinely display significantly elevated Aβ deposition and impaired Aβ clearance compared to conspecifics expressing alternative APOE isoforms (Buttini et al., 2002; Deane et al., 2008; Dolev & Michaelson, 2004). Likewise, elevated oxidative modifications on levels cholesterol is hypothesized to facilitate the accumulation of toxic Aβ, provided that oxysterols (e.g 24-OH Chol & 27-OH Chol) are known to mediate the enzymatic processing of APP and influence Aβ production (Famer et al., 2007; Marwarha, Raza, Prasanthi, & Ghribi, 2013).

A growing body of literature suggests lipid dysregulation upon HIV infection may contribute to HAND, including alterations amongst the most vulnerable lipid species such as ceramide, sphingomyelin and cholesterol (Bonfanti et al., 2007; Bowman et al., 2019; Funderburg & Mehta, 2016). Dyslipidemia is indeed a hallmark of HIV infection and is routinely associated with an individual's state of viremia (Oka et al., 2012; Rose et al., 2008). In regards to ceramide and sphingomyelin, aberrantly elevated levels of these lipid species have been reported in the brain tissue and CSF of HIV infected individuals displaying significant cognitive impairments; moreover, *in vitro* assessment of these altered lipids suggested their causal role in facilitating significantly increased levels of oxidative stress and ensuing neuronal apoptosis (Haughey et al., 2004). Similar results have been reported in a separate cohort of HAND patients, where dysregulated ceramide and sphingomyelin levels were associated with decreased performance in mnemonic capacities (Mielke, Bandaru, McArthur, Chu, & Haughey, 2010). Moreover, following the reduction in plasma ceramide due to drug treatment, individuals diagnosed with HAND illustrate improved cognitive performance across several specific domains (e.g. sequential reaction time task, letter number sequencing) (Sacktor et al., 2018).

Along with ceramide and sphingomyelin, the dysregulation of cholesterol metabolism in both neuronal and non-neuronal cells following HIV infection has been examined and may contribute to HAND (Waheed & Freed, 2009). For instance, HAND patients who maintain significantly decreased CSF levels of cholesterol compared to other lipid species (e.g. sphingomyelin) also maintain significantly impaired performance across several cognitive tasks (e.g. Rey Auditory Verbal Learning Test, Delayed Recall) (Mielke et al., 2010). Similarly, significant reductions in multiple forms of cholesterol in the CNS can classify infected HIV individuals from uninfected controls, while further reductions in esterified cholesterols in particular can predict subsequent likelihoods for developing cognitive decline (Bandaru et al., 2013). Furthermore, exposure to HIV viral proteins (i.e. Tat) can induce the dysregulation of neuronal cholesterol pathways, which consequently compromise homoeostasis and viability across neuronal and non-neuronal cells of the CNS (Ahooyi et al., 2018; Cotto et al., 2018).

5.4 Implications for Treatment

Taken together, results presented here suggest the regulation of oxidative stress in conjunction with PQC modulators may result in novel therapeutic opportunities in HAND. Specifically, data gathered from HIV proteins indicated antioxidants could partially preserve neuronal electrophysiology and BAG3 levels. This is consistent with the otherwise adverse consequences of Tat and Nef, which include the generation of abnormal ROS and impairments in PQC (Agrawal et al., 2012; G. R. Campbell, P. Rawat, R. S. Bruckman, & S. A. Spector, 2015; Fields et al., 2015; Ivanov et al., 2016). While the reliability of antioxidant therapeutics remains debated, some reports suggest their application in combination with other treatments can result in enhanced outcomes for neurodegenerative conditions, including AD (Cornelli, 2010; Kabir et al., 2020). As the selective augmentation BAG proteins has proved efficacious in the context of other age-related neurological diseases, targeted PQC therapies in combination with antioxidant therapies may provide novel treatments amongst our aging HIV population (Y. L. Cao et al., 2017; Carra, Seguin, Lambert, & Landry, 2008; Kermer et al., 2015; Lei, Brizzee, & Johnson, 2015).

In addition, future investigations are encouraged to validate if the existing evidence suggesting the benefits of enhanced BAG3 functioning are maintained in HAND. For instance, BAG3 overexpression has been shown to mitigate α-synuclein concentrations (i.e. implicated in PD) and increases its translocation to perinuclear compartments in conjunction with Hsc70 and p62 (Y. L. Cao et al., 2017). Along with a subsequent elevation in HSPB8 binding, the augmentation BAG3 similarly results in significantly reduced levels of polyQ repeats (i.e. implicated in HD) (Carra et al., 2008). Additionally, increasing BAG co-chaperone's (i.e. Hsc70) capacity to recognize aggregate-prone substrates, via the stabilization of its ADP-bound conformation, inhibits the formation insoluble cytosolic aggregates by promoting the clearance of α-synuclein as well as polyQ repeats (Roodveldt et al., 2009; A. Wang et al., 2013). Moreover, in primary neurons, such BAG3 overexpression is capable of significantly decreasing levels of pTau (Lei et al., 2015). The validation of such results would encourage the consideration of targeted PQC modulators as novel therapeutic options in HAND.

As exemplified by emerging data in accelerating aging syndromes, particularly from Hutchinson–Gilford progeria (HGPS) research, the augmentation of PQC processes represent a reliable method for effectively inhibiting aberrant protein aggregation and preserving cellular homeostasis. HGPS is characterized by the abnormal aggregation of the protein progerin, and the rapid induction of cellular conditions commonly associated with aging (Ghosh & Zhou, 2014). Striking results from cultured HGPS cells demonstrate the direct enhancement of autophagy mechanisms, via the application of rapamycin and ensuing inhibition of mTOR1, significantly reduces concentrations of progerin (particularly its insoluble conformation), inhibits the accumulation of progerin puncta in the nucleoplasm, and significantly enhances cell viability (K. Cao et al., 2011). Similar results were obtained in a separate cohort of HGPS fibroblasts, where the induction of autophagy machinery (i.e. via rapamycin treatment) lead to significantly decreased progerin aggregation, and inhibited the deleterious consequences of progerin accumulation (i.e. damage to chromatin from a comprised nuclear envelope) (Cenni et al., 2011). Thus, provided that both HAND and HGPS are fundamentally characterized by their abnormal PQC, further investigations are encouraged to determine if the targeting of PQC mechanisms in accelerated aging disorders might serve as a broader model in advancing treatment options for HAND (Burtner & Kennedy, 2010).

The potential role of HIV (i.e. a neurotropic virus) to facilitate neuronal dysfunction reveals several additional therapeutic targets that merit consideration, including polymerase inhibitors, transcriptase inhibitors and transferrin-targeting delivery vehicles. For example, adherence to a DNA polymerase inhibitor (i.e. valacyclovir) can reduce the risk of dementia by 10-fold in HSV-1 infected individuals and preserve neuronal viability otherwise compromised following HSV reactivation (Doll, Hoebe, Thompson, & Sawtell, 2020; Tzeng et al., 2018). Examination of nucleoside reverse transcriptase inhibitors (NRTIs) may also prove efficacious, provided that the antiretroviral medication lamivudine can mitigate age-related neuroinflammation that is implicated in AD and HAND (De Cecco et al., 2019). Such genomic-regulatory approaches in treatment seem particularly appealing, given that the delivery of select transcription factors was recently shown to revert aging and AD-associated mechanisms (Sarkar et al., 2020). Bioengineering advances that enhance CNS drug delivery should also be considered, given that the efficacy of antiretrovirals in the CNS is inherently dependent upon penetration across the BBB (Letendre et al., 2008). In particular, the development of transferrin-targeting immunoglobulins may serve as a reliable drug delivery vehicle to the brain and enable the amelioration of neuroviral contributions to neuronal dysfunction in HAND (Kariolis et al., 2020; Ullman et al., 2020).

CHAPTER 6

MATERIALS AND METHODS

6.1 Primary Neuronal Culture and Cell Treatment

Tissue preparation and cell cultures were performed according to Temple University's Institutional Animal Care and Use Committee and the National Institute of Health (NIH) guidelines. As described previously, primary neuronal cultures were prepared using dissected E18 prenatal rat embryonic brains (Mohseni Ahooyi, Shekarabi, Decoppet, et al., 2018; Mohseni Ahooyi et al., 2019). After digestion in trypsin solution (0.25%), neurons were plated on tissue culture plates, slides or microelectrode arrays, MEAs (see below), that were coated with poly-D-lysine (Sigma Aldrich, St. Louis, MO) as well as laminin (Invitrogen 23017, USA). Cells were maintained 14 days (25 days for MEA studies) in vitro (DIV) prior to treatments, at which point experiments were initiated. All experiments were completed in triplicate to confirm the reliability of results. For 6-well plates, approximately 1.5 x 10^6 cells were applied per well; for chamber slides and MEAs, approximately 1.5 x 10^5 cells were applied per well. Cell populations were estimated using disposable slides (C10228; ThermoFisher, Waltham, MA) and an automated cell counter (AMQAF1000; ThermoFisher, Waltham, MA). Cells were initially incubated for 24 hours in first day media (Neurobasal Media, 2% fetal bovine serum, 2% B27 supplement, 1% Penicillin-Streptomycin, .25% Glutamax supplement). This first day media is formulated to provide ample nutrition for developing neurons while minimizing the growth of non-neuronal cells. After 24-hour incubation, all first day media will be replaced with second day media (Neurobasal Media, 2% B27 supplement, 1% Penicillin-Streptomycin, .02% Glutamax supplement). Second day media is formulated to provide sufficient nutrition for developing and mature neurons while eliminating the potential growth of non-neuronal cells. To ensure viability of cells, cells were maintained at constant temperature (37C) and oxygen (95% O_2, 5% CO_2).

Neurons were transduced with Ad-Null (Vector Biolabs, Malvern, PA), Ad-Tat (made in-house), Ad-Nef (made in-house), Ad-siBAG3 (Vector Biolabs, Malvern, PA) and Ad-BAG3 (Vector Biolabs, Malvern, PA). To produce viral constructs in-house for transduction, cDNA was cloned from HIV strain 89.6 into compatible restriction sites of the shuttle plasmid pDC515(IO) and subsequently rescued by co-transfection with pBHGfrtDeltaE1,E3FLP in 293 IQ cells (Microbix Corporation, Mississauga, Ontario Canada). The Ad was then plaque purified, amplified, and subsequently purified on cesium chloride (CsCl) gradient centrifugation. Plaque purified virus was dialyzed against elevated salt and MgCl2 for further purification, provided that CsCl does not generate entirely pure preparations. Collected viral particles were diluted for concentration measurement at OD260, while the virus concentration was calculated based on number of particles/ml. MOI was maintained at 1 for all vectors throughout procedures. Transduction durations were maintained in accordance with previous experiments in our laboratory (72 Hr). To ensure no degradation of reagents, H_2O_2 treatments proceeded using indicated dilutions prepared from stock immediately prior to their application (VWR BDH7690, USA). To assess the combined effect of H_2O_2 with viral proteins, H_2O_2 treatments were initiated after the transduction period; this time point remained consistent for all H_2O_2 experiments. Antioxidant (AOX) treatments were applied in a manner previously described (T.-S. Li & Marbán, 2010; Luo et al., 2014);

here, a proprietary antioxidant solution at recommended dilutions was applied in culture for 24 hours following transduction (A1345; Sigma Aldrich, St. Louis, MO).

6.2 Animal Models

Animal procedures and protocols were performed according to Temple University's Institutional Animal Care and Use Committee and the National Institute of Health (NIH) guidelines. Adult male mice (18–28 g) were singly housed in a temperature (21–23°) and humidity-controlled vivarium with constant airflow on a reverse 12-h light/dark cycle (lights on/off at 09:00 EST). Food and water were available *ad libitum*. Two mouse models previously in our laboratory were utilized in the present experiments. Tg26 HIV transgenic mice are a widely used and well-described mouse model that encode the entire pNL4–3 HIV-1 genome, except a segment of gag/pol genes. This model expresses HIV viral proteins such as Tat and Nef, while exhibiting neuropathology observed upon HIV infection, including neurocognitive deficits. Doxycycline (DOX)-inducible GFAP promoter driven HIV Tat transgenic mice were provided by the Comprehensive NeuroAIDS Center. This model conditionally-expresses the HIV Tat protein in an astrocyte-specific manner under the control of a GFAP-driven Tet-on promoter, which is activated in the presence of DOX. Both iTat and their control littermates received doxycycline hyclate (DOX) orally over the course of 6 weeks (S3888; Bioserv, Flemington, NJ). C57BL/6J mice maintained in our laboratory's colony were utilized as controls. All mice were euthanized at similar time points (approximately 6 months old) and brain tissue was harvested for subsequent analyses. While the right and left hemispheres of Tg26 animals were processed for immunoblotting/qPCR and immunohistochemistry, respectively, iTat analyses was restricted to immunoblotting/qPCR from right hemisphere homogenates due to the limited availability of tissue. After the right hemisphere was dissected (i.e. frontal cortex, whole hippocampus) and homogenized, the total volume from each region was split in equal parts for protein and RNA analysis.

6.3 Metabolic and Viability assays

To assess general metabolic activity, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dihhenyltetrazolium bromide) assay was used. Following treatment, primary rat neurons were incubated for 2 hr at 37C in solution containing MTT according to the manufacturer's recommendations (CT015; Sigma Aldrich, St. Louis, MO). Measures from each sample were determined as the difference between 595nm and 620nm wavelength values obtained by spectrophotometry. To gauge neuronal cell death, the trypan blue exclusion procedure was used. Here, cell solutions were prepared by equal dilution with trypan blue dye (15250061;ThermoFisher, Waltham, MA) followed by quantification using disposable slides (C10228; ThermoFisher, Waltham, MA) and an automated cell counter (AMQAF1000; ThermoFisher, Waltham, MA).

6.4 Immunoblotting

For immunoblotting experiments, neuronal and mouse brain samples were homogenized and lysed with RIPA buffer (25mM Trizma base pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, .1% SDS) containing a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Protein concentrations were assessed by means of a standardized Bradford assay (Bio-Rad, Hercules, CA). SDS-polyacrylamide gels, 10–12%, and nitrocellulose membranes (LI-COR, Inc., Lincoln, NE) were used for electrophoretic protein separation and transfer, respectively.To reduce non-specific binding and background, membranes were blocked in Odyssey (LI-COR) blocking buffer (1 Hr, room temperature). Primary (1:1000, overnight, 4C) and secondary (1:10000, 1 Hr, room temperature) antibodies were applied followed by imaging on the Odyssey CLx Imaging System (LI-COR). Protein levels were assessed and standardized to appropriate loading controls by means of optical density analysis using Image Studio acquisition software. The following primary antibodies were used: ANT (Santa Cruz; SC11433), β-Tubulin (Sigma; T8578), Bcl-2 (Sant Cruz; SC7382), BAG1 (Santa Cruz; SC939), BAG2 (Novus Biologicals; NBP159086), BAG3 (Proteintech; 105991AP), BAG5 (Proteintech; 266281AP), BAG 6 (R&D Systems; AF6438), BAX (Santa Cruz; SC493), GAPDH (Santa Cruz; SC32233), G3BP (Abcam; ab181150), Hsc/Hsp 70 (Sant Cruz; SC24), Histone (CellSignaling;9715), LC3 (Santa Cruz; L8918), Nef (Abcam; ab42355), Tat (NIH Aids Reagent Program; R705), TIA (Proteintech; 121332AP), VDAC1 (Santa Cruz; SC8017), YBX1(Proteintech; 203391AP).

6.5 Immunocytochemistry

Following fixation (4% paraformaldehyde), blocking (1% BSA), and washing, neurons were labeled with the following primary antibodies (1:100, overnight, 4C): β Tubulin (Sigma; T8578), BAG1 (Santa Cruz; SC376848), BAG3 (Proteintech; 105991AP) MAP2 (Cell Signaling; 4542), G3BP (Abcam; ab181150), TIA (Proteintech; 121332AP), YBX1(Proteintech; 203391AP). Alexa Fluor®secondary antibodies (1:500) (ThermoFisher, Waltham, MA) and VectaShield with DAPI medium (Vector Laboratories, Burlingame, CA) were used for labeling and mounting, respectively. Imaging utilized a fluorescence microscope (BZ-X710; Keyence, Osaka, Osaka, Japan) and image processing employed the Hybrid Cell Count module within the BZ Image

Analyzer software (Keyence) to obtain optical density measurements expressed in μM per cell.

6.6 Immunoprecipitation

For immunoprecipitation procedures, neurons were lysed in conjunction with a protease inhibitor cocktail as previously described. After protein concentrations were assessed, extracts were incubated with appropriate concentrations of the Tat antibody (1:50 dilution) in specialized buffer (20mM Trizma base pH 8.0, 137 mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA pH 8.0) at 4C overnight. Here, incubation with normal rabbit serum served as the control condition (Invitrogen; 10510). After thorough washing and purification, immunoglobulin binding beads (LSKMAGAG02; Sigma Aldrich) were then incubated with the sample-antibody solution for 4 hours at 4C. Following centrifugation, antibody-bound beads were the applied to gel electrophoresis and immunoblotting to identify if target proteins (i.e. TIA, G3BP) interacted with the antibody of interest (i.e. Tat).

6.7 Cytoplasmic/Nuclear Protein Extraction

Sample for cytoplasmic and nuclear extractions initially utilized a specialized Buffer A (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 1mM DTT and 1 mM PMSF, pH 7.6) containing a protease inhibitor cocktail, followed by an acute incubation on ice (10 min.). Appropriate volume of NP-40 was then added (1%) followed by mild vortex, low-speed centrifugation and removal of the supernatant (i.e. cytoplasmic extract). Subsequent freeze and thaw cycles with a higher dilution of NP-40 (10%) followed by harsh vortexing and high-speed centrifugation yielded a second and distinct supernatant (i.e. nuclear extract).

6.8 Immunohistochemistry

Prior to immunohistological procedures, whole brain tissue was processed for formalin fixation and paraffin embedding. After extraction, brains were stored in 10% formalin for a minimum of 24 hours. Following incubation, tissue was placed in an automated processor (Citadel 2000, Shandon) to ensure infiltration of paraffin. Processing consisted of 1 hr incubations in 10% formalin, 70% ethanol, 100% ethanol, 100% xylene, and melted paraffin. Tissue was then further encased into paraffin blocks to accommodate subsequent slicing. Brain slices were cut in sagittal sections at 5μM on a rotary microtome (HM315, Microm) and mounted onto precleaned, charged microscope slides (Superfrost Plus, Fisher Scientific).

At the commencement of procedures, paraffin was melted from slides through a 15 minute incubation at 55C. Tissue was then deparaffinized and hydrated through a series of incubations consisting of 100% xylene, 100% ethanol, 90% ethanol, 70% ethanol and distilled H_2O. Antigen retrieval was then performed using sodium citrate heated to 90C for 20 minutes. Following PBS washes, endogenous peroxidases were blocked by incubating slides in 3% H_2O_2 diluted in methanol. Following another series of PBS washes, tissues were placed in 5% serum solution (species serum dependent on host of secondary antibody) to reduce nonspecific binding following application of primary antibody. After blocking, sections will be incubated in primary antibody at 4C for 14-16 hours at a dilution of 1:200. Following primary antibody incubation, tissue was subjected to PBS washes followed by a 1 hour incubation in biotinylated secondary antibody at a dilution of 1:200. In order to form a biotin based complex necessary for chromogen staining, avidin and peroxidase was then be applied using a standardized solution

(Vectastain Elite ABC HRP kit, Vector Laboratories). After a 1 hour incubation, sections were then developed utilizing a diaminobenzidine (DAB) substrate (DAB HRP Kit, Vector Laboratories) for approximately 60 seconds. In addition, tissue was then counterstained with hematoxylin to distinguish nuclei (Vector Hematoxylin QS, Vector Laboratories). After a final set of dehydration washes in ethanol and xylene, coverslipping utilized permanent mounting media to ensure preservation of samples (Vectamount, Vector Laboratories). Imaging utilized a fluorescence microscope (BZ-X710; Keyence, Osaka, Osaka, Japan) and image processing employed the Hybrid Cell Count module within the BZ Image Analyzer software (Keyence) to obtain optical density measurements expressed in μM per cell.

6.9 ROS Quantification

To assess levels in reactive oxygen species (ROS), cells were incubated with the mitochondrial oxygen free radical indicator MitoSOX-red (Life Technologies, Carlsbad, CA) for 30 min at 37C. Slides were then mounted for confocal imaging in an open perfusion microincubator (PDMI-2; Harvard Apparatus) and images were obtained at 561 nm excitation by using a confocal microscope (810; Carl Zeiss, Oberkochen, Germany).

6.10 RNA Isolation and cDNA Preparation

Total RNA for each neuronal and mouse brain sample (frontal cortex) was processed using the Trizol (ThermoFisher) extraction protocol followed by RNA purification using Direct-zol™ RNA MiniPrep Plus (Zymo Research, Irvine, CA) in accordance with manufacturer specifications. cDNA synthesis on total RNA samples utilized with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

6.11 Real Time-Quantitative RT-PCR (qRT-PCR)

Sequence design for forward and reverse primers utilized the most to date reference genomes followed by submission to NCBI's Basic Local Alignment Search Tool (BLAST) to ensure the selected primers do not display similarity to other sequences (see below). The sensitivity and specificity of primers were checked by RT-PCR using FailSafe PCR Kit (Lucigen, Middleton, Wi). All qPCR reactions were conducted with the LightCycler96® (Roche) using the SYBR™ Green master mix (Applied Biosystems, ThermoFisher), according to the manufacturer's specifications. Relative quantity was normalized to Actin expression.

6.12 Primer Design

Sequence design for forward and reverse primers employed the most up to date reference genomes (for example, assembly Rnor_6.0). In brief, 18-22 bp sequences were selected that flank a selected 20bp sequence chosen from exons of sufficient length (> 100bp). Primer sequences contained 48-55% C/G concentrations, maintained similar melting temperatures, and maintained an amplicon size of greater than 100bp but less than 500bp. Following selection, sequences will be submitted to NCBI's Basic Local Alignment Search Tool (BLAST) to ensure the selected primers are not substantially similar to other sequences. Primer sequences were as follows: TIA (Forward 5'-ACTGTACCGTGTACTGTGGA-3'; Reverse 5'-TTCCATAGGCAGGTACTTGC-3'), G3BP (Forward 5'-GTTGCTGATGACTCTGGAA-3'; Reverse 5'-GTCCTCAAGTCCTCCTGT-3'), YBX1 (Forward 5'-CTCCACGCAATTACCAGCAA-3; Reverse 5'-ATTGAAGTTGCGGCGATACC-3'), Actin (Forward 5'-CAGGTCCAGACGCAGGATGGC-3'; Reverse 5'-CTACAATGAGCTGCGTGTGGC-

3’), Tat Forward (5’-CGGTGGGAGGCCTATATAAGC-3’; Reverse 5’-AACTGCAGTTATTCCTTCGGGCCTGTCGG-3’), BAG1 (Forward-5′-AGATGGTCCAGACGGAGGAA-3′; Reverse-5′-CAGATACCTCCAAGTCCTTCAGC-3′), BAG 3 (Forward-5′-GGCCCTAAGGAAACTGCAT-3′; Reverse-5′-GGGAATGGGAATGTAACCTG-3′).

6.13 MicroElectrode Array (MEA)

Neuronal activities were recorded pre-treatment (Day 0) and 96hrs (Day 4) as well as 168hrs (Day 7) after transduction. To ensure the assessment of AOX effects were temporally consistent with AOX effects observed in other experiments, recordings were conducted 24 hours after AOX was added to half of the cultures post-transduction (i.e. 96 hours). Media with or without AOX was then maintained until the final recording session on Day 7 to assess potential chronic effects of AOX treatment on neuronal functioning. In each recording session, multiple minutes of recordings were performed to acquire local field potential (LFP) of neuronal extracellular action potential at 2000 Hz (2kHz) from 60 electrodes simultaneously. To avoid confounding measures with electrical interference in the beginning or conclusion of experiments, stabilized signals from the middle of the recording session (i.e. three, 20-second averages) were used for statistical analyses. Recorded data were then transferred as numerical values in microvolts to MATLAB (Mathworks, Natick, MA) for pre- and post-processing. Low-pass filtering with cut-off frequency f_c=300 Hz was applied to all recording data prior to data analysis.

LFPs of different experimental groups were visualized in MATLAB with representative channels in a 60,000 ms course (120,000 sampled points for each signal). To depict the network-wide activation and provide a measure of activity beyond a single electrode, raster plots were generated based on the time at which a spike was detected for all channels, and visualized for all time points as well as experimental groups. To show the rate at which neuronal activation travels from the source across populations, the cross-correlation values between pairs of electrodes and their distance were used to compute conduction velocities (cm/s). These data were then normalized (nondominsionalized) with respect to initial conditions to reflect the treatment effects on wave propagation rates. For technical details refer to (Mohseni Ahooyi, Shekarabi, Decoppet, et al., 2018).

6.14 Statistical Analyses

Statistical procedures were optimized based on the between-subject experimental designs as well as the consistent, continuous nature of the measured variables across assays. Consistent with experimental methods used to assess variation in biological systems, primary results were replicated across at least three primary cultures. However, as conditions can vary across cultures (e.g. exact cell count) and the absolute values is some techniques are not operationally relevant (e.g. raw optical densities of immunoblot), results were normalized and primarily expressed as relative ratios (i.e. fold change) to control for conditions within each experiment as well as maintain validity (Adler & Alon, 2018). Independent samples *t*-tests were primarily employed to compare measures between control and experimental conditions across assays. While such comparison of means in the restricted sample sizes characteristic of biomolecular experiments limits the power of analyses, it nonetheless enables the interpretation of experimental findings for biological relevance that would otherwise be obscured by the use of non-parametric (e.g. Mann-Whitney-U) or discrete (e.g. chi-square) alternatives (i.e. due to the inherently limited variation in rankings/levels between groups). All statistical analyses were conducted utilizing SPSS v 22 (IBM, Armonk, NY) or MATLAB (Mathworks).

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