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Analysis of the Effects of Therapeutic Compounds for the Treatment of HIV Infection and Multiple Sclerosis in Neuroglial Cells

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Analysis of the Effects of Therapeutic Compounds for the Treatment of HIV Infection and Multiple Sclerosis in Neuroglial Cells

Abstract
HIV-Associated Neurocognitive Disorders (HAND) continue to afflict approximately half of HIV-infected patients, despite effective viral suppression through antiretroviral therapy (ART). Synaptodendritic damage and white matter pathologies persist; suggesting that toxicities from antiretroviral drugs combined with viral protein effects from CNS reservoirs may be at fault. We hypothesized that ART compounds activate cellular stress pathways leading to dysfunction of neuroglial cells, and that attenuating this stress could ameliorate the dysfunction. Previous studies have demonstrated abundant oxidative stress in HAND patients, and antiretroviral compounds are capable of generating reactive oxygen species (ROS) to invoke this stress in peripheral cell populations. Here we have established that antiretroviral compounds produce robust levels of oxidative stress in neurons and oligodendrocytes in vitro. In neurons, increased ROS leads to mitochondrial depolarization and apoptotic cell death, which was successfully rescued by treatment with fumaric acid ester monomethyl fumarate (MMF) through activation of the endogenous antioxidant response (EAR). Oligodendrocytes were also negatively affected by specific antiretroviral agents. While not directly toxic, protease inhibitors invoked dose-dependent, reversible decreases in the differentiation from oligodendrocyte precursor to mature oligodendrocytes. ROS were produced, but MMF did not rescue the maturation deficit. Further investigation concluded that MMF, the active metabolite of the drug Tecfidera, which is approved for treatment of Multiple Sclerosis, does not activate the EAR in oligodendrocytes. In fact, the EAR was also not activated by any oxidant compound tested, suggesting that precursor cells which are known to have low levels of antioxidant/detoxifying enzymes may also be susceptible to oxidative stress because they cannot appropriately activate this response. In vivo, both synaptodendritic damage and myelin loss were observed in rodent models of antiretroviral administration. In higher primates, synaptodendritic damage was evident in SIV-infected pigtail macaques that had received an ART regimen, and myelin basic protein levels were reduced in human HIV patients with HAND who had been ART-medicated for at least one year. Together these results strongly implicate antiretroviral compounds as a contributing factor for the persistence of HAND, and necessitate the development of less toxic antiretroviral therapeutics and adjunctive therapies which can minimize the CNS side-effects of currently utilized compounds.

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ANALYSIS OF THE EFFECTS OF THERAPEUTIC COMPOUNDS FOR THE TREATMENT OF HIV INFECTION AND MULTIPLE SCLEROSIS IN NEUROGLIAL CELLS

Brigid Kathleen Jensen

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ANALYSIS OF THE EFFECTS OF THERAPEUTIC COMPOUNDS FOR THE TREATMENT OF HIV INFECTION AND MULTIPLE SCLEROSIS IN NEUROGLIAL CELLS

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Brigid Kathleen Jensen

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ABSTRACT

ANALYSIS OF THE EFFECTS OF THERAPEUTIC COMPOUNDS FOR THE TREATMENT OF HIV INFECTION AND MULTIPLE SCLEROSIS IN NEUROGLIAL CELLS

Brigid Kathleen Jensen
Kelly L. Jordan-Sciutto
Judith B. Grinspan

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Introduction

1.1 OVERVIEW

In addition to effects from HIV infection and viral proteins, the antiretroviral compounds used to treat HIV have major implications for cell populations of the central nervous system (CNS) which likely contribute to the continued prevalence of HIV-Associated Neurocognitive Disorders (HAND) in roughly half of infected individuals. In addition to studying the effects of these compounds in neurons and astrocytes, the work presented here is novel in that it also focuses heavily on oligodendrocytes and a potential adjunctive therapy that may help to ameliorate antiretroviral-mediated side-effects in the CNS. This introduction will therefore provide the background for our studies on the effects of ART on these cell populations.

We will begin with an introduction of HAND, mechanisms of CNS neuropathology, and the persistence of neurological dysfunction despite therapeutic intervention (1.2). We will then proceed to review antiretroviral therapies: their development, suggested regimens, and side-effects (1.3). From this knowledge, we hypothesized that ART compounds activate cellular stress pathways in neuroglial cells leading to dysfunction, and that attenuating this stress could ameliorate these effects. One prominent cellular stress found in HAND is oxidative stress, which is generated by viral infection, viral proteins, and antiretroviral agents. This is presented next, as it is a likely mechanism for much of the neuroglial damage observed in patients (1.4). While our preliminary experimental endeavor is in neurons, a majority of the investigations and implications presented in this body of work regard oligodendrocytes. As such, the specific pathologies observed in HAND and susceptibility of oligodendrocytes to oxidative stress are also presented, and are followed by a detailed introduction to oligodendrocyte differentiation and myelin maintenance (1.5, 1.6). Due to the potential for oxidative stress operating as a mechanism for dysfunction in HAND and oligodendrocyte dysfunction, the cellular stress response pathway of the endogenous antioxidant response is summarized, and is followed by the consequences of dysfunction of this pathway in
disease (1.7, 1.8). Potential avenues for countering oxidative stress in HAND are then described (1.9). Finally, taking all of these areas into consideration our rationale and objectives are outlined (1.10).

1.2 HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS (HAND)

According to the most recent reporting in 2014, approximately 36.9 million people worldwide are infected with human immunodeficiency virus-1 (HIV-1) (UNAIDS, 2014). This disease, which is characterized by depletion of CD4+ T-cells and progression into acquired immunodeficiency syndrome (AIDS), also frequently leads to behavioral, cognitive, and motor disturbances collectively termed HIV-Associated Neurocognitive Disorders (HAND) (Kaul, 2005, Ozdener, 2005, UNAIDS/WHO, 2009). Before the advent of combined antiretroviral therapy (cART) in the late 1990s, approximately 25% of HIV-infected individuals were afflicted by a severe neurocognitive dysfunction termed HIV-Associated Dementia (HAD), with rampant neuronal death observed in frontal lobes, hippocampi, and basal ganglia (Navia, 1986, McArthur, 1987, 1994, Glass, 1995).

Neuroinvasion occurs early in the course of HIV infection, and likely enters the central nervous system (CNS) within 1-2 weeks post-infection during the time of peak viremia (Chakrabarti et al., 1991, Williams et al., 2001). The mechanism by which this occurs is still under some speculation, as this requires viral migration across the blood brain barrier (BBB) and blood cerebral spinal fluid barrier, which are normally non-permissive to invading pathogens (Atluri et al., 2015). There is accumulating evidence that the virus can cross into the CNS within infected monocytes traversing the barrier, as well as through direct transcytosis across the endothelial cell barrier (Roberts et al., 2010, Dohgu et al., 2012, Williams et al., 2012). The “Trojan Horse” model, originally proposed by Haase and colleagues for the spread of visna virus, and which has been supported by many in vitro and in vivo studies for other lentiviruses, proposes that neuroinvasion and establishment of CNS viral reservoirs occurs as infected monocytes transmigrate across the BBB, and subsequently differentiate into macrophages. This pool of infected macrophages can then spread infection to neighboring brain-resident microglia, perivascular macrophages, and astrocytes, quickly establishing a viral reservoir in this compartment (Peluso et al., 1985, Haase, 1986, Gendelman et al., 1994, Liu et al., 2002, Gonzalez-Scarano, 2005, Kaul, 2005). Importantly, neurons themselves are not
able to be infected by HIV, as they lack the CD4 receptor required for viral entry (Kaul, 2005). With this in mind, several factors play a role in the neuronal damage occurring in those individuals with HAND. The first proposed mechanism holds that direct injury occurs from HIV proteins gp120, Tat, and Vpr, which are released from infected macrophages and microglia, and interact with receptors on neurons. The second, termed the “bystander effect”, suggests that the damage is instead caused by the immune response mounted by infected cells. These macrophages, microglia, and astrocytes release a myriad of cytotoxic molecules, including reactive oxygen species, nitric oxide, glutamate, and pro-inflammatory cytokines and chemokines which culminate in neuronal damage and dysfunction in addition to activation of neighboring uninfected immune cells which further secrete toxic factors (Gonzalez-Scarano, 2005). Classic cellular hallmarks in response to a neuroinflammatory environment were readily apparent in HAND patients prior to therapeutic interventions, including multinucleated giant cells, microglial nodules, astrogliosis, and myelin pallor (McArthur et al., 2003). Elevated levels of inflammation can also lead to an excitotoxic state, where excess extracellular glutamate chronically activates N-methyl-D-aspartate (NMDA) receptors on neurons, raising intracellular calcium levels and causing the activation of calcium-dependent proteases calpains and caspases, ultimately leading to apoptotic death. This mechanism of HIV-mediated neuronal toxicity has been well documented and characterized (O’Donnell et al., 2006). Both direct viral protein effects and indirect bystander effects of viral infection have been demonstrated, with the observed changes in patients likely resulting from a combination of the two mechanisms.

Since the introduction of cART, there has been a dramatic reduction in HAD from 25% to only 2% of patients (Heaton, 2010). While this was a promising and hopeful development, unfortunately there is now an increased prevalence of a broader spectrum of dysfunction in HAND, which includes categories of asymptomatic neurocognitive impairment, and mild neurocognitive disorder. Current estimates based on clinical findings suggest that approximately half of infected individuals suffer from some level of HAND (Gray, 2003, Brew, 2004, Heaton, 2010, 2011). Additionally, a recent study has reported that HIV-positive individuals aged 50 years or older with undetectable viral loads were greater than seven times more likely to have mild cognitive impairment than age-matched seronegative controls (Sheppard et al., 2015).
Changes in pathology have also been discovered following widespread access to antiretroviral therapy, with the subcortical pathology evident in untreated individuals shifting to cortical manifestations in treated patients (Gray, 2003, Brew, 2004, Gonzalez-Scarano, 2005, Heaton, 2011). While drastic cellular responses to neuroinflammation are no longer commonly discovered in cART-mediated HAND patients, abundant clinical and pathological studies have demonstrated that synaptodendritic damage and white matter pathologies persist (Masliah et al., 1997, Everall et al., 1999, Zheng et al., 2001, Langford et al., 2003, Ellis et al., 2007, Everall et al., 2009, Muller-Oehring et al., 2010, Tate et al., 2010). Additionally, while extensive neuronal death is no longer observed, evidence for continued neuronal damage in the form of dendritic pruning and reduction in synaptic density remain (Everall et al., 2005). Finally, over the course of lifetime infection, HIV severely disrupts the integrity of the BBB through a variety of mechanisms, including reducing the expression of structural components required for intercellular junctions, transporters, and metabolizing enzymes in endothelial cells (Atluri et al., 2015, Shawahna, 2015). Therefore in addition to early CNS viral entry, over the lifetime of infected individuals, continuing effects resulting from the escape of drug resistant viral species and direct antiretroviral toxicities also need to be considered.

Finally, another critically important area to highlight is the pediatric HIV-infected population. In infected children neurological complications are common, with pathologies including progressive multifocal leukoencephalopathy, encephalopathy, and cerebrovascular events (Wilmshurst et al., 2006). Additionally, there is increasing documentation of neurocognitive deficits, developmental delays, and emerging motor, cognitive, and behavioral deficits in young patients (Crowell et al., 2014, Whitehead et al., 2014, Wilmshurst et al., 2014). It is urged by the World Health Organization that all infected children under the age of 5 receive antiretroviral therapy (WHO, 2013). Despite this mandate for treatment, very little is known about the effects of HIV and antiretrovirals on the developing brain. As these children will have lifelong effects of both virus and therapy, it is crucial to understand the cellular ramifications of these xenobiotic insults during the critical periods for neurological development and cortical myelination.

Through suppression of plasma viremia and reconstitution of immune system function, cART was anticipated to resolve HIV-mediated neurological pathologies. While explicit mechanisms remain unclear,
it is thought that the early CNS invasion and establishment of a persistent viral reservoir which perpetuates an inflammatory and excitotoxic environment underlies continuing neurocognitive deficits despite effective peripheral viral suppression in HIV patients, at least in part (Anthony, 2005, Gonzalez-Scarano, 2005). Another area of interest, which is the prominent focus of this work, is the potential for CNS side-effects of antiretroviral compounds to also play a significant contributing role to the persistence of neurocognitive impairment in the ART-era. As it is undeniable that neurologic deficits remain in treated patients, further investigation into mechanisms underlying dysfunction is imperative (Palella, 1998, Antinori, 2007, Broder, 2010, Heaton, 2010, 2011).

1.3 ANTIRETROVIRAL THERAPY

Due to the error-prone process of reverse transcription during replication, HIV-1 is highly susceptible to mutations. This feature of the virus underlies the emergence of drug resistant mutations in patients over time, leading to viral resurgence and the ultimate failure of single drug antiretroviral therapy as introduced in the early 1990s to combat progression to AIDS. This led to the revision of antiretroviral therapy to a multiple drug treatment approach termed ART, which was implemented to simultaneously target multiple stages of replication, employing knowledge of HIV replication and its ability to undergo RNA mutational selection (Broder, 2010, NIH, 2011). As of March 2015, of the nearly 37 million individuals globally infected with HIV, approximately 15 million were currently accessing antiretroviral therapy translating to 41% of HIV-positive adults, 32% of HIV-positive children, and 73% of HIV-positive pregnant women which serves to prevent vertical transmission to their babies (UNAIDS, 2014).

Currently recommended ART regimens include a cocktail of nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse-transcriptase inhibitors (nNRTIs), protease inhibitors (PIs), and the more recently developed entry and integrase inhibitors (NIH, 2011, AIDSTAR-One, 2012, AIDSInfo, 2015). These most recent guidelines recommend treatment of all infected children under the age of 5 years, irrespective of CD4+ T-cell count. For adults, it is urged that all individuals with severe or advanced HIV disease corresponding to clinical stage 3 (advanced) or stage 4 (severe) and those with a CD4 count \( \leq 350 \) cells/mm\(^3\) to immediately initiate ART (see Table 1.1 for full HIV clinical staging details). It is also
suggested for all individuals with a CD4 count between 350 and 500 cells/mm$^3$, and is strongly recommended for those with active tuberculosis, those with HBV co-infection with evidence of chronic liver disease, as well as those with serodiscordant partners to reduce transmission to the uninfected individual (AIDSInfo, 2015).

Components of suggested ART regimens vary according to age of the patient, previous antiretroviral experience, and co-morbidities for which treatment produces drug interactions with certain classes of ART compounds. For adults and adolescents 10yrs and older weighing more than 35kg, first-line ART should consist of two NRTIs supplemented with a nNRTI to inhibit viral reverse transcriptase, thereby suppressing pro-viral DNA generation from viral RNA. When second-line ART is necessary due to acquired drug resistance, the primary recommendation is two NRTIs + a Ritonavir-boosted PI, which bind the active site of HIV-aspartyl protease and prevent maturation of viral gag-pol precursor proteins (Broder, 2010, NIH, 2011, WHO, 2013). While the NRTIs that make up this secondary regimen differ based on the makeup of the original regimen, Darunavir/ritonavir and Lopinavir/ritonavir are the preferred boosted PI options (AIDSInfo, 2015). Currently, all infected children under 3 years of age should receive a Lopinavir/ritonavir based regimen for first-line ART (WHO, 2013).


While the criteria for ART initiation have now been made clear based on patient CD4 counts, AIDS-defining illnesses, and specific co-morbidities, strategies for eradication of CNS viral reservoirs and for decreasing the risk of developing HAND have not yet been well-refined. A likely cause for the persistence of CNS viral effects is due to limited penetration of ART compounds into brain tissue through actions of
the blood brain barrier (BBB). The major proposed method to eliminate viral reservoirs and reduce inflammation is to implement therapies with drugs that would attain therapeutic concentrations in the CNS of patients. While HIV can circumvent the BBB, the actions of efficient drug efflux and transport proteins in the endothelial cells comprising this structure results in reduced levels of ART concentrations in cerebral spinal fluid (CSF) and brain parenchyma as compared with blood plasma (Berger, 2004, Letendre, 2008, Varatharajan, 2009). To address this issue, researchers have established the CNS penetrance effectiveness (CPE) score, which gives a numerical value following an algorithm which takes into account the chemical structures, pharmacokinetic, and pharmacodynamic data of antiretroviral compounds (Letendre, 2008, Tozzi et al., 2009, Letendre et al., 2010). Unfortunately results of clinical studies incorporating predicted highly CNS penetrant regimens utilizing these CPE scores have not yielded consistent results in terms of neurological function. Several studies yielded inconclusive outcomes, others reported a positive correlation between higher CPE and better neurocognitive outcome, and the study undertaken by Marra and colleagues revealed that regimens containing compounds with higher CPE scores may in contrast be associated with worse neurocognitive performance (Letendre, 2008, Cysique and Brew, 2009, Marra, 2009, Tozzi et al., 2009, Garvey et al., 2011, Lanoy et al., 2011, Smurzynski, 2011, Baker et al., 2015).

While these studies were well designed, investigating the effects of CPE scores on neurological outcomes in human patients comes with inherent caveats. Due to technical and ethical limitations, in living patients clinical studies cannot directly assess brain parenchymal drug concentrations, and rely instead on drug levels found in the CSF, usually after a single-dose administration. CSF and parenchyma have very different hydrophobic/hydrophilic compositions, which will greatly impact drug solubility and binding to cellular proteins and result in dissimilar concentration readings. In addition, other drug-related factors such as poor ART adherence or the impact of additional medications taken by the patient can alter the concentrations obtained by clinicians and confound the measured outcomes. Efforts to assess the impact of high CPE on neurological outcomes is also hindered by CNS reservoirs established early in infection with viral species harboring many iterations of rapidly acquired mutations, as well as later escape of drug-resistant viral species to the protected CNS environment (Chakrabarti et al., 1991, Gonzalez-Scarano, 2005). Moreover, comorbidities such as drug and alcohol abuse and infections should be considered when
assessing CNS drug availability and establishing and evaluating CPE scores, as all of these factors have been demonstrated to alter BBB integrity independent of HIV infection (Shiu et al., 2007, Ramirez et al., 2009). Another crucial factor to consider given the interest of implementing high CNS-penetrant ART regimens long-term in patients is the potential for direct toxicities of antiretroviral drugs in the CNS, as side-effects in the rest of the body are pervasive.

Unfortunately, peripheral side-effects of antiretroviral compounds are widespread and include metabolic syndrome, lipodystrophy, atherosclerosis and peripheral neuropathy (Carr, 1998a, Dalakas, 2001, Cohen, 2005, Parker, 2005, Ellis et al., 2008, Vidal et al., 2010). These functional deficits have been linked to various cellular effects of ART compounds of multiple drug classes. Through careful study in hepatocytes, T-cells, macrophages, adipocytes, lymphocytes, a cell line derived from a human laryngeal cancer, and purified human proteasomes, activation of ER stress, the unfolded protein response, and proteasome inhibition have been documented following PI exposure (Andre, 1998, Carr, 1998b, Piccinini M, 2002, Parker, 2005, Zhou, 2005, 2006, Gupta et al., 2007). Dysfunction through these mechanisms leads to pathologies culminating in atherosclerosis, hypercholesterolemia, lipodystrophy, and insulin resistance (Carr, 1998a, b, Cohen, 2005, Parker, 2005, Zhou, 2005). While not yet studied in the CNS, side-effects of antiretrovirals have been demonstrated in the peripheral nervous system (PNS). A debilitating peripheral neuropathy has been described in patients, stemming from NRTI and to a lesser extent PI usage (Dalakas, 2001, Ellis, 2008). Toxicity caused by compounds of the NRTI class arises by competition with cellular thymidine triphosphate for DNA pol-γ, resulting in the termination of mitochondrial DNA synthesis (Dalakas, 2001). Ensuing decreases in mitochondria number and oxidative phosphorylation enzymes leads to electron transport chain uncoupling and an increase in mitochondrially-derived intracellular reactive oxygen species (ROS) resulting in mitochondrial depolarization, and ultimately culminates in activation of the apoptotic cascade and neuronal death (Dalakas, 2001).

An interesting development was the finding that ART compounds themselves produce oxidative stress, even in the absence of virus (Mondal et al., 2004, Blas-Garcia et al., 2011). It has also recently been reported that AZT can modulate gene expression through ROS produced in the mitochondria in
cardiomyocytes. This study showed that the ROS diminished DNA methylation, which altered gene expression and promoted the pathophysiological changes associated with cardiomyopathy (Koczor et al., 2015). ART may therefore also be a contributing factor to the persistence of chronic oxidative stress observed in patients with well-controlled viral titers, and this stress may cause not only direct cellular toxicity but also changes in gene expression resulting in alterations of cellular function.

Toxic effects of ART in the CNS have not been rigorously studied, however as antiretroviral-associated toxicities are well documented in the periphery, these observations suggest that it is plausible that these compounds may contribute to cellular damage and death leading to continued manifestations of HAND in the ART-era. In the past several years the idea of direct ART toxicity has begun to be appreciated, as both we and others have demonstrated neurotoxic effects in primary neuronal cultures in response to ART compounds (Chapter 3) (Robertson et al., 2012).

1.4 OXIDATIVE STRESS IN HAND

Chronic oxidative stress is thought to play a significant role in the cellular damage resulting in the manifestation of HAND, as has also been suggested for many other neurodegenerative diseases (Mariani, 2005, Romano, 2010). Even in HIV-positive patients with well-controlled viral infection, evidence for persistence of oxidative stress is profound (Blas-Garcia et al., 2011). This topic has been thoroughly explored in Chapter 2, which has been excerpted from a book chapter we published reviewing the Persistence of HAND in the Era of ART. Key evidence demonstrating persistence of oxidative stress in patients, and oxidative stress induced by viral infection, viral proteins and antiretroviral therapy have been summarized and included here.

Despite effective viral control, lipid peroxidation remains pervasive in HIV-infected individuals, with persistently elevated levels of hydroperoxides, isoprostanes, and malondialdehyde detected in ART-treated patients compared with seronegative controls (Jareno et al., 1998, Hulgan et al., 2003, Ngondi et al., 2006, Masia et al., 2007, Wanchu et al., 2009). Levels of peroxide species were more highly elevated in patients on PI-based regimens compared with those on nNRTI-based regimens, suggesting that the PI class may
induce or enhance levels of oxidative stress (Hurwitz et al., 2004, Masia et al., 2007). Additionally, through chemical-shift magnetic resonance spectroscopy and careful analysis of lipids, lactate, and creatine levels derived from patients, it was determined that the inflammation and oxidative stress initiated by HIV-infection was not ameliorated by suppressive ART (Roc et al., 2007). Through nuclear and mitochondrial staining, the oxidized DNA product 8-hydroxy-2’-deoxyguanosine (8-oxoG) was shown to be significantly elevated in autopsy tissue from frontal cortex of HAND patients, suggesting that ongoing infection and/or ART promote oxidative modification of DNA (Zhang et al., 2012). Furthermore, clastogenic factors, which cause chromosomal breaks and DNA damage and may be released from cells undergoing oxidative stress, were present in plasma of all HIV-infected individuals regardless of ART status (Edeas et al., 1997). Finally, circulating levels of glutathione are markedly reduced in ART-medicated HIV patients, with the ratio of oxidized to reduced glutathione also remaining out of balance (Walmsley et al., 1997, Wanchu et al., 2009, Gil et al., 2010). Glutathione is a critically important antioxidant molecule in the cell, which acts as a reducing agent through its thiol groups and is able to donate an electron to unstable molecules such as reactive oxygen and nitrogen species (Espinosa-Diez et al., 2015). Once oxidized, it reacts with another similarly oxidized glutathione molecules to become glutathione disulfide, and then is converted back to its reduced state by glutathione reductase (Espinosa-Diez et al., 2015). In cells, this enables glutathione to act as an efficient buffer for reactive oxygen species produced by normal cellular activity, with homeostatic conditions normally supporting a ratio of 1:9 oxidized to reduced glutathione (Halprin and Ohkawara, 1967). An increase in this ratio, such as is seen in ART-medicated patients described above, is indicative of oxidative stress, with buildup of cellular ROS contributing to activation of signaling molecules responsible for triggering the endogenous antioxidant response as will be discussed in Section 1.7 (Halprin and Ohkawara, 1967).

Therefore, while patients have effective viral suppression to undetectable levels, evidence for persistently robust levels of oxidative stress is abundant. Contributing factors to this chronic stress include consequences of viral infection, direct effects of viral proteins from brain-resident reservoirs, and side-effects of antiretroviral therapies, each of which will be discussed in further detail below.
1.4a: OXIDATIVE STRESS BY HIV INFECTION

In the decades since the discovery of HIV, extensive research has been conducted to determine the effects of the virus on infected cells and cytotoxic factors released by these cells. Infected macrophages secrete a variety of neurotoxic oxidative stress inducing compounds including glutamate, nitric oxide, and superoxide (Lipton, 1994, Bukrinsky et al., 1995, Jiang et al., 2001, Mollace et al., 2002, O'Donnell et al., 2006). In addition, activation and degranulation of macrophages by viral infection leads to release of enzymes that further produce free radicals, such as myeloperoxidase. This protein is released as a cellular response to infection as it behaves as a viricidal and inactivates HIV, but it also produces oxidative damage to surrounding cells through production of hypocholorous acid and oxidation of tyrosine residues causing protein cross-linking (Chase and Klebanoff, 1992, Heinecke et al., 1993, Hong and Banks, 2015). Furthermore, HIV infection of macrophages and microglia also results in deficiency of intracellular glutathione, and imbalance of the oxidized to reduced glutathione ratio (Lipton, 1994, Zhao et al., 2004, Erdmann et al., 2007, Huang et al., 2011). Increased malondialdehyde levels in astrocytes treated with supernatants from infected macrophages validated that secreted factors have negative oxidative stress-mediated consequences on neuroglial cells (Mollace et al., 2002). In addition to the oxidative consequences as a result of infection, viral proteins found on the HIV envelope or secreted from infected cells also need to be considered independently from their roles in viral entry and replication, as they too produce oxidative effects in brain resident cells.

1.4b: OXIDATIVE STRESS BY HIV PROTEINS

When HIV proteins gp120 and Tat were examined in vivo and in vitro in the absence of whole virus, both induced glutathione depletion, lipid peroxidation, and increase in malondialdehyde (Banerjee et al., 2010). Additionally, both gp120 and Tat alter lipid metabolism in primary cultured neurons, increasing levels of hydroxynoneal, sphingomyelin, and ceramide (Haughey et al., 2004). Independent studies investigating gp120 effects on astrocytes revealed an accumulation of ROS and activation of the antioxidant response (Viviani et al., 2001, Reddy et al., 2011). Studies investigating the oxidant species produced following gp120 application determined that production of both nitric oxide and superoxide ions contribute to neuronal toxicity (Dawson et al., 1993, Walsh et al., 2004). Exogenously applied Tat protein also triggers
the accumulation of ROS in a variety of cell types including microglia, brain microvascular endothelial cells, and neurons (Flores et al., 1993, Kruhan et al., 1998, Toborek et al., 2003). Tat protein directly injected into rat striatum also significantly increases levels of both oxidative modifications and carbonyl additions to proteins (Aksenov et al., 2001). Finally, microglia expressing viral protein R (Vpr) have been demonstrated to have activated oxidative stress pathways (Deshmane et al., 2009). Studies investigating Vpr have shown that exogenous application to human fetal astrocytes resulted in decreased intracellular glutathione and ATP levels, and skewed the balance of the remaining glutathione towards the oxidized rather than reduced form (Ferrucci et al., 2012).

It has been well documented through these *in vivo* and *in vitro* studies that viral proteins can generate oxidative stress in multiple cell populations from the periphery and CNS. These findings highlight the importance for effective suppression of viral replication via ART to minimize the oxidative damage caused by infection. Emerging evidence also suggests that an additional source of oxidative stress includes the antiretroviral compounds themselves, which will be discussed in the next section.

**1.4c: OXIDATIVE STRESS BY ART**

Through a combined effort of many laboratories, it has been clearly demonstrated that a variety of drugs from the PI and NRTI drug families alone or in combination result in accumulation of ROS, hydrogen peroxide, and factors promoting the recruitment of monocytes in a wide range of cell populations. The compounds shown to cause these effects in human adipocytes, monocytes, aortic endothelial cells, and myeloid cell lines included the PIs: Ritonavir, Saquinavir, Lopinavir, Indinavir, Nelfinavir, and Atazanavir, as well as the NRTIs: Zidovudine (AZT), Stavudine (d4T), and Didanosine (Mondal et al., 2004, Ferraresi et al., 2006, Lagathu et al., 2007, Chandra et al., 2009, Touzet and Philips, 2010, Manda, 2011, Brandmann et al., 2012). Combined antiretroviral treatment also led to increased oxidative stress and mitochondrial dysfunction in BBB endothelial cells (Manda, 2011). Thus, in many cell populations antiretroviral drugs of varying classes show evidence for generation of oxidative stress. The individual effects of specific drug classes will be discussed in more detail below.
The NRTI AZT has been extensively studied, as it was the first available antiretroviral drug. Within five years of clinical approval, AZT-induced deficits in mitochondrial enzymes and electron transport chain (ETC) uncoupling had been reported (Lewis et al., 1992). ETC disruption causes the accumulation of intracellular ROS and mitochondrial DNA oxidation, which has been demonstrated following AZT exposure in both acute and chronic experimental paradigms (de la Asuncion et al., 1999, Bialkowska et al., 2000, de la Asuncion et al., 2004, Gao et al., 2011). Subsequently, AZT has also been shown to deplete intracellular glutathione, increase mitochondrial lipid peroxidation, and ultimately induce caspase-3 dependent apoptotic cell death (Yamaguchi et al., 2002, de la Asuncion et al., 2004, Gao et al., 2011). Another NRTI, d4T, behaves very similarly to AZT. d4T also causes ROS accumulation, mitochondrial stress and oxidized DNA, and altered activity of mitochondrial oxidative phosphorylation enzymes (Gerschenson et al., 2001, Velsor et al., 2004). The nNRTI drug class includes the compounds Efavirenz, Nevirapine, and Rilpivirine, and has also been implicated in the production of oxidative stress in cells. Application of Efavirenz to a human hepatoblastoma cell line resulted in superoxide generation, depletion of cellular glutathione, and loss of mitochondrial function and membrane potential (Apostolova et al., 2010). Efavirenz has been linked to neuropsychological side effects, and has since been removed from treatment recommendations (Decloedt and Maartens, 2013). It is widely held that the oxidative stress effects described above play a role in the neurotoxicity of this compound (Apostolova et al., 2010, Ciccarelli et al., 2011). Within the PI drug class, Ritonavir has been demonstrated to increase nitrotyrosine levels, and both Ritonavir and Amprenavir invoke noted increases in superoxide anion production in porcine coronary arteries (Chai et al., 2005). In astrocytes and pancreatic beta cells, both Indinavir and Nelfinavir induce time- and concentration- dependent depletion of intracellular glutathione (Chandra et al., 2009, Brandmann et al., 2012). Currently, potential oxidative effects of the entry and integrase inhibitor antiretroviral drug classes have not been explored. Similarly the lack of studies investigating the effects of antiretroviral compounds on the neuroglial cell populations affected in HAND is surprising, despite the abundant evidence which supports a role for antiretroviral compounds in oxidative stress-induced cellular dysfunction and damage.
As has been summarized here, it is clear that pervasive oxidative damage occurs in HIV patients despite effective viral control. Therefore it is of upmost importance to recognize the cellular effects of viral enclaves and antiretroviral drugs, and how they may be perpetuating these effects.

1.5 OLIGODENDROCYTES IN HAND

Prior to the introduction of antiretroviral therapy, alterations in white matter including myelin pallor, gliosis, and leukoencephalopathy were prominent in HIV-infected patients (Gray et al., 1996, Everall et al., 2005). Now even with effective viral suppression in the ART-era, while severe forms of leukoencephalopathy have been diminished, the prevalence of white matter pathologies in patients has increased, with amount of white matter damage positively correlated with the degree of neurocognitive impairment observed (Langford et al., 2003, Everall et al., 2005, Muller-Oehring et al., 2010, Tate et al., 2010). MRI and diffusion tensor imaging studies have revealed that these effects include dramatic thinning of the corpus callosum, reduction in blood flow to white matter, and loss of both structural integrity and volume from white matter structures including: the superior longitudinal fasciculus, superior corona radiata, and the internal capsule (Pomara et al., 2001, Ragin et al., 2004, Gongvatana et al., 2009, Wohlschlaeger et al., 2009, Hoare et al., 2010, Tate et al., 2011, Kelly et al., 2014). A transcriptome analysis determined genes which were altered following HIV-infection and which remained dysregulated in individuals who attained successful viral suppression through ART but were diagnosed with HAND (Borjabad et al., 2011). Among the persistently altered transcripts were myelin transcription factor 1, myelin basic protein, and myelin-associated oligodendrocyte basic protein, which are critical for oligodendrocyte maturation, myelination, and maintenance (Borjabad et al., 2011). Thus, white matter pathologies remain prevalent in HIV-infected patients despite viral suppression through ART, with dysregulation of myelin mRNAs and loss of structural integrity of myelin evident by imaging technologies.

Similar to their neuronal counterparts, oligodendrocytes lack the CD4 receptor required for HIV entry, and thus, changes in these two cell populations must be caused by indirect effects of viral infection of other cell populations, rather than direct consequences of infection of neurons or oligodendrocytes (Sattentau et al., 1986, Wiley et al., 1986). While molecular mechanisms have not yet been investigated, oligodendrocytes
exhibit elevated p53 and BAX levels in postmortem brain samples from frontal cortex of HIV-infected individuals who had progressed to AIDS, indicating the activation of apoptotic cell death pathways (Jayadev et al., 2007). However, in patients with end stage disease of fully developed AIDS, many of whom suffered from HIV-Associated Dementia and HIV-encephalitis, it is difficult to attribute specific mechanisms leading to neuronal or oligodendrocyte death.

As was discussed in section 1.4, chronically high burdens of oxidative stress are abundant in HIV patients even following suppression of viral replication. Oligodendrocytes are exceptionally susceptible to oxidative stress-mediated cell death due to low glutathione and antioxidant levels, coupled with high energy demands required to efficiently produce and maintain myelin to insulate surrounding neurons (Back et al., 1998, Back et al., 2002, Folkerth et al., 2004, Jana and Pahan, 2007). In rodent models of hypoxia/ischemia, oxidative stress generated by the insult led not only to immediate death of oligodendrocyte precursor cells (OPCs), but also to a subsequent reduction in number of mature oligodendrocytes and hypomyelination of white matter (Levison et al., 2001, Reid et al., 2012). In addition to direct OPC dropout due to oxidative stress-mediated toxicity, we found in vitro that at sub-toxic doses, oxidative stress caused by oxidant insult halts oligodendrocyte differentiation (French et al., 2009). In vitro studies have also demonstrated a maturation-stage specific vulnerability of this cell population, as mature oligodendrocytes are far more resilient than OPCs and immature oligodendrocytes due to higher glutathione and antioxidant enzyme levels (Halliwell, 1992, Back et al., 1998, Fern and Moller, 2000, Baud et al., 2004). Several demyelinating disorders including MS and periventricular leukomalacia involve oxidative stress as a contributing factor to pathology (Haynes et al., 2003, Gilgun-Sherki et al., 2004, van Horssen et al., 2008). With this in mind, it is likely that the chronically high-levels of oxidative stress still observed in ART-mediated HAND patients also contributes to the persistence of white matter pathologies in this population.

Despite these striking pathological findings in HIV-infected individuals, there is a notable lack of molecular studies detailing the cellular effects of secreted factors from HIV-infected macrophages or direct effects of ART on oligodendrocyte precursors or mature oligodendrocytes. However, the laboratory of Pamela Knapp has recently published an article investigating the effects on oligodendrocytes in a rodent
model of inducible astrocyte-driven Tat expression. After 3 months of Tat-transgene expression, animals displayed disruption of myelin in the caudate-putamen, aberrant oligodendrocyte morphology in corpus callosum and anterior commissure, and significantly decreased levels of myelin basic protein and myelin-associated glycoprotein in striatum (Zou et al., 2015). Additionally, when cells in vitro were exposed to exogenously applied Tat protein, viability of immature oligodendrocytes was significantly decreased and surviving cells displayed significant deficits in maturation as evidenced by decreased branching processes and myelin membrane extensions (Zou et al., 2015). This pioneering study has provided the groundwork that viral proteins can affect oligodendrocyte survival and extent of myelination. It is of crucial importance now to further understand the effects of HIV on oligodendrocytes in the context of the entire virus and in infection. This is extremely challenging due to imperfect animal systems that do not fully recapitulate human cellular attributes. In addition to direct effects of viral proteins, evidence has suggested that factors secreted by macrophages and astroglia, both which are infected or non-infected but activated due to the presence of viral infection, may also contribute to negative neuroglial effects. Therefore the model of human macrophage infection and application of supernatants to cells in culture may be utilized to provide insight into additional aspects of neuropathology. Furthermore, effects of antiretroviral compounds on oligodendrocytes have not been considered in the context of chronic HIV infection. As this cell population is vitally important to ensure proper neuronal signaling and cognition, effects of viral infection, inflammation, oxidative stress, and antiretrovirals on this cell population need further consideration. Oligodendrocytes are frequently overlooked in the study of diseases; however, because myelin formation and maintenance occur both during human development and adulthood, the consequences are profound when these processes are disrupted.

1.6 THE IMPORTANCE OF OLIGODENDROCYTE DEVELOPMENT AND MYELIN MAINTENANCE

Myelin, produced by oligodendrocytes in the CNS and Schwann cells in the PNS, provides the basis for the propagation of rapid neuronal impulses, which is required for proper functioning of the human nervous system. This process, known as salutatory conduction, is facilitated by the ensheathment of axons by multiple layers of specialized glial membrane, which insulates the neuronal axonal plasma membrane by
reducing the transverse capacitance and increasing the resistance. By effectively restricting action potentials to small unmyelinated axon segments known as nodes of Ranvier, velocity of signal conduction is accelerated up to 100-fold when compared with an unmyelinated axon of the same diameter (Landahl and Podolsky, 1949, Frankenhaeuser, 1952, Nave and Werner, 2014). In addition to their function in insulating axons, oligodendrocytes also provide trophic support to the axons their myelin membranes surround, which is crucially important for maintaining cellular functioning and signaling at long extended distances from the neuronal soma (Lappe-Siefke et al., 2003, Edgar and Garbern, 2004). In contrast, loss of myelin integrity can disrupt proper signaling, resulting in neuronal degeneration and neurological dysfunctions (Criste et al., 2014, Mighdoll et al., 2015).

Mature oligodendrocytes are generated from oligodendrocyte precursors cells (OPCs) according to a well-characterized progression, allowing for assessment according to stage-specific antigens (Figure 1.1) (Miller, 2002). In rodents, at embryonic day 12.5, OPCs originate in ventricular zones and migrate both dorsally and radially, expressing markers such as A2B5, platelet-derived growth factor receptor A (PDGF-A), and NG2. OPCs transition to immature oligodendrocytes expressing galactocerebroside (GalC) and begin to extend actin filaments to elaborate processes and adopt the morphology of mature oligodendrocytes. During this period, oligodendrocyte precursor cells (OPCs) decrease levels of Inhibitor of DNA binding (ID) transcription factors, allowing the pro-differentiation transcription factors Olig1 and Olig2 to promote upregulation of proteins required for maturation into oligodendrocytes, as well as myelin component mRNAs (Miller, 2002, Chen et al., 2012). Following contact with neurons, these maturing cells continue to extend processes, upregulate expression of myelin proteins, and ultimately ensheathe neuronal axons with the lipid- and protein-based myelin membrane (Grinspan, 2002, Miller, 2002). A majority of myelin proteins are translated in the perinuclear endoplasmic reticulum and trafficked via the secretory pathway for ultimate insertion into the forming myelin membrane (Colman et al., 1982). In contrast, mRNA for myelin basic protein is trafficked intracellularly via microtubules to sites proximal to membrane insertion. Translation into protein then occurs on free-ribosomes and is quickly followed by insertion into the nearby developing myelin membrane (Colman et al., 1982, Carson et al., 1997). In vivo, myelin is highly enriched in lipids, which constitute approximately 75% of membrane constituents, with the
remaining 25% being myelin proteins. Chief among the protein components are proteolipid protein (17%), myelin basic protein (8%), cyclic nucleoside phosphodiesterase (CNPase, 4%), myelin oligodendrocyte glycoprotein (MOG, 1%), and myelin-associated glycoprotein (MAG, 1%) (Jahn et al., 2009).

Myelin maintenance and replacement is a continually ongoing process over the course of an individual’s lifetime, which is essential for optimal neuronal functioning and survival (McLaurin and Yong, 1995). In addition to baseline myelin maintenance, remyelination of axons from which damaged myelin has been stripped off has been extensively documented, primarily through clinical and molecular studies investigating the pathology of Multiple Sclerosis (Hartley et al., 2014, Kutzelnigg and Lassmann, 2014). In notable recent studies, while myelination was previously viewed solely as an early life event, it has been shown that active synthesis and formation of new myelin is required for motor learning in adulthood (Long and Corfas, 2014, McKenzie et al., 2014).

Myelin is damaged or improperly formed in a variety of conditions, which primarily result in motor dysfunction. Factors contributing to these diseases include genetic mutations of myelin components termed leukodystrophies, demyelinating diseases caused by inflammation such as Multiple Sclerosis, and hypomyelination or myelin damage caused by injury such as in perinatal white matter injury in preterm infants (Volpe, 2001, Garbern, 2007, Lee et al., 2010, Markowitz, 2013). Myelin deficits have now also been implicated in neurodegenerative diseases such as ALS, and neurological diseases with cognitive and behavioral consequences such as autism and schizophrenia (Kang et al., 2013, Chavarria-Siles et al., 2015, Libero et al., 2015). While OPCs persist in the CNS in most of these diseases, they are unable to differentiate and effectively form new myelin.

Two of these disorders highlight the contribution of oxidative stress to myelin dysfunction. In Multiple Sclerosis, while the mechanism driving initial autoimmune targeting of myelin is not understood, inflammation and oxidative stress are thought to drive much of the continuing pathology (Friese et al., 2014, Mahad et al., 2015). Symptoms arise from disruption of myelin integrity in white matter tracts within the brain, resulting in improper transmission of neuronal impulses (Markowitz, 2013, National Multiple
In addition to destruction of myelin sheaths and death of associated oligodendrocytes, cellular pathology also includes axonal damage and subsequent cell death of the demyelinated neuron (Criste et al., 2014, Mallucci et al., 2015). As was mentioned previously, impaired myelination can hasten neuronal axonal degeneration by altering trophic support provided by the ensheathing oligodendrocytes (Lappe-Siefke et al., 2003, Edgar and Garbern, 2004, Criste et al., 2014).

Oxidative stress negatively impacts oligodendrocytes on multiple levels, as it can perturb myelination in mature oligodendrocytes, and concomitantly both depletes the OPC pool and prevents effective differentiation of the remaining OPCs for replacement of the damaged mature oligodendrocytes to remyelinate affected areas (Leison et al., 2001, French et al., 2009, Reid et al., 2012). A devastating loss of myelination also occurs in pre-term infants who suffer from perinatal white matter injury (PWMI), which frequently results in considerable neurologic dysfunction and diagnosis of cerebral palsy (Volpe, 1981, 2001). This brain damage targets white matter specifically, and while it can be triggered by several independent events, one of the leading causes is lack of cerebral oxygenation (Back and Rosenberg, 2014). This causes the generation of robust levels of oxidative stress, and cell death of the particularly vulnerable OPC pool as discussed in section 1.5, ultimately resulting in hypomyelination of the cortex in the developing child (Volpe, 2001, Back and Rosenberg, 2014). The evidence of oxidative stress-mediated damage to oligodendrocytes in these conditions highlights the necessity for reduction of this stress in order to facilitate recovery or attenuate dysfunction in demyelinating disorders.

1.7 THE ENDOGENOUS ANTIOXIDANT RESPONSE (EAR)

In response to an imbalance of redox homeostasis, cells activate a cellular stress pathway known as the endogenous antioxidant response (EAR) in order to reduce levels of oxidative stress. This response is mediated by the activity of a ubiquitously expressed transcription factor of the Cap’n’Collar family, NF-E2 (nuclear factor (erythroid-derived 2))-related factor-2 (Nrf2) (Motohashi, 2004b). Nrf2 has been demonstrated to be essential for a transcriptional response to oxidative stress (Chan, 1999, Leung, 2003). Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm by a Kelch-like ECH-associated protein-1 (Keap1) dimer, in complex with Cul3/Rbx/E3 ligase which facilitates constitutive ubiquitination and proteasomal protein degradation (Figure 1.2A) (Itoh, 1999, Zipper, 2002, Zhang DD, 2003, Zhang,
Cytoplasmic retention of Keap1/Nrf2 complexes are ensured by dual actions of Keap1 through its nuclear export signal and actin binding via its Kelch repeat domain (Kang, 2004, Velichkova, 2005).

Under pathway activating conditions, the interaction between Nrf2 and Keap1 is altered, with release from Keap1-mediated repression allowing for Nrf2 nuclear translocation (Li, 2009). Two methods of pathway activation occur, through either modification of Keap1 cysteines or Nrf2 phosphorylation (Figure 1.2B). Several cysteine residues on Keap1 are critically responsible for effective basal ubiquitination of Nrf2, which targets the protein for proteasomal degradation (Dinkova-Kostova, 2002, Zhang DD, 2003). During pathway activation, modification of these residues results in a conformational change of the Keap1 homodimer sequestering Nrf2 in the cytoplasm (Wakabayashi, 2004, Eggler, 2005, Tong, 2007). In this way, the altered Keap1 dimer retains the Nrf2 molecule bound in complex, but ubiquitin transfer to Nrf2 is disrupted, no longer successfully targeting it for degradation. As a result, there is an accumulation of unbound de novo translated Nrf2 which can enter the nucleus and activate transcription (Zhang DD, 2003, Wakabayashi, 2004, Eggler, 2005, Kobayashi, 2006, Purdom-Dickinson, 2007, Tong, 2007). Pathway activation by this method also switches the ubiquitin transfer to the Keap1 molecules, instead targeting Keap1 for degradation and further enhancing the available Nrf2 pool (Hong, 2005, Zhang, 2005). Alternatively, Nrf2 can be phosphorylated by Protein Kinase C or PKR-like ER kinase, which directly disrupts the association of Keap1 and Nrf2 (Huang, 2000, 2002, Bloom, 2003, Cullinan, 2003).


While still incompletely understood, two mechanisms have been proposed for cessation of the antioxidant response. In the absence of Nrf2, the Keap1 complex can bind prothymosin-α, driving nuclear import (Figure 1.2Ci) (Karapetian, 2005, Padmanabhan, 2008, Niture, 2009). Once nuclear, Keap1 switches prothymosin-α for Nrf2 binding (Niture, 2009). This nuclear Keap1-Nrf2 complex can either be directed to Crm-1 mediated export to resume normal cytoplasmic repression activity, or alternatively can remain nuclear and target Nrf2 for internuclear proteasomal degradation (Nguyen, 2005, Velichkova, 2005, Sun, 2007, Niture, 2009). The second proposed mechanism involves glycogen synthase kinase 3β (GSK3β) (Figure 1.2Cii). Free cytoplasmic Nrf2 can be phosphorylated by active GSK3β, which prevents nuclear import and targets for Keap1-independent ubiquitination by the SCF/β-TrCP/Culll/Rbx/E3 ubiquitin ligase complex leading to subsequent proteasomal degradation (Salazar, 2006, Rada, 2011). Activated GSK3β can also phosphorylate cytoplasmic Fyn kinase, causing its translocation and phosphorylation of nuclear Nrf2, which once modified in this manner becomes fated for nuclear export and degradation (Jain, 2006, 2007).

While this pathway has been well described in many cell populations, investigation into EAR activity has been somewhat limited in lineages of the central nervous system (Li, 2009). Through many elegant studies the laboratory of Johnson and colleagues has demonstrated Nrf2-mediated EAR activity in astrocytes (Kraft, 2004) and reviewed in (Vargas and Johnson, 2009). Similarly, it has been well documented that upregulation of Nrf2-mediated antioxidant response proteins can occur in macrophages (Itoh et al., 2003, Li et al., 2004). Through these studies, it has been shown that activating the EAR in both astrocytes or macrophages can provide protection to neurons (Kraft, 2004, Li et al., 2004). Neurons are particularly susceptible to toxicity from oxidative stress, with many findings concretely demonstrating that in an in vivo
context other cell populations likely provide a supportive buffer (Bell, 2013). In contrast to other cell populations, very little is known about the EAR in oligodendrocytes. Studies using OLN-98 cells and primary rat oligodendrocytes showed HO-1 protein upregulation following hydrogen peroxide exposure (Goldbaum and Richter-Landsberg, 2001, Stahnke et al., 2007). While this suggested EAR pathway activation, this insult also culminated in loss of mitochondrial function and apoptosis, suggesting an ineffective or incomplete response which was insufficient to protect the cells (Goldbaum and Richter-Landsberg, 2001). In summary, little is known about the EAR pathway in neurons and oligodendrocytes, two cell populations that have been suspected to primarily draw on macrophages and astrocytes for cytoprotective support. In support of this, both macrophages and astrocytes have been demonstrated to robustly activate the EAR pathway.

1.8 NRF2 PATHWAY DYSFUNCTION IN DISEASE

Given the pervasive oxidative stress found in many neurodegenerative diseases, better understanding of the EAR pathway in CNS cell populations is of vital importance (Gilgun-Sherki et al., 2004, Mariani, 2005, Ramsey, 2007, Romano, 2010). In the absence of Nrf2 activity there is heightened susceptibility not only to direct oxidative stress, but also to acetaminophen hepatotoxicity, carcinogens, ischemia, and hyperoxic lung injury, as well as a general suppression of immune responses (Chan, 2001, Enomoto, 2001, Ramos-Gomez, 2001, Cho, 2002, Shih, 2005, Thimmulappa, 2006). Postnatal lethality occurs under conditions of Keap1 deletion in knockout mice, resulting from Nrf2 pathway dysregulation (Wakabayashi, 2003). As these studies indicate, balance of Nrf2 and Keap1 functions are critical for maintaining cellular redox homeostasis, with alterations in either component resulting in drastic cellular consequences. Additionally, changes in subcellular localization of these proteins can also impact function, as Nrf2 activity is highly dependent on its ability to activate transcription of EAR targets.

In many neurodegenerative pathologies, Nrf2 and other transcription factors undergo aberrant cellular localization, precluding appropriate transcriptional regulation in response to activation of their respective signaling pathways (Chu, 2007, Patel, 2011). Several lines of evidence suggest that dysregulation of the EAR through inappropriate localization of Nrf2 may contribute to disease progression. In Alzheimer
Disease (AD), levels of nuclear Nrf2 are significantly reduced in affected patients compared to age-matched controls, even in neurons displaying abundant oxidative damage, suggesting a defect in nuclear Nrf2 translocation (Ramsey, 2007). Nrf2 is highly enriched in the nucleus of affected neuronal cell populations in Parkinson Disease (PD), suggesting that the EAR has been activated but the response is not capable of overcoming the levels of oxidative stress to provide cellular protection (Ramsey, 2007). Recently, high levels of nuclear expression of Nrf2 and downstream target HO-1 were found in oligodendrocytes in active demyelinating regions from Multiple Sclerosis (MS) patients (Licht-Mayer et al., 2015). However, in a similar finding to cells in PD, the highest enrichment of nuclear Nrf2 was found in degenerating oligodendrocytes in the MS lesions (Licht-Mayer et al., 2015). Preliminary evidence from our laboratory has suggested that Nrf2 localization may also be affected in individuals with HAND. In comparison to ART-treated, HIV-positive neurocognitively normal individuals, ART-treated individuals with HAND have elevated cytoplasmic Nrf2 levels. This abnormal elevation does not indicate a productive antioxidant response, as nuclear Nrf2 is required for EAR-based gene upregulation. Increased evidence of oxidative damage (4-HNE) is also seen in the HAND patients compared to seronegative age-matched neurocognitively normal controls, supporting the notion that while oxidative stress is accumulating with HIV and ART, an effective protective response is not mounted. However, it is challenging to draw conclusions regarding stress response proteins in post-mortem tissue derived from patients with a chronic degenerative condition. Cells frozen in time at the point of autopsy which display highly enriched levels of a particular response protein could be chronically and effectively combatting a stressor, or conversely could be ineffectively attempting to overcome a non-survivable level of stress and are instead fated for cell death.

1.9 COUNTERING OXIDATIVE STRESS IN HAND

Nrf2 pathway upregulation has been suggested as a potential avenue for therapy in ALS, Alzheimer Disease, and Parkinson disease (Calkins, 2009, Tufekci, 2011, Kumar et al., 2012, Petri et al., 2012). In MS, where oxidative stress is a key pathological feature of the disease, therapies aimed at reducing this stress burden have been approved and are used currently in the clinical setting (FDA, 2013, Friese et al., 2014, Dubey et al., 2015, English and Aloi, 2015, Mahad et al., 2015). Due to the vast amount of experimental evidence demonstrating that both HIV and ART induce oxidative stress, it has also been
proposed that boosting the EAR through promoting Nrf2 pathway activity may be an effective adjunctive therapeutic option for all individuals living with HIV, not only those already affected by HAND.

Encouragingly, multiple compounds activating the Nrf2 pathway have proven effective in preventing oxidative consequences of viral infection, viral proteins, and antiretroviral compounds in vitro. Chief among these compounds are dimethyl fumarate, resveratrol, and curcumin (Chai et al., 2005, Touzet and Philips, 2010, Cross et al., 2011, Gao et al., 2011). Other antioxidants also show promise in preventing oxidative stress invoked by exposure to antiretroviral drugs in vitro, but their actions have not been confirmed to be Nrf2-pathway dependent. These compounds include: acetyl-L-carnitine, dihydroxybenzyl alcohol, and glutathione mimetic tricyclodecan-9-yl-xanthogenat (D609) (Ferraresi et al., 2006, Opii et al., 2007, Weakley et al., 2011). In a slightly different approach, the compound diphenylene iodonium was used to specifically inhibit NADPH oxidase in adipocytes. Through the action of this compound, the generation of oxidative stress was prevented for the PI drug class, but not for the NRTIs (Lagathu et al., 2007). This finding suggests that different classes of antiretroviral agents may lead to the generation of ROS through different mechanisms, requiring adjunctive therapeutics that target these specific cellular pathways.

Of the currently proposed potential adjunctive options, dimethyl fumarate is of particular interest, due to its dual functionality of activating the Nrf2-mediated EAR, as well as suppressing levels of HIV-replication in macrophages (Cross et al., 2011). This compound could potentially become a vital adjunctive therapy for HIV, which would lower viral titers in addition to lowering oxidative stress generated by viral proteins and co-administered antiretrovirals. DMF has recently been FDA approved for the treatment of MS under the trade name Tecfidera and successfully reduces relapse rates (FDA, 2013, Limmroth, 2013, English and Aloi, 2015). Surprisingly, while suspected to act as an antioxidant on the basis of previous reports demonstrating activation of the Nrf2-pathway, the mechanism of action by which Tecfidera is effective in MS has not yet been elucidated. In the body, DMF is rapidly converted to monomethyl fumarate (MMF), so that in blood plasma only MMF is ever detected (Litjens et al., 2004, Fox et al., 2014). While the effects of MMF on oligodendrocytes, the primary cell population affected in MS pathology currently remains
unexplored, antioxidant effects of MMF have been reported in neurons and macrophages through upregulation of Nrf2-mediated EAR target genes, similar to findings using the parent compound DMF (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012).

A second alternative approach to combatting oxidative stress is direct scavenging of reactive oxygen and nitrogen species through dietary supplementation of antioxidants such as Vitamin C (ascorbate). This has been demonstrated to have beneficial outcomes in patients when supplemented to their ART regimens, and has protected against effects of HIV protein gp120, PI Nelfinavir, and NRTI AZT in vitro (de la Asuncion et al., 2004, Vincent et al., 2004, Walsh et al., 2004). Supplementation with a variety of other antioxidants and micronutrients such as vitamins A and E, selenium, coenzyme Q10, and alpha-lipoic acid have also demonstrated partial protection from oxidative stress induced by HIV/SIV and ART in vivo, through assessment of restored blood glutathione levels (Delmas-Beauvieux et al., 1996, Batterham et al., 2001, de la Asuncion et al., 2004, Jariwalla et al., 2008). While still in early stages of determining their efficacy, both the NMDA-receptor blocker memantine and monoamide oxidase type B inhibitor selegiline have been proposed as potential adjunctive therapeutics for the treatment of HAND (Lindl et al., 2010, Zhao et al., 2010). Memantine, which is currently approved for Alzheimer Disease, may benefit patients by reducing glutamate-mediated excitotoxicity (Kutzing et al., 2012). Selegiline, which is utilized for treatment of Parkinson Disease, may aid in reducing the oxidative stress burden in HAND through direct reduction of oxygen-based free radicals (Nagatsu and Sawada, 2006). Going forward, it is imperative to identify and develop adjunctive therapies that can effectively attenuate the oxidative stress caused by HIV infection and antiretroviral therapy. Until antiretroviral compounds with less toxic side effects can be designed and successfully targeted to all areas of established viral enclaves, oxidative stress will continue to be a persistent burden in this patient population who are now living with their infection long-term, over greatly enhanced life expectancies.

1.10 RATIONALE AND OBJECTIVES

Antiretroviral compounds are quite successful at attenuating HIV-replication, yet despite peripheral viral titers below the limit of detection, approximately half of infected individuals suffer from some level of
impairment within the spectrum of dysfunction characterized as HIV-Associated Neurocognitive Disorders (HAND) (Gray, 2003, Brew, 2004, Heaton, 2010, 2011). Peripheral toxicities of these compounds have been well characterized, yet the toxic effects of these compounds on the major cell types of the CNS, which have potential implications for the persistence of HAND, have been overlooked (Carr, 1998a, Dalakas, 2001, Cohen, 2005, Parker, 2005, Ellis et al., 2008, Vidal et al., 2010). We anticipated that antiretroviral compounds, which activate several cellular stress pathways in the periphery, are likely to affect cell populations within the brain, which may contribute to loss of cognitive function (Parker, 2005, Zhou, 2005, 2006, Gupta et al., 2007). In chapter 2, we extensively review the evidence for oxidative stress in HIV-infected individuals both before and after the development and widespread use of therapy, the contributions of the virus and of antiretrovirals to this continued stress, and potential adjunctive therapeutic interventions to combat these effects.

In chapter 3, we then test the hypothesis that antiretroviral compounds negatively impact neuronal function in vivo and in vitro. We found that in both pigtail macaques and rats, in vivo antiretroviral administration resulted in synaptodendritic damage. In vitro studies demonstrated that exposure to antiretrovirals led to accumulation of ROS and slight activation of the EAR, yet culminated in neuronal damage and death. Use of the fumaric acid ester monomethyl fumarate (MMF) to potently upregulate the cellular antioxidant response prevented both the accumulation of ROS and subsequent neuronal toxicity. These findings suggest that oxidative stress is a major contributor underlying neuronal toxicity following antiretroviral treatment, and could inform potential adjunctive therapies to ameliorate cellular damage caused by these compounds.

In chapter 4, we explore the white matter loss prevalent in HIV patients on ART by directly testing the effect of antiretroviral compounds on the survival, maturation, and maintenance of the myelin-producing cells of the CNS, oligodendrocytes. We discovered that compounds in the PI drug class invoked reversible dose-dependent decreases in the maturation of oligodendrocyte precursor cells. No deficits were noted following NRTI treatment. Unlike the rescue from toxicity found in neuronal cultures, MMF was unable to restore maturation to normal levels when co-administered with PIs. In vivo administration of Ritonavir
resulted in reduced myelin protein levels in frontal cortex. Similarly, prefrontal cortex from HIV-positive individuals with HAND who had received antiretroviral therapy displayed a significant decrease in myelin basic protein levels compared to HAND individuals who had not received treatment, as well as HIV-negative control individuals. These findings demonstrate that antiretrovirals in the PI class have damaging effects in oligodendrocytes, and have clinical implications for treatment regimen recommendations both in juvenile HIV patients who are in the process of initial cortical myelination, as well as maintenance of myelin in adults on longitudinal lifelong therapy.

Intrigued by our finding that MMF failed to rescue oligodendrocyte maturation in the presence of antiretrovirals where it had succeeded in rescuing neuronal toxicity, we next pursued a thorough investigation into the activity of the EAR in oligodendrocytes following oxidant and antioxidant treatment (Chapter 5). In vitro application of oxidant tBHP to mouse oligodendrocyte precursors and mature oligodendrocytes resulted in robust accumulation of ROS, yet target genes of the antioxidant response were not upregulated. As we noted in previous studies (French et al., 2009, Reid et al., 2012), oxidant treatment also resulted in a significant reduction in maturation of precursors into maturing oligodendrocytes. Application of MMF successfully reduced ROS accumulation induced by multiple oxidants, but also did not activate the EAR or rescue maturation deficits in oligodendrocytes. Quite surprisingly, oligodendrocytes did not activate the EAR under conditions of oxidative stress to restore homeostasis, even after treatment with MMF. In addition to these implications in HAND, this study also aids in explaining the mechanism behind the recently FDA-approved drug Tecfidera (DMF), which has proven to be effective in reducing relapse rates in Multiple Sclerosis. Based on our findings, the therapeutic effect of the primary metabolite MMF in MS patients is not likely a direct antioxidant effect on oligodendrocytes, but rather an indirect effect by promoting this response in other CNS cell types, or through anti-inflammatory actions that we have not yet investigated.

Finally, we incorporate our findings into the context of previous studies (Chapter 6). We provide a summary of this body of work, an interpretation of the significance of our findings, and considerations for both clinical ramifications as well as potential future avenues of investigation.
<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>HIV-Associated Symptoms</th>
<th>Clinical Event</th>
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| 1              | Asymptomatic           | No HIV-related symptoms  
|                |                        | Painless enlarged lymph nodes persisting 3+ months |
| 2              | Mild                   | Involuntary loss of body weight (<10%)  
|                |                        | Recurrent upper respiratory tract infections  
|                |                        | Herpes zoster (Shingles)  
|                |                        | Recurrent oral ulceration  
|                |                        | Papular pruritic lesions  
|                |                        | Seborrhoeic dermatitis |
| 3              | Advanced               | Involuntary loss of body weight (>10%)  
|                |                        | Chronic diarrhea persisting 1+ months  
|                |                        | Persistent fever-intermittent or constant for 1+ months  
|                |                        | Persistent oral candidiasis, Oral hairy leukoplakia  
|                |                        | Pulmonary tuberculosis  
|                |                        | Severe bacterial infection (pneumonia, meningitis, etc)  
|                |                        | Acute necrotizing ulcerative gingivitis or necrotizing ulcerative periodontitis  
|                |                        | Unexplained anaemia, neutropaenia  
|                |                        | or chronic thrombocytopenia |
| 4              | Severe                 | HIV wasting syndrome  
|                |                        | Pneumocystis pneumonia  
|                |                        | Recurrent bacterial pneumonia  
|                |                        | Chronic or disseminated- herpes simplex virus infection, mycobacteria infection, cytomegalovirus, isosporiasis. mycosis, cryptosporidiosis, CNS toxoplasmosis, or atypical leishmaniasis.  
|                |                        | Extrapulmonary cryptococcosis (including meningitis)  
|                |                        | Extrapulmonary tuberculosis  
|                |                        | Recurrent non-typhoid Salmonella bacteraemia  
|                |                        | Lymphoma (cerebral or B-cell non-Hodgkin)  
|                |                        | Invasive cervical carcinoma  
|                |                        | Kaposi sarcoma  
|                |                        | Progressive multifocal leukoencephalopathy  
|                |                        | HIV encephalopathy  
|                |                        | HIV-Associated nephropathy  
|                |                        | HIV-Associated cardiomyopathy |

**Table 1.1 HIV Clinical Staging**

Adapted From “Criteria for HIV Staging Events”, and “WHO clinical staging of HIV/AIDS for adults and adolescents with confirmed HIV infection” (WHO, 2007). Clinical staging score is determined by level of HIV-Associated symptoms according to occurrence of specific clinical events in patients.
Oligodendrocyte Lineage Progression

Figure 1.1 Oligodendrocyte Lineage Progression

Oligodendrocyte maturation stage can be assessed through expression of stage-specific protein markers. The top panel shows immunofluorescent images of typical cells in each of the three identified oligodendrocyte lineage stages. These cells, isolated from newborn rat pups, were labeled with antibody to the ganglioside marker A2B5 for precursors, galactocerebroside (GalC) for immature oligodendrocytes, and proteolipid protein (PLP) for mature oligodendrocytes. In the lower panel are illustrations denoting process length and complexity with maturation. Under these drawings are the typical markers found at each stage in the lineage progression. In addition to PLP, myelin protein components: 2’,3’-cyclic nucleotide phosphodiesterase (CNP), myelin-associated glycoprotein (MAG), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG) begin to be expressed where indicated.

Adapted from oligodendrocyte differentiation figures (Grinspan, 2002, See, 2009).
Figure 1.2 Complex Nrf2 Regulation Occurs Under Varying Cellular Conditions

A model of Nrf2 pathway regulation is presented as described in the text. A) Basal conditions, B) Pathway activation, C) Pathway attenuation with two proposed mechanisms Ci) Keap1 shuttling and Cii) Fyn shuttling and Nrf2 phosphorylation.
1.11 REFERENCES


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factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. Cancer Res 61.


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CHAPTER 2

Excerpted from:

Persistence of HIV-Associated Neurocognitive Disorders in the Era of Antiretroviral Therapy

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2.1 OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASES

Oxidative stress is a shared pathological finding in a myriad of neurodegenerative diseases including Alzheimer Disease, Parkinson Disease, Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS), and HIV-Associated Neurocognitive Disorder (HAND) (Reynolds et al., 2007, Shukla et al., 2011). While it is clear that chronic levels of oxidative stress which will overwhelm the protective capacities of the cellular endogenous antioxidant responses may be responsible in part for the neuronal death occurring in these conditions, in many neurodegenerative diseases it is difficult to ascertain whether oxidative stress is the causative factor for disease pathology and progression or rather a resultant downstream event of other cellular dysfunctions (Ramsey, 2007, Reynolds et al., 2007, Melo et al., 2011, Shukla et al., 2011). Nonetheless, in HAND several lines of evidence suggest that both HIV and antiretroviral compounds may result in oxidative and nitrosative stress in the periphery and in the CNS (Lipton, 1994, Mondal et al., 2004, Walsh et al., 2004, Lagathu et al., 2007, Li et al., 2008, Chandra et al., 2009, Blas-Garcia et al., 2011). Deficits in total antioxidant levels and increases in the markers for oxidative stress are still observed in individuals on stable ART regimens with undetectable viral titers, necessitating the need for adjunctive therapies to ameliorate this imbalance (Hulgan et al., 2003, Mandas et al., 2009). As has been suggested in a variety of neurodegenerative conditions, antioxidant supplementation or upregulation of the endogenous antioxidant response in cells of the CNS may ameliorate the neuronal damage and death contributing to HAND (Reynolds et al., 2007, Calkins, 2009, Lindl et al., 2010, Melo et al., 2011).

2.2 OXIDATIVE STRESS IN THE ERA OF ART

The evidence of disrupted antioxidant balance and oxidative stress represent a continued concern for HIV-infected individuals even when virus is successfully controlled by ART (Blas-Garcia et al., 2011). To understand the magnitude and implications of this problem, it is important to understand the initial disruptions to the antioxidant system that were observed in infected individuals, and determine whether ART contributed to the resolution or the exacerbation of these problems.

2.2a Oxidative stress prior to ART: As early as 1988, while researchers were just beginning to understand the HIV virus, Sönnerborg and colleagues determined that plasma levels of malondialdehyde in
adults with HIV infection were elevated up to 30% when compared with controls (Sonnerborg et al., 1988). Malondialdehyde is the breakdown product of polyunsaturated lipids by reactive oxygen species, and is a mainstay in terms of a biological marker for measuring the relative levels of lipid peroxidation and oxidative stress in individuals (Moore and Roberts, 1998). A multitude of studies followed, showing significant increases in the levels of free radicals, hydroperoxides, hydroxynoneal, and oxidation of thiols in infected individuals, and confirming that malondialdehyde is increased significantly in infected adults, as well as children (Favier et al., 1994, Malvy et al., 1994, Repetto et al., 1996, Walmsley et al., 1997, Allard et al., 1998, Jareno et al., 1998, McLemore et al., 1998, Turchan, 2003, Haughey et al., 2004, Sacktor, 2004, Suresh et al., 2009). The metabolic synthesis of lipids is closely tied to the oxidative state of the cell and the lipids residing in the cell membrane, as certain enzymes such as sphingomyelinase, are sensitive to the oxidative status of the cell and regulate their activity based on cellular need (Haughey et al., 2008). Studies looking into the production of sphingolipids illustrated an overproduction of both sphingomyelin and ceramides with HIV-infection, suggesting a lipid imbalance caused by the virus (Haughey et al., 2004, Sacktor, 2004, Haughey et al., 2008). Additionally, studies showed remarkable deficiencies in antioxidant micronutrients including zinc, selenium, Vitamin C, Vitamin D, Vitamin E and beta-carotene (Vitamin A) (Dworkin et al., 1986, Droge et al., 1994, Favier et al., 1994, Allard et al., 1998, Ogunro et al., 2006, Srinivas and Dias, 2008, Suresh et al., 2009, Bilbis et al., 2010, Oliveira et al., 2011, Sudfeld et al., 2012). Conflicting reports exist on perturbation of total antioxidant status prior to ART, with several clinical studies reporting decreased total antioxidant capacity (Jareno et al., 1998, McLemore et al., 1998, Suresh et al., 2009), while Repetto et al. described an increase in the overall antioxidant capacity as individuals progressed to AIDS (Repetto et al., 1996). The discrepancies between these findings are likely based on the assays utilized to determine “total antioxidant capacity”, as different enzymatic approaches target different portions of the antioxidant system.

Activity levels of superoxide dismutase (SOD), which catalyzes the detoxification of the oxidant superoxide into hydrogen peroxide and water, were assessed by a variety of laboratories. Elevated SOD activity was observed in all evaluated HIV-infected individuals, with further increases occurring with disease progression to AIDS. In addition, increases in SOD mRNA levels were reported in individuals with
HAD, as compared to those who were neurocognitively normal (Delmas-Beauvieux et al., 1996, Repetto et al., 1996, Boven et al., 1999). These changes in SOD were observed in microglial cells, as well as in HIV-infected macrophages, suggesting a virus-triggered induction (Boven et al., 1999). An essential antioxidant found in the brain which buffers many reactive oxygen species is glutathione. In HIV-infected patients, overall glutathione levels were found to be reduced, and the remaining glutathione was greatly skewed to the oxidized versus reduced form (Buhl et al., 1989, Aukrust et al., 1995, Delmas-Beauvieux et al., 1996, Repetto et al., 1996). The enzyme glutathione peroxidase, which promotes the conversion of hydrogen peroxide to water through the use of glutathione, was also decreased, illustrating a severe imbalance in this system whose goal is to maintain cellular redox homeostasis (Ogunro et al., 2006). Another deleterious consequence of rampant pro-oxidants within cells is oxidative modification of DNA bases. Increased levels of 5-hydroxyuracil, 5-hydroxycytosine, 8-hydroxyadenine and 8-hydroxyguanine were found when comparing DNA isolated from lymphocytes of HIV-infected individuals versus uninfected controls (Jaruga et al., 1999). One other common product of an imbalanced oxidative state which is beginning to gain more interest and research focus is peroxynitrite. This compound, which is formed when superoxide reacts with nitric oxide, is detectable through its nitrotyrosine moiety, and is found at higher levels and with more frequency in the brains of patients with HAD, compared with those who are neurocognitively normal (Boven et al., 1999). In addition to the generation of superoxide as a direct result of HIV infection, the virus also increases mRNA expression of inducible nitric oxide synthase (iNOS), which enables a precipitous accumulation of this deleterious oxidation product (Boven et al., 1999). Further research in the Nath laboratory has illustrated that thirteen proteins with nitrotyrosine modifications are present in the CSF of individuals with HIV-infection. Individuals with dementia had the highest levels of these nitrites and nitrates. Importantly, three of these proteins were significantly elevated in individuals who showed declines in neurocognitive assessment over a period of 6 months (Li et al., 2008).

While it appears that there is not an all-or-nothing increase or decrease in antioxidant capacity, the evidence is clear that the components of the antioxidant defense system prior to ART were greatly affected by HIV-infection, and that the capabilities of endogenous antioxidant response were not able to alleviate damaging oxidative alterations to proteins and DNA.
2.2b Persistence of oxidative stress in the era of ART: In the ART era, oxidative stress is still pervasive in individuals living with well-controlled HIV-infection (Blas-Garcia et al., 2011). In 2007, a group at the University of Pennsylvania sought to ascertain whether ART had an effect on inflammation and oxidative stress in the brains of HIV-infected individuals through utilization of chemical-shift magnetic resonance spectroscopy. Through careful analysis of lipid, lactate, and creatine levels, they determined that the inflammation and oxidative stress initiated by the HIV infection was not ameliorated in ART-treated individuals, compared to seronegative controls (Roc et al., 2007). While these effects were observed in all HAND patients, the levels of oxidative stress markers were higher in those with more severe cognitive deficits (Roc et al., 2007).

Lipid peroxidation is still rampant despite effective viral control. While a couple of studies report decreased levels in markers of lipid peroxidation in ART-medicated patients, as compared with those not receiving ART, multiple studies have shown persistent statistically significant increases in hydroperoxides, isoprostanes, and malondialdehyde in ART-treated HIV-infected individuals, compared to seronegative controls (Jareno et al., 1998, Hulgan et al., 2003, Flourie et al., 2004, Ngondi et al., 2006, Masia et al., 2007, Wanchu et al., 2009, Gil et al., 2010, Ibeh et al., 2011, Ibeh and Emeka-Nwabunnia, 2012). Interestingly, two independent groups have determined in patient blood samples that the levels of peroxide species and oxidative stress are higher in patients on protease inhibitor (PI) based regimens, as compared with those in individuals on non-nucleoside reverse transcriptase inhibitor (nNRTI) based regimens, implicating a role for the protease inhibitor class in induction or exacerbation of oxidative stress (Hurwitz et al., 2004, Masia et al., 2007).

Micronutrient deficiencies are still problematic with ART, and while most patients have adequate plasma concentrations of vitamins C, D, and E, reported levels are still considered sub-optimal and lower than seronegative patients (Stephensen et al., 2006, Sudfeld et al., 2012). While subsequent reports have indicated that zinc and selenium deficits are no longer observed in individuals on ART, further definitive confirmation of these findings is necessary (Stephensen et al., 2007, Sundaram et al., 2008). Additionally, in studies which evaluated the serum of adults and the saliva of children, the total antioxidant status was
found to be decreased in ART treated HIV-patients when compared to HIV-negative controls, mirroring findings in studies conducted in the pre-ART era (Mandas et al., 2009, Padmanabhan et al., 2010).

While several studies have indicated that introduction of ART has been accompanied by an improvement in overall glutathione status, this effect is not totally rectified and imbalances still occur (Aukrust et al., 1995). In particular, numerous groups have shown that circulating glutathione levels are still markedly reduced in HIV-infected individuals, when compared with age-matched controls, with the ratio of oxidized to reduced glutathione remaining out of balance (Walmsley et al., 1997, Flourie et al., 2004, Wanchu et al., 2009, Gil et al., 2010, Awodele et al., 2012). Unfortunately, studies investigating nitrosative stress and nitrosylated proteins in HIV-infected individuals on ART are still lacking; however, the Nath, Hammond, and Sutliff laboratories have been investigating nitrosative stress in HIV, and it is likely that such reports are forthcoming.

It has been reported that HIV-positive individuals do not have altered levels of 8-hydroxy-2’-deoxyguanosine [8-oxoG] in their urine regardless of ART or lipodystrophy status (Paul et al., 2003). While these findings appear to be promising, in a recent study, autopsy tissue from frontal cortex was stained for both nuclear and mitochondrial 8-oxoG. The levels of this oxidized DNA product were significantly increased in cases of HAND, suggesting that ART, or ART in combination with ongoing infection may promote DNA oxidative modification, cellular dysfunction damage and death (Zhang et al., 2012). Additionally, the presence of clastogenic factors, which cause chromosomal breaks and DNA damage and which may be released from cells under conditions of oxidative stress, was observed in the plasma of all HIV-patients tested by Edeas et al.. This was true of patients that were both asymptomatic and symptomatic for AIDS-defining pathologies, and was independent of ART status (Edeas et al., 1997). The effects of these clastogenic factors appear to persist in multiple cell populations implicated in HAND pathogenesis. For example, in leukocytes obtained from HIV-infected individuals, the percentage of cells exhibiting DNA fragmentation was increased in individuals on ART, as compared with those who were ART-naïve (Gil et al., 2010). However, the results of this study have not addressed the possible contribution of latent or low level of infection to the findings.
Due to pervasive oxidative damage and antioxidant imbalance despite effective long-term viral control in patients, it is now imperative to recognize the direct effects that viral enclaves and antiretroviral drugs themselves may have on perpetuating these effects. Further studies are needed to investigate the independent effects of ART and the virus on oxidative damage in the CNS, as well as in the periphery in order to better determine therapeutic interventions to resolve these dysfunctions.

2.3: OXIDATIVE STRESS BY HIV

Exhaustive research has been conducted in order to determine the effects of the HIV on infected cells and the cytotoxic factors released from these cells. As addressed earlier, it is now clear that HIV-infected macrophages secrete a variety of neurotoxic substances including glutamate, nitric oxide, and superoxide (Lipton, 1994). Within actively infected human myeloid-monocytic cell lines or monocyte-derived macrophages, HIV induces an increase in superoxide anions, with a concomitant increase in superoxide to combat these factors (Kimura et al., 1993, Boven et al., 1999). Mollace and colleagues have demonstrated that the supernatants from HIV-infected human primary macrophages induced oxidative stress in astrocytes, as indicated by increases in malondialdehyde levels (Mollace et al., 2002). They further showed that these supernatants, which contained excess superoxide, induced astrocytic apoptosis, confirming HIV-mediated toxicity of this secreted product (Mollace et al., 2002). In a similar fashion, in HIV-infected monocytes, an induction of nitric oxide synthase and subsequent increase nitric oxide was observed (Bukrinsky et al., 1995). It is also interesting to note that elevated oxidative stress in the form of intracellular singlet oxygen is capable of reactivating latent HIV through long terminal repeat (LTR) transactivation in infected monocytes or lymphocytes, suggesting that the virus may rely on oxidative stress signaling cascades for continuation of long-term infection or viral rebound (Piette and Legrand-Poels, 1994).

The amino acid and neurotransmitter glutamate is normally secreted from neurons into the synaptic cleft, and is quickly removed and recycled through the actions of astrocytes. This molecule normally activates the N-methyl D-aspartate (NMDA) receptor on neurons, and allows for Ca2+ entry into the cell. However, it has been clearly demonstrated that excessive extracellular levels of glutamate resulting from
overstimulation of neurons, impaired reuptake by astrocytes, or release from other cell populations within the brain can lead to hyperactivation of NMDA channels and subsequent increases in Ca2+ levels in the neuronal cytoplasm, resulting in excitotoxic neuronal death (Szydlowska and Tymianski, 2010). In 2001, Jiang et al. demonstrated through a series of elegant experiments that the molecule responsible for the neurotoxicity observed on neuronal cultures was a molecule of less than 3,000 kilodaltons, was not sensitive to trypsin digestion, and that its neurotoxic effect was blocked by a selective NMDA receptor antagonist, MK-801. It was through this study, as well as a subsequent study by O'Donnell et al. that the increased levels of extracellular glutamate secreted by HIV-infected macrophages may be a major factor in HIV-infected macrophage mediated indirect neuronal death (Jiang et al., 2001, O'Donnell et al., 2006). The increases in extracellular glutamate appears to be an effect of dysregulation of the glutamate synthesis pathway, as inhibition of the mitochondrial glutaminase enzyme blocks the production and the secretion of glutamate from HIV-infected macrophages (Zhao et al., 2004, Erdmann et al., 2007, Huang et al., 2011). As was distinctly noted in human patient samples, a marked deficiency of glutathione and an imbalance between the oxidized and the reduced glutathione was observed. Within cells, glutaminase is the enzyme which converts glutamine to glutamate. It is tempting to consider that the oxidative stress resulting from the lack of the antioxidant properties of glutamine may be very tightly coupled to a depletion of glutamine from cells by hyperactivation of glutaminase enzymes, precipitating an overproduction of glutamate and triggering an excitotoxic neuronal death pathway.

Similar to effects of whole virus in vivo, when gp120 and Tat were injected into the brains of rats, both of these viral proteins induced lipid peroxidation and glutathione depletion (Banerjee et al., 2010). Additionally, both of these proteins significantly reduced intracellular glutathione, and increased malondialdehyde in immortalized brain endothelial cells, showing that the oxidative status of these cells would also be directly affected by the presence of virus. This is of particular importance as the altered oxidative status of these endothelial cells comprising the BBB will have potential impact on not only the integrity of the BBB, but also on the monocyte/macrophage transmigration to the CNS, an important factor in the persistence of HAND (Price et al., 2005). Further, when applied to neurons in culture, gp120 and Tat induce disruptions in the lipid metabolism, leading to increased levels of sphingomyelin, ceramide, and
hydroxynoneal, paralleling disruptions in these pathways observed in the neurons of HAND patients (Haughey et al., 2004).

Studies that investigated gp120 separately have revealed that it is capable of inducing ROS formation, and activating the antioxidant response in astrocytes (Viviani et al., 2001, Reddy et al., 2011). Studies looking into the production of oxidant species by gp120 have shown that superoxide ions as well as nitric oxide are involved in neuronal toxicity (Dawson et al., 1993, Walsh et al., 2004). Mechanistically, gp120-induced nitric oxide formation is dependent on a mannose-specific endocytic lectin in macrophages, while gp120-induced expression and upregulation of iNOS selectively occurs in astrocytes in human fetal neuroglial cultures astrocytes (Pietraforte et al., 1994, Walsh et al., 2004). Multiple studies have shown that in neurons undergoing gp120-induced toxicity, a significant increase in intracellular Ca2+, likely released from intracellular stores, preceded death, suggesting a mechanism of activation of calpains or other pro-death cellular machinery (Viviani et al., 2001, Agrawal et al., 2010).

Studies investigating Tat-induced neuronal death have revealed that this viral protein triggered the accumulation of ROS when exogenously applied to a variety of cell types, including lymphocytes, microglia, brain microvascular endothelial cells (BMECs) and neurons, as well as in HeLa cells expressing Tat (Flores et al., 1993, Israel and Gougerot-Pocidalo, 1997, Kruman et al., 1998, Toborek et al., 2003). When directly injected into striatum of rats, Tat produced dramatic increases in protein oxidative modifications and protein carbonyls (Aksenov et al., 2001). Protein carbonyls were also markedly increased in HeLa cells expressing Tat, supporting in vivo data (Flores et al., 1993). In addition, decreased levels of glutathione were observed in cardiac myocytes and BMECs exposed to recombinant Tat protein, Tat-expressing transgenic mice, and in Tat-expressing HeLa cells (Westendorp et al., 1995, Choi et al., 2000, Raidel et al., 2002, Toborek et al., 2003). Two studies pinpointed the involvement of manganese-dependent superoxide dismutase (Mn-SOD) as a key player in Tat-induced cellular changes. By expressing Tat in HeLa cells, the laboratories of Lehman and McCord convincingly showed that Tat suppressed the RNA, protein, and activity levels of Mn-SOD, while inducing no changes in the CuZn SOD enzyme levels (Flores et al., 1993, Westendorp et al., 1995). In HIV-infected individuals, plasma levels of SOD were

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increased in plasma, and these changes were in parallel with disease progression to AIDS. If the results from these HeLa cell studies can be expanded to other cell populations, it is tempting to speculate that perhaps this overall increase in SOD activity is a compensatory mechanism resulting from the Tat-induced alterations in Mn-SOD activity and the subsequent increases in superoxide ions that cannot be eliminated. The overexpression of Tat in HeLa cells also led to decreases in overall glutathione levels, with a lower ratio of reduced to oxidized glutathione in the remaining supply, mimicking the effect seen in HIV infected individuals in vivo and in HIV-infected macrophages in vitro (Flores et al., 1993, Westendorp et al., 1995).

Finally, studies focusing on microglia have shown that the expression of HIV viral protein R (Vpr) can induce oxidative stress pathways, and can activate HIV latent gene expression (Deshmane et al., 2009). When exogenously applied to human fetal astrocytes, the oxidative stress caused by Vpr causes decreases in intracellular ATP and glutathione, and skews remaining glutathione in favor of the oxidized versus reduced form (Ferrucci et al., 2012).

In summary, exhaustive in vivo and in vitro studies indicate that HIV viral proteins themselves can induce, precipitate, and augment oxidative stress in multiple cell types, of both peripheral and CNS tissue. These findings further emphasize the importance of complete inhibition of viral replication in alleviating oxidative stress via antiretroviral therapy. However, emerging evidence suggests that antiretroviral drugs themselves might inadvertently lead to oxidative stress.

2.4: OXIDATIVE STRESS BY ART

Due to the requirement for lifelong adherence to antiretroviral regimens to prevent viremia and immune system compromise, it is necessary to investigate the effects of these compounds in a cellular context. Many of these drugs are associated with negative side-effects, and have been linked to the metabolic syndrome, atherosclerosis, lipodystrophy, proteasome inhibition and the unfolded protein response (Zhou, 2005, 2006, Vidal et al., 2010). Additionally, it has become apparent that these compounds themselves produce oxidative stress, even in the absence of virus, and may in fact be contributing to the persistence of oxidative stress in patients with well-controlled viral load (Mondal et al., 2004, Blas-Garcia et al., 2011).
HIV is highly susceptible to mutations, mostly due to the error-prone reverse transcription step during replication, and underlies the emergence of drug resistant mutations over time in patients treated with single antiretroviral drugs in the early 1990s. Additionally, the development of more sensitive methods for HIV RNA detection revealed the presence of viral reservoirs in multiple tissues, including the CNS. These findings led to the revision of antiretroviral therapy, which, until that time included mostly single antiretroviral drug regimens. A multiple drug treatment approach, termed ART, was implemented to aim at different steps in the HIV replication. Currently recommended ART regimens include a cocktail of nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse-transcriptase inhibitors (nNRTIs), protease inhibitors (PIs), and to a lesser extent, entry inhibitors and integrase inhibitors (Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Available at http://aidsinfo.nih.gov/). This approach has led to improved immune function, long-term viral suppression, and underlies the reductions in HIV-associated morbidity and mortality in the era of ART. The recently updated guidelines recommend ART initiation to all HIV-infected, ART-naive individuals irrespective of CD4+ counts. The impact of early initiation of ART on HIV-associated neurological complications remain to be seen. While a consensus has been reached regarding the time to initiate ART based on CD4 cell counts, AIDS-defining illnesses, and certain co-morbidity factors; the panel did not clearly outline strategies to best eradicate the viral reservoirs in the CNS, and to decrease the risk of developing HAND among infected patients.

In the meantime, one major approach to better control HAND has been to implement therapies that include drugs which achieve therapeutic concentrations in the CNS. While HIV can circumvent the BBB barrier, complex drug transport and efflux mechanisms at this junction hampers the achievement of effective antiretroviral concentrations in the brain parenchyma. Recent efforts to address this hurdle have led to the establishment of CNS penetrance effectiveness (CPE) score, an algorithm based on the chemical structure, pharmacodynamic and pharmacokinetic data of antiretroviral drugs (Letendre et al., 2008, Tozzi et al., 2009, Letendre et al., 2010). In summary, an antiretroviral drug with high CPE score is small in size (molecular mass below 400-500 kDa), has high lipid solubility and low protein binding, and it is not a substrate for drug transport or efflux proteins. Unfortunately, results of several clinical studies which
incorporated CPE into the design and the analysis of outcomes are not conclusive (Letendre et al., 2008, Cysique et al., 2009, Marra et al., 2009, Tozzi et al., 2009, Garvey et al., 2011, Lanoy et al., 2011, Smurzynski et al., 2011). While a positive correlation between CPE scores and neurological outcomes are observed in several studies, one study revealed that ART regimens with higher CPE scores might be associated with worse neurocognitive performance (Marra, 2009).

These studies investigating the effect of CPE scores on neurological outcomes have inherent caveats. First, due to limited methodologies, clinical studies cannot assess the drug concentrations in the brain parenchyma, and instead depend on the CSF levels, which are usually based on measurements after a single-dose administration of the drug. Several factors, such as poor drug adherence and the impact of co-prescribed drugs on the pharmacokinetics of antiretroviral drugs can impact CNS concentrations and can confound the measured outcomes. Additionally, the escape of drug-resistant viral species into the CNS and their establishment in viral reservoirs early during infection can lead to the rise of drug-resistant HIV species in the CNS, and can hinder the efforts to assess the impact of CPE scores on neurological outcomes. Further, co-morbidity factors impacting the integrity of the BBB should also be considered in assessing drug availability in the CNS. Among these factors are cancer, infections, and drug and alcohol abuse, all of which are shown to alter BBB integrity independent of HIV infection, and should be considered when CPE scores are established and evaluated (Shiu et al., 2007, Qin et al., 2008, Ramirez et al., 2009). One final factor to consider in evaluating the long-term effectiveness of ART is the potential for direct toxicities of antiretroviral drugs in the CNS, especially given the interest in implementation of ART regimens with better CNS penetrance and possibly developing nanoART as part of treatment plans. Antiretroviral drugs have known side-effects in the periphery, including dyslipidemia, and lipo hypertrophy. Furthermore, antiretroviral drug-associated toxicity is well documented in the peripheral nervous system, and potential CNS toxicities secondary to ART exist. Oxidative damage elicited by antiretroviral drugs is of particular interest, given ample evidence of ongoing oxidative stress in the HAND brain, as described above.
In several cavalier studies, researchers eloquently demonstrated in several cell populations (human adipocytes, monocytes, myeloid cell lines, and human aortic endothelial cells) that a variety of drugs from the PI and NRTI families, alone or in combinations, induced the production of ROS, hydrogen peroxide, and factors promoting monocyte recruitment. The compounds reported to induce these changes included PIs: Indinavir, Nelfinavir, Lopinavir, Ritonavir, Saquinavir, Atazanavir and NRTIs: Stavudine (d4T), Zidovudine (AZT), and Didanosine (Mondal et al., 2004, Ferraresi et al., 2006, Lagathu et al., 2007, Chandra et al., 2009, Touzet and Philips, 2010, Manda, 2011, Brandmann et al., 2012). The compounds Amprenavir (PI) and Abacavir (NRTI) were consistently reported as lacking these effects, making these drugs good candidates for inclusion in regimens to be prescribed to patients with HAND (Lagathu et al., 2007).

As the first available antiretroviral drug, AZT has been extensively studied. While AZT may not be a mainstay drug of choice for customized optimal regimens, as the primary ART compound available in resource-limited developing countries, it is still of importance to understand its cellular effects. AZT was approved for treatment in 1987, and as early as 1992 the Papoian laboratory reported deficits in mitochondrial enzymes and uncoupling of the electron transport chain (ETC) (Lewis et al., 1992). The disruption of the ETC is a primary cause of mitochondrial-based intracellular ROS accumulation and mitochondrial DNA oxidation, an effect since expounded upon in multiple laboratories after acute AZT exposure in isolated heart mitochondria and primary human cardiomyocytes, and after chronic gestational AZT exposure in mouse liver and kidney and in the lung and brain of fetal patas monkeys (de la Asuncion et al., 1999, Bialkowska et al., 2000, de la Asuncion et al., 2004, Gao et al., 2011). This compound has also been shown to increase mitochondrial lipid peroxidation, deplete intracellular glutathione, and lead to oxidation of remaining glutathione, ultimately inducing a caspase-3- and caspase-7-dependent apoptotic death (Yamaguchi et al., 2002, de la Asuncion et al., 2004, Gao et al., 2011). When another NRTI, d4T was investigated, it also was shown to produce ROS, mitochondrial oxidative stress, oxidized mitochondrial DNA, and altered activity of mitochondrial oxidative phosphorylation enzymes (Gerschenson et al., 2001, Velsor et al., 2004). Similarly, NRTI Zalcitabine (ddC) also induces oxidative stress, as evidenced by the accumulation of protein carbonyls and nitrotyrosine modifications. Interestingly, this study also reported
that the better-tolerated cytidine analog Lamivudine (3TC) did not produce these effects, suggesting that 3TC may be considered as an alternative to reduce oxidative stress, and that future compounds generated from this structural base may behave similarly (Opie et al., 2007). Interestingly, in contrast to studies in human lymphoid cells, Brandmann and colleagues have recently reported that in astrocytes AZT, Lamivudine, Efavirenz, and Nevirapine do not appear to reduce intracellular levels of glutathione (Brandmann et al., 2012). Whether or not these compounds behave similarly in neurons and other cell populations in the CNS remains to be elucidated.

In the non-nucleoside reverse transcriptase inhibitor (nNRTI) drug class, Efavirenz applied to a human hepatoblastoma cell line resulted in superoxide generation, depletion of intracellular glutathione, and decrease in mitochondrial function and membrane potential that was independent of mitochondrial DNA replication (Apostolova et al., 2010). In addition, Efavirenz has also been linked to neuropsychological side effects in HIV-infected patients, and it is probable that the specific oxidative stress effects on mitochondria and glutathione may play a role in the neurotoxicity of this compound, as neurons are particularly sensitive to perturbations in the antioxidant system (Apostolova et al., 2010, Ciccarelli et al., 2011).

Within the protease inhibitor class, both Ritonavir and Amprenavir have been associated with increased superoxide anion production, while Ritonavir has been shown to cause increases in nitrotyrosine levels in porcine coronary arteries (Chai et al., 2005). On the other hand, both Indinavir and Nelfinavir have been shown to induce a time and concentration dependent depletion of intracellular glutathione in astrocytes as well as in pancreatic beta cells (Chandra et al., 2009, Brandmann et al., 2012). Nelfinavir is of particular interest, as it suppresses cytosolic, rather than mitochondrial superoxide dismutase levels, and induces a necrotic rather than apoptotic cell death cascade in an adipocyte cell line (Vincent et al., 2004, Chandra et al., 2009). Future studies of this compound will undoubtedly prove interesting and may have important implications in patients with regard to their neurocognitive outcomes associated with this compound. Finally, it is interesting to note that multiple studies investigating the oxidative effects of the thymidine analogs in the NRTI class have reported increased ROS, hydrogen peroxide, and nitric oxide intermediates but no superoxide anions (Ferraresi et al., 2006, Amatore et al., 2010).
Currently, there is no information on possible oxidative effects of the entry inhibitor, integrase inhibitor, and mutation inhibitor drug classes. Similarly, the lack of published studies for the neuroglial cell populations in the brains of HAND patients is surprising. In order to design custom drug regimens which will not precipitate, or can ameliorate oxidative stress, cellular dysfunction and death, it is critical to have an understanding of the cellular effects of each currently approved antiretroviral compound, and to design future compounds with minimal oxidative effects. Further, it is possible that the continued dysfunction in superoxide production and superoxide dismutase levels in patients may be due to the effects of more than one drug in a multi-drug regimen. Thus, designing future combinations which do not precipitate oxidative stress is of utmost importance in efforts to resolve the persistence of HAND observed in the ART era.

2.5 POTENTIAL THERAPEUTIC AVENUES FOR OXIDATIVE STRESS IN THE ART ERA

Since the manifestations of oxidative stress induced by HIV and ART have emerged, it has also become evident to scientists that boosting the endogenous antioxidant response may be a valid and encouraging adjunctive therapeutic option. Within the cell, the antioxidant response is mediated through the activation of transcription factor NF-E2 (nuclear factor (erythroid-derived 2))-related factor-2 (Nrf2) and its myriad of effector phase II and III detoxifying enzymes. Relevant to the previously discussed aberrations in oxidant detoxification, this pathway upregulates superoxide dismutase, peroxiredoxins, thioredoxins, and multiple glutathione biosynthesis enzymes (Shih, 2003, Li, 2009).

In vitro, multiple compounds which act upon the Nrf2 pathway have proven effective in ameliorating the oxidative effects of viral infection, viral proteins, or antiretroviral drugs. Among these compounds are resveratrol, dimethyl fumarate, N-acetylcysteine, and curcumin (Chai et al., 2005, Touzet and Philips, 2010, Cross et al., 2011, Gao et al., 2011, Manda, 2011). Antioxidants which have not yet been shown to act through the Nrf2 pathway, but which have similar effects in vitro include dihydroxybenzyl alcohol, water soluble vitamin E (trolox), glutathione mimetic tricyclodecan-9-yl-xanthogenat, and acetyl-l-carnitine (Ferraresi et al., 2006, Opii et al., 2007, Apostolova et al., 2010, Weakley et al., 2011). An alternative approach to specifically inhibit the actions of NADPH oxidase through the compound diphenylene iodonium, was able to specifically prevent the effects of PIs, but not NRTIs in human adipocytes (Lagathu
et al., 2007). This interesting finding suggests that the different antiretroviral classes may lead to ROS production through different mechanisms, and that specific therapeutics targeting individual oxidant-producing enzymes or pathways may need to be considered for specific ART regimens.

An alternative approach to activating Nrf2 pathway is supplementation with the antioxidant vitamin C (ascorbate), which is capable of directly scavenging reactive oxygen and nitrogen species. This method has been shown to be effective in counteracting the deleterious effects of gp120, as well as Nelfinavir *in vitro*, and has been shown to have beneficial outcomes in patients, when supplemented to ART regimens (de la Asuncion et al., 2004, Vincent et al., 2004, Walsh et al., 2004). Along these same lines, supplementation with a variety of other antioxidants and micronutrients including minocycline, glutathione replenishing peptide alpha-lipoic acid, selenium, vitamin A, Vitamin E, and a multivitamin regimen including vitamins A, C, E selenium and coenzyme Q10 have demonstrated partial protection from the deleterious oxidative effects of HIV/SIV and ART *in vivo*, with particular emphasis on restoration of the total blood glutathione levels (Delmas-Beauvieux et al., 1996, Batterham et al., 2001, de la Asuncion et al., 2004, Jariwalla et al., 2008, Meulendyke et al., 2012). Finally, the NMDA-receptor blocker memantine, and monoamido oxidase type B inhibitor selegiline, approved for treatment in Alzheimer and Parkinson Diseases, respectively, have also been proposed as potential therapeutics for HAND (Batterham et al., 2001, Lindl et al., 2010). Memantine might exert therapeutic effects through reduction of residual virus-mediated glutamate excitotoxicity, and selegiline has been reported to be capable of reducing oxygen-based free radicals (Nagatsu and Sawada, 2006, Kutzing et al., 2012). The use of these compounds and their efficacy in reducing oxidative damage in HAND are still in early stages. Initial studies were confounded by the fact that much damage was already present in late stage HAD patients and a protective effect at this stage was not evident (Schifitto et al., 2009). Further work with these compounds will require identifying patients with ANI or MND to enroll in longitudinal studies to determine whether these compounds will have long-term benefit.

Going forward, it is imperative that the oxidative stresses imposed by HIV and ART are targeted by adjunctive therapies. As research progresses with the quest for eliminating viral reservoirs and designing
more effective, less toxic antiretroviral compounds, it is important to keep in mind that oxidative stress is and will continue to be a persistent burden, especially in a patient population with a significantly enhanced life expectancy.


CHAPTER 3

Antiretroviral Drugs Induce Oxidative Stress and Neuronal Damage in the Central Nervous System

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3.1 ABSTRACT
HIV-associated neurocognitive disorder (HAND), characterized by a wide spectrum of behavioral, cognitive, and motor dysfunctions, continues to affect approximately 50% of HIV(+) patients despite the success of combination antiretroviral drug therapy (cART) in the periphery. Of note, potential toxicity of antiretroviral drugs in the central nervous system (CNS) remains remarkably underexplored, and may contribute to the persistence of HAND in the cART era. Previous studies have shown antiretrovirals (ARVs) to be neurotoxic in the peripheral nervous system in vivo and in peripheral neurons in vitro. Alterations in lipid and protein metabolism, mitochondrial damage, and oxidative stress all play a role in peripheral ARV neurotoxicity. We hypothesized that ARVs also induce cellular stresses in the CNS, ultimately leading to neuronal damage and contributing to the changing clinical and pathological picture seen in HIV-positive patients in the cART era. In this report, we show that ARVs are neurotoxic in the CNS in both pigtail macaques and rats in vivo. Further, in vitro, ARVs lead to accumulation of reactive oxygen species (ROS), and ultimately induction of neuronal damage and death. While ARVs alone caused some activation of the endogenous antioxidant response in vitro, augmentation of this response by a fumaric acid ester, monomethyl fumarate (MMF), blocked ARV-induced ROS generation and neuronal damage/death. These findings implicate oxidative stress as a contributor to the underlying mechanisms of ARV-induced neurotoxicity, and will provide an access point for adjunctive therapies to complement ARV therapy and reduce neurotoxicity in this patient population.
3.2 INTRODUCTION

Despite introduction of combination antiretroviral therapy (cART), HIV-associated neurocognitive disorder (HAND) continues to affect approximately 50% of HIV(+) patients (Dore et al., 1999, Heaton et al., 2010). Further, in the cART era, the underlying neuropathology has shifted from overt subcortical involvement to a more insidious cortical damage (Gannon et al., 2011). Various factors, such as poor adherence to drug regimen, emergence of resistant virus species, and residual viral DNA in the CNS, may contribute to these changes (Gannon et al., 2011). However, another likely contributor to HAND in the cART era is the virtually unstudied potential for antiretroviral (ARV)-related toxicity in the CNS. cART has decreased HIV-related morbidity and mortality by limiting T-cell loss and controlling opportunistic infections. However, cART regimens are associated with potentially serious side-effects, including dyslipidemia, lipohypertrophy, and increased risk of atherosclerosis (Vidal et al., 2010). Additionally, cART-associated toxicity in the peripheral nervous system is well-documented, and it is likely that cART would trigger similar responses in the CNS. Pharmacokinetic studies suggest limited ARV penetrance into the CNS and indicate low cerebrospinal fluid (CSF) and parenchymal drug concentrations (Yilmaz et al., 2004, Yilmaz et al., 2009). However, direct blood-brain barrier (BBB) compromise by viral proteins and neuroinflammation and indirect BBB impairment due to concomitant factors, such as coexisting infections, can lead to increased CSF and parenchymal drug concentrations. Thus, the impact of ARVs in the CNS of HIV(+) patients is clinically relevant and must be examined.

Initial cART usually includes two nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs) in combination with a non-nucleoside reverse-transcriptase inhibitor (nNRTI) or with a protease inhibitor (PI) boosted with a low dose of a second PI, Ritonavir. NRTIs and nNRTIs bind to the HIV reverse transcriptase enzyme and inhibit pro-viral DNA synthesis; PIs inhibit viral proteases needed for virus maturation and assembly. Despite some crossover, certain ARV classes are more highly associated with particular side effects and toxicities than are other classes. PIs alter lipid metabolism and induce the endoplasmic reticulum stress response in macrophages, linking PIs to increased risk of atherosclerosis (Touzet and Philips, 2010). NRTIs inhibit DNA polymerase-γ and lead to decreased mitochondrial DNA, loss of mitochondrial membrane potential and oxidative phosphorylation, consequently precipitating
oxidative stress (Nolan and Mallal, 2004). Previous studies exploring possible side effects of ARVs in the CNS are scarce and mostly involve cell lines (Cui et al., 1997). Due to mutations and aberrations in immortalized cell lines, these studies may not reflect the ARV toxicity potentially occurring in biological settings. Peripheral dorsal root ganglia neurons are the only primary cell type of neural lineage previously studied for ARV toxicity (Werth et al., 1994). In this report, we examined effects of ARVs in primary CNS neurons both in vivo and in vitro. Our findings suggest that cART induces oxidative stress and neurotoxicity in the CNS, and that the patients on long-term cART regimens would benefit from adjunctive therapies that include antioxidant strategies to overcome deleterious effects of cART in the CNS.

3.3 MATERIALS AND METHODS

normal antibody diluent. Thermo Scientific (Waltham, MA): goat anti-rabbit horseradish peroxidase (HRP) antibody and goat anti-mouse HRP antibody, SuperSignal West Dura extended duration substrate. Tocris Bioscience (Ellisville, MO): thapsigargin. The antibody against calpain-cleaved spectrin was a generous gift from Dr. Robert Siman (Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA).

**Primary Cortical Neuroglial Cultures.** Primary rat cortical neuroglial, pure neuronal, and pure astrocytic cultures were isolated from embryonic day 17 Sprague Dawley rat pups, with modifications of protocols previously described (Wilcox et al., 1994). Briefly, the cortical cell suspensions isolated from rat pups following standard protocols were plated on poly-L-lysine coated T25 tissue culture flasks (2 x 10⁶ cells/flask), 96-well tissue culture plates (0.5 x 10⁵ cells/well), or glass coverslips (0.25 x 10⁶ cells/well). These cultures were maintained in neurobasal media with B27 supplement at 37°C with 5% CO₂ for the generation of neuroglial cultures, as described previously (Wang et al., 2010, Akay et al., 2011b, White et al., 2011). Half of the media was replaced with fresh media every 7 days and the experiments were performed at 21 days in vitro (DIV), at which time the cultures contain approximately 85-90% neurons and 10-15% astrocytes/glia. For the preparation of pure neuronal cultures, cultures were treated with 10µM Ara-C 48 hours after plating and maintained in neurobasal media with B27 supplement at 37°C with 5% CO₂; at 21 DIV, the age at which the experiments were conducted, no astrocytes are detectable by staining for GFAP. Pure astrocytic cultures were prepared by initially plating the cortical cell suspensions in 10% fetal bovine serum (FBS) in DMEM and maintaining at 37°C with 5% CO₂ for 7-12 days, after which time astrocytes constitute more than 90% of the cultures. These pure astrocytic cultures are then re-plated in T25 flask or poly-L-lysine-coated glass coverslips in DMEM/10% FBS and the experiments are conducted 3-5 days after re-plating.

**SIV/pigtail macaque model of HIV CNS Disease.** Juvenile pigtailed macaques (*Macaca nemestrina*) were inoculated with SIV/DeltaB670 and SIV/17E-Fr, as described previously (Zink et al., 1999). Beginning 12 days after inoculation, animals were treated daily with a four ARV drug combination (cART) until necropsy (range, day 161–175). The treatment consisted of the NRTI Tenofovir (Gilead) at 30 mg/kg
subcutaneously every day; the PIs Saquinavir (Roche) and Atazanavir (Bristol-Myers Squibb) at 205 and 270 mg/kg orally twice a day, respectively; and the integrase inhibitor L-870812 (Merck) (Hazuda et al., 2004) at a dose of 10 mg/kg orally twice a day (Zink et al., 2010). The Tenofovir dose was determined on the basis of previous studies (Tsai et al., 1997), while Atazanavir and Saquinavir doses were determined by pharmacokinetic experiments conducted in pigtailed macaques, and reflected those that resulted in the same area under the curve as detected in humans treated with Atazanavir and Saquinavir (Zink et al., 2010). The dose of the integrase inhibitor was based on a previous study conducted in rhesus macaques (Hazuda et al., 2004).

**Rodent model of antiretroviral-induced neurotoxicity.** All surgical procedures were performed with the approval of the Institutional Animal Care and Use Committee. Adult male Sprague Dawley rats were catheterized via jugular vein, as described previously (Thrivikraman et al., 2002). The drug cocktail composed of AZT (100mg/kg/day), Ritonavir (20mg/kg/day), and Saquinavir (25mg/kg/day) was administered twice daily for 7 days by continuous intravenous injection. Each drug dose was based on previously published studies in rodents (Shibata et al., 2002, Huisman et al., 2003, Manda et al., 2010, Pistell et al., 2010, Waring et al., 2010, Yang et al., 2010, du Plooy et al., 2011, Fontes et al., 2011, Lledo-Garcia et al., 2011, Wagner et al., 2011, Mak et al., 2013, Reyskens and Essop, 2013a, b, Reyskens et al., 2013). The catheters were flushed with 0.3 ml heparin (50 IU/ml) in PBS until the end of treatments. At time of euthanasia, preceding decapitation and tissue harvest, catheter patency was re-verified by response to pre-euthanasia sedation (100mg/kg ketamine/ 10mg/kg xylazine). The brains were removed, the frontal cortex and the hippocampus were dissected on ice, and the tissue samples were stored at -80 °C until immunoblot analysis.

**Immunofluorescence Staining of Tissue.** Tissue slides of paraffin-embedded tissue sections from hippocampus of male and female pig-tailed macaques (*Macaca nemestrina*) were prepared for immunofluorescent staining, as described previously (Akay et al., 2011a). Briefly, glass slides containing paraffin-embedded sections (10 mm) were heated to 55°C for at least 30 min, deparaffinized in Histoclear, and rehydrated with consecutive 100%, 95%, 90% and 70% ethanol washes. Endogenous peroxidase
activity was blocked with 3% H$_2$O$_2$ in methanol and antigen unmasking was achieved with target retrieval solution at 95°C for 1 h. Tissue sections were blocked with 10% normal goat serum in phosphate-buffered saline solution (PBS). Mouse monoclonal antibodies to synaptophysin, MAP2, and rabbit polyclonal antibody to GFAP were used at empirically defined dilutions (synaptophysin at 1:500, MAP2 at 1:100; GFAP at 1:80), and DAPI was used to stain nuclei. The tyramide amplification system was used to detect synaptophysin. Slides were mounted in Citifluor AF1, and for each specimen, 5-10 randomly selected areas within the CA1-CA3 layer of the hippocampus were scanned along the z-axis to create z-stack images at high-magnification (600X) by laser confocal microscopy on a Biorad Radiance 2100 equipped with Argon, Green He/Ne, Red Diode, and Blue Diode lasers (Biorad, Hercules, CA). Post-acquisition analysis was conducted using MetaMorph 6.0 (Universal Imaging, Downingtown, PA). Total intensity for synaptophysin, MAP2 and GFAP were determined by the measurement of integrated pixel intensity for synaptophysin, MAP2 or GFAP per z-stack image, where the integrated pixel intensity is defined as total pixel intensity per image times the area of pixels positive for synaptophysin, MAP2 or GFAP. Averages are expressed as mean ± SEM. All data was analyzed by Prism 5.0 (GraphPad Software, San Diego, CA).

**MAP2 Cell-Based ELISA.** A MAP2 cell-based ELISA was performed to quantify neuronal damage/death as previously described (White et al., 2011). Briefly, primary rat cortical neuroglial cultures plated in 96-well plates were fixed for 30 min with 4% paraformaldehyde in 4% sucrose at the conclusion of treatments. After blocking for 1 hour with 5% normal goat serum in PBS, the plates were incubated with monoclonal MAP2 antibody overnight at 4°C, followed by washes with PBS with 0.1% Tween-20 (PBS-T). The plates were then incubated for 30 min with goat anti-mouse secondary antibody conjugated to beta-lactamase TEM-1 at room temperature, washed with PBS-T, and incubated in the dark at room temperature for 1 hour in fluorocillin green substrate. Fluorescence intensity was measured using a Fluoroskan Ascent fluorometer plate reader (Thermo Electron, Waltham, MA) with excitation at 485 nm and emission at 527 nm.

**Hand-Counting of MAP2-Positive Cells.** As a complementary method to MAP2 ELISA, neuronal survival was verified by hand-counting of MAP2-positive neurons. 15mM propidium iodide was added to primary cortical neuroglial cultures grown on coverslips 15 minutes before the end of the treatments. The
coverslips were washed once with PBS and fixed for 30 min with 4% paraformaldehyde in 4% sucrose, followed by blocking and permeabilization in 0.2% BSA + 0.1% Triton X-100 in PBS for 1 hour at room temperature (RT). The coverslips were then washed twice with PBS and incubated with anti-MAP2 antibody (1:100) in normal antibody diluent for 2 hours at RT. After two washes with PBS-T, the coverslips were incubated in a fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (1:200) for 30 min at RT. The coverslips were mounted on slides and alive/dead neurons were hand-counted based on MAP2 and PI-positive staining using a Nikon Eclipse E400 fluorescent microscope (Nikon Corp, Tokyo, Japan) equipped with Olympus DP70 digital camera (Olympus Corp, Tokyo, Japan). Live cells stain negative for PI, while dead cells retain it in their nuclei. The number of surviving MAP2-positive neurons will be positive for MAP2 and negative for PI staining. The percentage of MAP2-positive cells ± SEM were calculated from blinded counting of six fields at 200X in two adjacent vertical columns through the center of each coverslip, proceeding from top to bottom and bottom to top. For each condition, three coverslips were counted from two or more independent experiments.

**Quantification of Synaptophysin.** Cultures grown on coverslips, as described above, were immunofluorescently stained for synaptophysin (1:1000), MAP2 (1:100), and DAPI. Tyramide amplification was used for the detection of synaptophysin. For each treatment condition, images of 10 randomly selected areas from 3 coverslips from 3 independent experiments were captured by fluorescence microscopy. Post-acquisition analysis was performed using the NIH ImageJ program (V1.36b, Bethesda, MD). Briefly, synaptophysin-positive puncta were detected by background subtraction and manual thresholding. The number of synaptophysin-positive puncta was determined by dividing the total number of puncta within a dendrite segment by the length of the segment.

**Measurement of Reactive Oxygen Species.** The superoxide indicator dihydroethidium (DHE, Invitrogen) was used to detect the presence of reactive oxygen species in vitro. 3mM DHE was added to culture media 15 min prior to conclusion of treatments. Cells were then washed with PBS, fixed with 4% paraformaldehyde in 4% sucrose at RT for 8 min, and stained for DAPI. The coverslips were then mounted on slides with CytoSeal and visualized using fluorescent microscopy, as described above. Post-acquisition
analysis was performed using MetaMorph to determine the fluorescence intensity of DHE normalized to the area of DAPI signal.

**Immunoblotting.** Whole cell extracts of rat tissue samples were prepared by homogenization in ice-cold tissue extraction buffer (50mM Tris pH 7.5, 0.5M NaCl, 1% NP-40, 1% SDS, 2mM EDTA, 2mM EGTA, 5mM NaF, 0.4mM Na$_3$VO$_4$, 1mM dithiothreitol (DTT) and 1:100 protease inhibitor cocktail), followed by centrifugation at 12,000 g at 4°C for 20 min. Whole cell extracts of primary rat cortical cultures were prepared with ice-cold cell lysis buffer (50mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 10 mM EDTA, 0.4 mM Na$_3$VO$_4$, 100 mM DTT and 1:100 protease inhibitor cocktail), followed by centrifugation at 14,000 g at 4°C for 10 min. The protein concentrations of the collected supernatants were determined using the Bradford method and 25-50 µg of protein was loaded into each lane of a 4-12% Bis-Tris gradient gel for separation. A broad range molecular weight ladder was run on each gel. Subsequent to separation, proteins were transferred onto PVDF membranes, and blocked in tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA) for 1 hour at room temperature. The membranes were incubated with the primary antibodies in TBS-T with 5% BSA at 4°C overnight, washed with TBS-T, followed by incubation with corresponding HRP-conjugated secondary antibodies. The membranes were developed using SuperSignal West Dura extended duration substrate. Loading controls were obtained by staining the membranes and the gels with the Biosafe Coomassie Stain for 20 min, followed by destaining with deionized water for 30 min. For densitometric analysis, autographs were scanned into Adobe Photoshop (Adobe Systems, San Jose, CA) and regions of interests (ROI) of equal size were determined for each band. The pixel intensities of ROIs were quantified using the NIH ImageJ program (V1.36b, Bethesda, MD). Target band intensities were normalized to gel and membrane coomassie stain controls to account for protein degradation and loading discrepancies, and the normalized target band intensities were used to quantify fold changes over controls.

**Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).** The expression of HO-1 and NQO-1 genes in rat neuroglial cells was quantified by qRT-PCR. Custom TaqMan® Gene Expression Assays were purchased from Applied Biosystems for the genes: NQO-1 (Rn00566528_m1) and HO-1
Approximately 5ng cDNA was used per reaction. StepOne™ Software v2.0 was used to construct the experimental protocol and the qRT-PCR took place in the StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Data was normalized using both b-actin (Rn00667869_m1) and 18S (Hs99999901_s1) and was analyzed according to the ΔΔC_T method. All samples were run in triplicate from three biological replicates.

**Statistical analysis.** All data was analyzed by Prism 5.0 software (GraphPad Software, San Diego, CA). Values are expressed as mean ± SEM, and values of p < 0.05 were considered significant for all statistical analyses performed.

### 3.4 RESULTS

**Antiretroviral drugs lead to neuronal damage in vivo**

cART-induced peripheral neuropathy is well-documented (Power et al., 2009), and it is likely that cART triggers similar damage to neurons in the CNS. To determine the neurotoxic potential of cART in the CNS, we assessed the effects of an ARV regimen on the expression of synaptophysin and MAP2, indicators of synaptic damage and neuronal loss, respectively, utilizing post-mortem tissue from a well-characterized SIV/pigtail macaque model of HIV CNS disease, which was designed to address the efficacy of CNS penetrant antiretroviral therapy in reducing viral load in the CNS (Zink et al., 2010). In this study, animals infected with SIV either received no cART or received early cART treatment that included Tenofovir (NRTI), Atazanavir (PI), Saquinavir (PI), and L-870812a (integrase inhibitor) 12 days after the virus inoculation. Without cART, 90% of animals develop neurologic disease within 3 months post-inoculation (p.i.). In contrast, animals receiving cART do not develop SIV encephalitis. Rather, they show a rapid reduction in their plasma and CSF viral load followed by continued suppression of SIV replication with maintenance of CD4+ T cell counts until elective euthanasia around day 160 p.i. Additionally, cART-treated animals do not exhibit any outward signs of neurological deficits. Further, our quantitative immunofluorescent analysis of hippocampal tissue sections revealed reduced astrogliosis in the hippocampus of SIV-infected, cART-treated animals (SIV(+)/cART), compared with SIV-infected macaques that did not receive cART ((SIV(+)/placebo)) (Figure 3.1B). However, we observed statistically
significant decreases in synaptophysin expression in the hippocampi of the SIV(+)/cART group, compared with that in either the uninfected or the SIV(+)/placebo group (Figure 3.1A and 3.1D). In addition, examination of the expression of a second marker of synaptodendritic integrity, calmodulin kinase II (CaMKII), by immunoblotting showed that CaMKII levels were significantly lower in the frontal cortex in the SIV(+)/cART macaques than in their SIV(+)/placebo counterparts (Figure 3.1E and 3.1F). CaMKII is highly expressed in neurons of macaque hippocampus and frontal cortex, whereas its expression in other cell-types, including microglia, infiltrating macrophages, and multinucleated giant cells, is minimal; thus, the differences in CaMKII expression in frontal cortex of the animals from our experimental groups are neuron-specific (Gupta et al., 2010). Our results demonstrate synaptic injury in the presence of cART despite effective control of SIV replication in the periphery and CNS. Interestingly, we did not observe changes in MAP2 fluorescence in the hippocampus of infected and/or cART-treated animals compared with untreated/uninfected animals (Figure 3.1A and 3.1C).

As studies of uninfected, cART-treated macaques have not been performed to determine the contribution of cART to neuronal damage independent of SIV infection, we administered combinations of ARVs intravenously to adult rats. In patients, initial cART usually includes two nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs) in combination with a non-nucleoside reverse-transcriptase inhibitor (nNRTI) or with a protease inhibitor (PI) boosted with a low dose of a second PI, Ritonavir (Rit). Thus, we used Zidovudine (AZT), an NRTI, along with two PIs, Saquinavir (Saq) and Rit, at doses based on previously published pharmacokinetic studies of these ARVs (Busidan and Dow-Edwards, 1999, Kageyama et al., 2005, Pistell et al., 2010). In agreement with previous studies (Waring et al., 2010), the animals showed no overt signs of distress during the course of the treatment. However, via immunoblotting, we observed decreases in hippocampal synaptophysin expression in cART-treated rats compared with vehicle-treated rats (Figure 3.1G), complementing our findings of synaptic damage in SIV(+)/cART macaques and further supporting a role for ARV-associated neuronal injury in the CNS. Of note, we also detected decreases in MAP2 levels in the cART-treated rat hippocampus (Figure 3.1G), which likely reflects the acute drug toxicity in this treatment paradigm, compared to the subtler synaptic injury observed in the cART-treated macaque brain.
Antiretroviral compounds in therapeutically relevant combinations are neurotoxic \textit{in vitro}

The only primary neural cell-type previously studied for ARV toxicity was dorsal root ganglia neurons in models of peripheral neuropathy (Werth et al., 1994). Here, to expand our studies on ARV neurotoxicity in the CNS, we used primary rat cortical neuroglial cultures aged 21 days \textit{in vitro} (DIV) (O'Donnell et al., 2006, Wang et al., 2007, White et al., 2011). We first evaluated the neuronal viability in response to increasing concentrations of AZT, Rit, or Saq. We based the range of doses on reported plasma and CSF levels of ARVs (Wynn et al., 2002, Anthonypillai et al., 2004). Importantly, animal studies predict brain parenchymal levels to be equal to or greater than CSF levels (Anthonypillai et al., 2004, Anderson and Rower, 2010). At 48 hours post-treatment with individual ARVs, Rit and Saq both led to dose-dependent decreases in MAP2-positive cells, as determined via hand-counting (Figure 3.2A and 3.2B, respectively). These results were confirmed with a cell-based MAP2 ELISA, which accurately reflects neuronal numbers, as well as neuronal damage (Figure 3.2D and 3.2E) (White et al., 2011). Furthermore, neuronal damage and death induced by Rit and Saq was time-dependent, as seen in Figure 2G, where the primary cortical cultures were exposed to individual ARVs for up to eight days. Additionally, both Rit and Saq led to dose-dependent decreases in synaptophysin expression after 16 hours of treatment, well before the loss of MAP2-positive cells occurred (Figure 3.2H and 3.2I). However, AZT did not lead to decreases in MAP2-positive cells, MAP2 fluorescence, or synaptophysin expression (Figure 3.2C, 3.2F, and data not shown, respectively). Next, we treated primary cortical cultures with drug combinations that included AZT, Rit, and Saq, alone or in combinations, for 48 hours, to assess acute neurotoxicity. As seen in Figure 3.2J and 3.2K, combination treatments that included Rit induced statistically significant neuronal damage/death, which were preceded by loss of synaptophysin detected at 16 hours post-treatment (Figure 3.2L). Additionally, we observed similar levels of neuronal damage in cortical cultures treated with a combination of d4T, Rit, and Saq (not shown). However, as d4T is no longer prescribed in most developed countries and the AZT/Rit/Saq combination reflects the first considered/prescribed combination in initial treatment plans, we focused on AZT/Rit/Saq combination treatments for further experiments. Next, we determined that treatment with either Rit or Saq, alone or in combination with AZT, lead to the activation of the Ca$^{2+}$-activated death protease, calpain, as evidenced by the increase of calpain-cleaved spectrin observed in these cultures (Figure 3.3). Interestingly, none of the treatment combinations caused increases in the cleaved, and
therefore active, form of caspase-3 (Figure 3.3), suggesting that neuronal death observed in our model may be a necrotic cell death rather than an apoptotic cell death. Together the findings presented here suggest that combination ARV treatments that include PIs are toxic to cortical neurons in vitro.

**Combination antiretroviral drug treatments induce oxidative stress in neurons**

An extensive number of studies have linked ARVs, especially PIs, to oxidative stress (Touzet and Philips, 2010). To determine whether neurons undergo oxidative stress when exposed to ARVs, we used dihydroethidium (DHE) as a marker for the presence of reactive oxygen species (ROS) (Rodriguez-Pallares et al., 2009). We included tert-Butyl hydroperoxide (tBHP), an organic pro-oxidant, at 100 mM, as a positive control. As seen in Figure 3.4A and quantified in Figure 3.4D, Rit/Saq and AZT/Rit/Saq combination drug treatments for 6 hours lead to ROS accumulation in cortical cultures, whereas ROS levels in vehicle-treated cultures were comparable to those in untreated cultures.

The cortical cultures generated for our studies contain 85-90% neurons and 10-15% astrocytes (Figure 3.4B); thus, we also determined the presence of ROS in pure neuronal and pure astrocytic cultures exposed to individual ARVs. As seen in Figure 3.4C and quantified in Figure 3.4E, Rit led to an early and sustained ROS production in pure neuronal cultures, while Saq-induced ROS accumulation occurred 24 hours after treatment. The transient ROS accumulation induced by AZT was resolved at 24 hours post-treatment. On the other hand, in pure astrocytic cultures, none of the three ARVs examined induced an appreciable sustained ROS generation, and only Rit caused a transitory ROS accumulation (Figure 3.5A and 3.5B). These data further suggest that the ARV-induced ROS production observed in primary cortical neuroglial cultures are occurring in neuronal cell populations.

**Endogenous antioxidant response activation by combination antiretroviral drugs**

Oxidative stress in cells triggers the transcriptional induction of oxidative stress responsive genes through the activation of the endogenous antioxidant response element (ARE) in their promoter regions (Chen and Kong, 2004). Thus, we determined the effect of ARVs on two such genes, NAD(P)H:quinone oxidoreductase-1 (NQO-1) and heme oxygenase-1 (HO-1). By quantitative reverse transcription
polymerase chain reaction (qRT-PCR), we observed increased mRNA levels of both NQO-1 and HO-1 in cultures treated with any of the tested combinations of ARVs, when compared with the levels in untreated cultures (Figure 3.6A and 3.6B, respectively), with the most striking increases observed in Rit/Saq-treated cultures. To determine whether the changes in the mRNA levels were reflected in protein levels, we used immunoblotting to examine protein levels of NQO-1 and HO-1. While we observed increases in NQO-1 and HO-1 protein levels at 16h (Figure 3.6C), the changes were more robust in cultures treated for 48 hours (Figure 3.6D). Further, in vivo, we detected increased levels of HO-1 protein in hippocampal lysates from cART-treated rats (Figure 3.6E and 3.6F). Interestingly, NQO-1 levels were not increased in cART-treated rat hippocampus (Figure 3.6E). Of note, we did not observe an antioxidant response in ARV-treated pure astrocytic cultures (Figure 3.5C, D and E). These data collectively suggest an activation of HO-1, as part of the endogenous antioxidant response, following ARV treatments in neurons.

Monomethyl fumarate (MMF) protects against ROS generation and induces activation of the endogenous antioxidant response

NQO-1 and HO-1 are targets of the fumaric acid ester, dimethyl fumarate (DMF), and of its hydrolyzed and active metabolite, monomethyl fumarate (MMF) (Linker et al., 2011). Our recent study (Cross et al., 2011) has shown that DMF and MMF reduce neurotoxin release from HIV-infected macrophages through induction of HO-1. We reasoned that augmented or earlier induction of NQO-1 and HO-1 in neurons by pre-treatment with the active metabolite, MMF, would provide protection in our in vitro model of ARV-induced neurotoxicity through an antioxidant effect. We first determined that MMF at 30-100 mM was not toxic in neuronal cultures, as determined by MAP2 ELISA (data not shown). Next, we pre-incubated primary cortical cultures with 100 mM MMF for 30 minutes before adding either Rit/Saq or AZT/Rit/Saq. Pre-incubation with MMF blocked ROS generation induced by ARV treatments lasting 6 hours (Figure 3.7A), 16 hours, and 48 hours (data not shown). Quantification showed ROS levels in cultures pre-incubated with MMF to be comparable to those detected in untreated and vehicle-treated cultures (Figure 3.7B). Further, we observed an early increase in HO-1 protein levels at 4 hours in cultures pre-incubated with MMF, whether or not pre-treatment was followed by ARV treatment (Figure 3.7C). This trend was sustained at 16 hours after combination ARV treatment, as well. Next, we determined whether augmenting
the endogenous antioxidant response by MMF blocks MAP2 loss induced by combination ARV treatments. As detected by the cell-based MAP2 ELISA, MMF blocked neuronal damage/death in cultures treated with Rit/Saq and AZT/Rit/Saq for 72 hours (Figure 3.7D). Finally, pre-treatment of the cultures with an HO-1 inhibitor, Sn(IV) mesophorphyrin IX dichloride (SnMP) (Zhao et al., 2004), reversed the MMF-induced decreases in ROS accumulation (Figure 3.7E), suggesting that MMF protection occurs through its augmentation of HO-1 expression.

3.5 DISCUSSION

Virus-related factors, such as resistant virus species and persistent viral DNA in the CNS, may contribute to the persistence of HAND in the post-cART era. One recent focus in HIV neurovirology is the development of ARVs with greater CNS penetrance (Letendre et al., 2008, Marra et al., 2009, Tozzi et al., 2009, Edén et al., 2010, Heaton et al., 2010, Garvey et al., 2011, Smurzynski et al., 2011). However, potential ARV toxicity in the CNS remains largely unexplored. Our study supports a possible contribution of the ARVs themselves to neuronal and synaptic damage observed in patients with HAND.

In this study, we show cART-induced synaptophysin loss, indicative of synaptic injury in two animal models. In our first model, in an in vivo model of SIV-infected pig-tailed macaques, we report decreased synaptophysin and CaMKII levels in the SIV(+)/cART group compared with uninfected or SIV(+)/placebo groups, indicating synaptodendritic damage. Interestingly, MAP2 levels did not change significantly across groups, which may be due to the relatively short duration of infection in this retrospective study cohort. While these data demonstrate potential effects of cART drugs in the presence of viral infection, there are three variables to consider in interpretation of these findings: 1) the time to euthanasia from the start of the experiments is different between SIV(+)/placebo and SIV(+)/cART groups, 2) persistent viral DNA in the CNS of SIV(+)/cART group, and 3) the lack of SIV(-)/cART group. It is not yet known whether brain SIV DNA is replication competent. As we utilized post-mortem samples obtained from a cohort of macaques enrolled in a previous study addressing the efficacy of CNS penetrant cART in reducing viral loads in the CNS, further experiments that include an additional control group receiving cART but not inoculation with
SIV will be instrumental to more clearly determine the contribution of viral DNA and cART to synaptic damage in this model.

In the second *in vivo* model presented here, adult rats received a therapeutically relevant combination of ARVs (NRTI+PI+Rit boost). In the small number of studies where pharmacokinetics and effects of ARVs in the CNS were examined, pathological read-outs of neuronal damage, such as MAP2 or synaptophysin loss, were not determined (Huisman et al., 2003, Anthonypillai et al., 2004, Anthonypillai et al., 2006).

Synaptic injury is a known indicator of neuronal damage and dysfunction in various neurodegenerative diseases, including HAND (Gupta et al., 2010) and synaptodendritic injury persists in HIV-infected individuals in the post-cART era (Xu and Ikezu, 2009). We observed decreases in synaptophysin and MAP2 protein levels in the hippocampus in response to ARV administration over 7 days. Thus, our model demonstrates that ARV-associated neurotoxicity warrants consideration in developing therapeutic regimens for HIV-infected patients.

We also show that the PIs, Rit and Saq, alone or in combinations with the NRTI, AZT, induce oxidative stress and neuronal damage/death in primary cultures at clinically relevant doses. Previous studies examined ARV-induced toxicity in cell lines, and Robertson et al. have provided the first evidence for ARV-induced neurotoxicity in primary rat neurons (Robertson et al., 2012). Here, we provide further evidence that PI-induced oxidative stress and neuronal death in primary neurons can be blocked by the activation of the endogenous antioxidant response. Interestingly, in our experimental paradigm, the NRTI, AZT, neither induced neuronal damage/death by itself, nor augmented PI-induced damage/death when used in combination. We observed similar effects from a combination using another NRTI, stavudine (d4T) (not shown). In agreement with our observations, a previous study presented similar findings, specifically that neither AZT nor d4T inhibited cell growth or neurite regeneration in PC-12 cells after long-term drug exposure (Cui et al., 1997). It should be noted that NRTIs are unequivocally tied to peripheral neuropathy, where the underlying pathology is mitochondrial toxicity and oxidative stress. However, NRTIs affect only certain cell populations, as they are formulated as pro-drugs and, to become active, need to be phosphorylated by two kinases, thymidine kinase 1 and 2 (TK1, TK2), and the expression of the
cytoplasmic TK1 is cell cycle-dependent (Bazzoli et al., 2010). Thus, in our model utilized to study post-mitotic neurons, AZT is most likely not converted to its active form, and thus does not contribute to neuronal damage/death. It is of note that our in vitro model of ARV-induced neurotoxicity utilized primary neuroglial cultures rather than cell lines. Primary cells are untransformed, and therefore more accurately reflect and predict ARV-associated effects occurring in the brains of patients on cART than would immortalized cell lines. The molecular pathways we investigated in this study are highly conserved from yeast to human cells; thus, the results obtained here in cells of rodent origin are likely conserved in human cells as well.

The drug concentrations used in this study are based on the plasma and cerebrospinal fluid (CSF) levels reported by various in vitro and in vivo studies (Huisman et al., 2003). As reported in such studies, AZT can be detected in the CSF at concentrations that are similar to those measured in the plasma (Wynn et al., 2002). Contrarily, as backed by various in vitro and in vivo studies (Wynn et al., 2002), both Rit and Saq are predicted to have limited CNS penetrance due to the strong tendency of these drugs to bind plasma protein because of their lipophilic nature and pharmacokinetic properties. However, a comprehensive study conducted in an in situ guinea pig model suggests that Rit can achieve high concentrations in choroid plexus and parenchyma through diffusion via the choroid plexus; in fact, the levels of Rit were comparable to levels measured in plasma (Anthonypillai et al., 2004). Further, the study showed that, surprisingly, the CSF levels of Rit were lower than the levels measured in the choroid plexus and the parenchymal compartments. Thus, the authors concluded that CSF concentrations of Rit may not necessarily reflect the parenchymal levels, which are indeed a better indicator of effective drug levels in the CNS. Rit and Saq concentrations in our experiments fall well within plasma ranges reported in patients receiving cART.

Evidence of oxidative stress has long been associated with the most severe forms of HAND (Ngondi et al., 2006, Mielke et al., 2010). Interestingly, despite systemic control of viral replication in cART-treated patients, markers of oxidative stress, such as elevated levels of lipid and protein oxidation, are still detectable in the brains of these individuals (Ances et al., 2008). Of note, oxidative stress is one of the underlying mechanisms involved in NRTI and PI-induced toxicity in the periphery. Our data suggest that
the sustained ROS accumulation in neurons due to prolonged exposure to ARVs might induce the oxidative stress associated with cART-induced toxicity in the CNS, leading to the observed changes in synaptophysin levels and the subsequent neuronal death.

Our data also suggests that astrocytes do not show either ROS accumulation or the endogenous antioxidant response in vitro. We also observe that astrogliosis in SIV-infected animals is resolved in cART-treated animals. While these findings suggest that astrocytes may not be highly impacted by ARVs in the short term, it is possible that prolonged exposure to ARVs might overwhelm astrocytes, which help buffer ROS accumulation in neurons under normal conditions, precipitating further neuronal damage.

We further show that the ARV drug-induced effects observed in this study can be blocked by MMF via activation of an endogenous antioxidant response. Cells respond to oxidative stress by activating the transcription of a subset of genes in an effort to clear excess ROS within the cell. Among these genes are NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) (Chen and Kong, 2004). Here, we show increased levels of NQO-1 and HO-1 mRNA and protein in ARV-treated neuronal cultures. Additionally, pre-treatment of cultures with MMF led to further increases in HO-1 protein levels in ARV-treated cultures and provided protection against ARV-induced toxicity. Finally, our finding that the protection provided by MMF is reversed with chemical inhibition of HO-1 further provides evidence that ARV toxicity is mediated via induction of oxidative stress. The neuronal damage and death that occurs despite the cellular initiation of the endogenous antioxidant response following exposure to ARVs suggests that this response may be insufficient or too delayed to protect cultures from ARV toxicity. In support of this explanation, we observed that MMF-mediated augmentation of antioxidant responses is strongly protective against the neurotoxic effects of ARVs. Further studies using this in vitro model are warranted to determine whether other clinically relevant ARV drug combinations induce neurotoxicity and, if so, to establish whether similar pathways are involved.

DMF, a psoriasis treatment used in Europe since 1994, is currently being tested as a disease-modifying agent for Multiple Sclerosis (MS) ((2011), Krieger, 2011, Linker et al., 2011). MMF is the active DMF
metabolite in vivo. Our report is the first to show MMF as a neuroprotectant against ARV-induced damage. Data from studies in MS patients show that DMF/MMF has good tolerability, can cross the blood-brain barrier efficiently, and has relatively few and minor side-effects. Further, we have recently shown that DMF suppresses HIV replication, induces the antioxidant response in macrophages, and blocks neurotoxin release from macrophages (Cross et al., 2011). Overall, our findings make this immunomodulatory and antioxidant agent a good potential adjunctive therapeutic for use in HAND.

One of the paradoxical outcomes of cART is the persistence of HAND, despite successful viral control. Moreover, there is a shift from overt dementia to more subtle neurocognitive impairments. Based on data presented here, we propose that different mechanisms by which HIV infection and cART induce neuronal damage underlie the changing neuropathology of HAND. Extensive studies have shown that during lentiviral infection, viral proteins and soluble factors secreted by infected cells lead to neuronal and synaptic damage via several direct as well as indirect mechanisms involving several cell types, including astrocytes. However, our data suggests that cART-mediated synaptic damage may involve direct mechanisms occurring specifically in neurons, such as oxidative stress, and that neurons, and not astrocytes, are the primary targets of cART-mediated damage in the CNS.

It is most likely that chronic exposure to cART regimens including neurotoxic ARVs over many years is associated with a slower, nonetheless insidious changes including synaptic damage in the absence of neuronal loss, and these neuronal perturbations may contribute to, and may even precipitate, some of the clinical and pathological changes observed in the chronic course of HAND in the cART era. While detrimental, such slow damage also suggests that alterations in cART regimens to include ARVs with low neurotoxicity profiles, such as NRTIs or nNRTIs may halt synaptic damage, and provide a point where previous damage may be reversed, either due to the withdrawal of the toxic drug, or with the help of an adjuvant, such as fumaric acid esters. Future studies, first in primates, then in humans, will be crucial to explore the specific impact of treatment interruption on recovery from cART-mediated neuronal damage and to determine the efficacy of potential adjunctive therapies necessary to mitigate the side effects of cART in the CNS.
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Figure 3.1 Antiretroviral drugs induce neuronal damage in vivo

(A, B, C and D) Formalin-fixed, paraffin-embedded tissue sections from hippocampus of pig-tailed macaques that were either uninfected (n=6), SIV-infected but not cART-treated (n=7), or SIV-infected and treated with cART (Tenofovir, Atazanavir, Saquinavir, and L-870812a) (n=4) were prepared for immunofluorescent analysis and were triple-labeled for MAP2 (red), synaptophysin (green) and GFAP. Sections were visualized by laser confocal microscopy and images were quantified for MAP2, synaptophysin and GFAP expression. (A) Representative composite images of two cases per group which were stained with MAP2 and synaptophysin are shown. Scale bar = 30µm. (B) Quantification shows the resolution of GFAP immunoreactivity in SIV(+)/cART group, compared with SIV(+)/placebo group (One-Way ANOVA, *p<0.05). No changes were observed in MAP2 expression between groups (C), but there were statistically significant decreases in synaptophysin immunoreactivity (D) in SIV(+)/cART group, as compared with SIV(+)/untreated and uninfected groups (One-Way ANOVA, *p<0.05, ns: not significant).

(E and F) Fresh-frozen tissue sections from the frontal cortex of pig-tailed macaques that were either uninfected (n=3), SIV-infected but not cART-treated (n=6), or SIV-infected and cART-treated (n=6) were used for standard protein extraction and subsequent immunoblotting for the expression of CaMKII. Actin was used as a loading control. A representative immunoblot is shown. Quantification shows statistically significant decreases in CaMKII in the cART-treated group, as compared with the uninfected group or the SIV(+)/untreated group (One-Way ANOVA, *p<0.05) (G) Whole-cell lysates prepared from hippocampus of rats treated for 7 days with AZT/Rit/Saq (n=4) or vehicle (n=2) were immunoblotted for synaptophysin and MAP2. A band from the coomassie blue staining is included to control for equal loading and protein degradation.
Figure 3.2 Therapeutically relevant combination antiretroviral drug treatments are neurotoxic in vitro

(A, B, and C) Primary rat cortical neuroglial cultures aged 21 days in vitro (DIV) on coverslips were exposed to Rit (A), Saq (B), or AZT (C) at increasing doses for 48 hours, followed by hand-counting for MAP2-positive cells. (n=3, vehicle: 0.04% DMSO, *p<0.05, One-Way ANOVA with post-hoc Newman-Keuls). (D, E and F) 21DIV primary neuroglial cultures grown in 96-well plates were treated with increasing doses of Rit (D), Saq (E), or AZT (F) for 48 hours, followed by MAP2 cell-based ELISA (n=2, vehicle: 0.04% DMSO, *p<0.05, One-Way ANOVA with post-hoc Newman-Keuls). (G) Primary neuroglial cultures were treated with AZT (25 mM), Rit (10 mM), or Saq (1 mM) at day zero. 90% of the media was changed with conditioned media supplemented with a fresh drug stock every two days and cultures were analyzed by MAP2 cell-based ELISA at days 4 and 8 (n=2, vehicle: 0.04% DMSO, *p<0.05, One-Way ANOVA with post-hoc Newman-Keuls). (H and I) Cultures grown on coverslips were exposed to the indicated treatments and synaptophysin-positive puncta were determined (n=3, vehicle: 0.04% DMSO, *p<0.05, One-Way ANOVA with post-hoc Newman-Keuls). (J and K) Primary neuroglial cultures were exposed to the indicated drug combinations (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) for 48 hours, followed by hand-counting for MAP2-positive cells (J) or MAP2 cell-based ELISA (K) (n=2, vehicle: 0.04% DMSO, *p<0.05, # p<0.01, One-Way ANOVA with post-hoc Newman-Keuls). (L) Primary neuroglial cultures that were exposed to the indicated drug combinations (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) for 16 hours were analyzed for synaptophysin-positive puncta (n=2, vehicle: 0.04% DMSO, *p<0.05, One-Way ANOVA with post-hoc Newman-Keuls).
Whole cell lysates were prepared from neuroglial cultures treated with the indicated single or combination drugs (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM), or with Thapsigargin (1mM) as a positive control, for 48 hours. Calpain activation was assessed using an antibody to detect the accumulation of calpain-cleaved spectrin and an antibody raised against the cleaved and active form of caspase-3 was used for detection of caspase activity. A band revealed by coomassie staining of the gel was used as a loading control (n=2, vehicle: 0.04% DMSO).
**Figure 3.4 Combination antiretroviral drug treatments induce oxidative stress in neurons.**

(A and D) Cortical neuroglial cultures grown on coverslips were treated for 6 hours with the indicated drugs (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) and the presence of reactive oxygen species was detected by DHE staining (red fluorescence).

(A) The images were captured with epifluorescent microscopy with uniform settings. (D) Quantification of DHE fluorescence was generated by measurement of DHE pixel intensity per DAPI area (n=3; *p<0.05, #p<0.01, One-Way ANOVA, post-hoc Newman-Keuls).

(B) Cortical neuroglial cultures grown on coverslips for 21 days and exposed to the indicated treatments (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) were immunofluorescently labeled for MAP2 (green) and GFAP (red). Note that the cultures are enriched for neurons.

(C) 21DIV pure cortical neuronal cultures grown on coverslips were treated with AZT (25 mM), Rit (10 mM), or Saq (1 mM) for 2, 12 or 24 hours. The presence of reactive oxygen species was detected by DHE staining (red fluorescence) and MAP2 was used to stain neurons (green fluorescence). (E) The images captured with confocal microscopy with uniform settings were analyzed for DHE pixel intensity per DAPI area (n=3; *p<0.05, One-Way ANOVA, post-hoc Newman-Keuls).
Figure 3.5 Antiretroviral drugs do not induce an endogenous antioxidant response in astrocytes.

(A) Pure astrocytic cultures grown on coverslips were treated with AZT (25 mM), Rit (10 mM), or Saq (1 mM) for 2, 12 or 24 hours. The accumulation of reactive oxygen species was detected by DHE staining (red fluorescence) and GFAP was used to label astrocytes (green fluorescence). (B) The images captured with confocal microscopy with uniform settings were analyzed for DHE pixel intensity per DAPI area (n=3; *p < 0.05, One-Way ANOVA, post-hoc Newman-Keuls). (C, D and E) Whole-cell lysates from pure astrocytic cultures treated with the indicated drugs (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) for 16h were immunoblotted for HO-1 and NQO-1. Representative blots from 3 independent experiments are shown in (C). Coomassie staining of gels were used as loading controls and fold changes over untreated lysates were determined. The quantification of HO-1 and NQO-1 band intensities from three independent experiments is shown in (D) and (E) (n=3; *p < 0.05, One-Way ANOVA, post-hoc Newman-Keuls).
Figure 3.6 Combination antiretroviral drug treatments induce the endogenous antioxidant response

(A and B) Cortical neuroglial cultures were exposed to the indicated treatments (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) for 6 hours to determine changes in NQO-1 (A) and HO-1 (B) mRNA levels. A representative of three experiments is shown. Actin was used as internal control and fold changes were determined by the ΔΔC₉₀ method (*p<0.0001, One-Way ANOVA, post-hoc Newman-Keuls). (C and D) Whole-cell lysates from cortical neuroglial cultures treated with the indicated drug combinations (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) for 16 and 48h were immunoblotted for NQO-1 (C) and HO-1 (D). Representative blots from 3 independent experiments are shown. Coomassie staining of gels were used as loading controls and fold changes over untreated lysates are indicated below each band of interest. (E and F) Whole-cell lysates prepared from hippocampus of rats treated for 7 days with AZT/Rit/Saq (n=4) or vehicle (n=2) were immunoblotted for HO-1 and NQO-1 (E) and fold change in HO-1 protein levels in the cART group compared with the vehicle group is shown (F) (*p<0.05, Student’s t-test).
**Figure 3.7** MMF induces activation of a cellular antioxidant response and blocks neuronal damage/death

(A) In the absence (upper panel) or presence (lower panel) of MMF (100 mM), 21 DIV rat neuroglial cultures on coverslips were treated for 6 hours with the Rit/Saq, AZT/Rit/Saq (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM), or were left untreated. ROS generation was detected by DHE staining (red fluorescence). (B) Quantification of nuclear DHE was done as described above (n=3, *p<0.01, One-Way ANOVA with post-hoc Newman-Keuls). (C) Whole cell lysates of cultures exposed to Rit/Saq or AZT/Rit/Saq treatments (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) in the absence or presence of MMF (100 mM) for 4 or 16 hours were immunoblotted for HO-1. A representative blot from 3 independent experiments is shown. A coomassie band from the gel was used as loading control. Quantification of band intensities is shown under each corresponding lane. (D) Primary cortical neuroglial cultures were either pretreated with MMF (100 mM) for 30 min or received no pre-treatment; cultures were then treated with Rit/Saq or AZT/Rit/Saq (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) for 72 hours to assess neuronal damage/death by MAP2 ELISA (n=3; vehicle=0.04% DMSO; *p<0.05 vs. untreated, @p<0.05 vs. Rit/Saq, #p<0.05 vs. AZT/Rit/Saq, One-Way ANOVA with post-hoc Newman-Keuls). (E) Primary cortical neuroglial cultures were pre-incubated with SnMP (20 mM) and/or MMF (100 mM) for 30 min before the addition of the indicated antiretroviral drugs (AZT: 25 mM, Rit: 10 mM, Saq: 1 mM) and were assessed for nuclear DHE accumulation at 16 (n=3; *p<0.01 vs. untreated, **p<0.05 vs. untreated, @p<0.01 vs. Rit/Saq, #p<0.05 vs. AZT/Rit/Saq, One-Way ANOVA with post-hoc Newman-Keuls).
3.7 REFERENCES


CHAPTER 4

Altered Oligodendrocyte Maturation and Myelin Maintenance: The Role of Antiretrovirals in HIV-Associated Neurocognitive Disorders

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4.1 ABSTRACT

Despite effective viral suppression through combined antiretroviral therapy (cART), approximately half of HIV(+) individuals suffer from HIV-Associated Neurocognitive Disorders (HAND). Studies of antiretroviral treated patients have revealed persistent white matter pathologies including diffuse myelin pallor, diminished white matter tracts, and decreased myelin protein mRNAs. Loss of myelin can contribute to neurocognitive dysfunction, as the myelin membrane generated by oligodendrocytes is essential for rapid signal transduction and axonal maintenance. We hypothesized that myelin changes in HAND are partly due to effects of antiretrovirals on oligodendrocyte survival and/or maturation. We showed that primary mouse oligodendrocyte precursor cell cultures treated with therapeutic concentrations of HIV protease inhibitors Ritonavir or Lopinavir displayed dose-dependent decreases in oligodendrocyte maturation. However, this effect was rapidly reversed following drug removal. Conversely, nucleoside reverse transcriptase inhibitor Zidovudine had no effect. Furthermore, in vivo Ritonavir administration to adult mice reduced frontal cortex myelin protein levels. Finally, prefrontal cortex tissue from HIV(+) individuals with HAND on cART showed a significant decrease in myelin basic protein compared with untreated HIV(+) individuals with HAND or HIV-negative controls. These findings demonstrate that antiretrovirals can impact myelin integrity, and have implications for myelination in juvenile HIV patients, and myelin maintenance in adults on lifelong therapy.
4.2 INTRODUCTION

Approximately 50% of patients infected with human immunodeficiency virus-1 (HIV) present with a broad spectrum of cognitive, motor, and behavioral disturbances collectively termed HIV-Associated Neurocognitive Disorders (HAND) (Kaul, 2005, Ozdener, 2005) despite effective viral control through combined antiretroviral therapy (cART) (Heaton, 2010, McArthur et al., 2010, Heaton, 2011, Sacktor and Robertson, 2014). cART is designed to simultaneously target multiple stages of HIV replication, thereby delaying viral mutational selection to acquire drug resistance. Most commonly prescribed regimens include viral reverse transcriptase inhibitors (nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs)), and HIV protease inhibitors (PIs) which prevent maturation of viral precursor proteins (WHO, 2013). While cART successfully reduces peripheral viral loads to undetectable levels in adherent individuals, inflammation and latent viral reservoirs remain in the central nervous system (CNS) (Chun, 1997, Finzi, 1997, Wong, 1997, Anthony, 2005, Persidsky, 2006).

Although pathology has shifted from subcortical to cortical manifestations since cART introduction, dendritic pruning and astrogliosis persist (Gray, 2003, Brew, 2004, Heaton, 2011, Sacktor and Robertson, 2014). Notably, white matter pathologies are prevalent, with the degree of neurocognitive impairment correlating with the amount of damage (Muller-Oehring et al., 2010, Tate et al., 2010). Imaging studies have revealed cortical myelin disruption, volume loss, and diminished structural integrity of the corpus callosum in HAND patients, which were even more pronounced in cART-treated individuals (Ragin et al., 2004, Tate et al., 2011, Kelly et al., 2014). Furthermore, transcriptome analysis in HAND patients on cART revealed dysregulation of genes critical to oligodendrocyte maturation and myelination, including myelin-associated oligodendrocyte basic protein, myelin transcription factor 1, and myelin basic protein (Borjabad et al., 2011).

In pediatric HIV patients, neurological complications such as encephalopathy and progressive multifocal leukoencephalopathy are frequent, with data suggesting emerging neurocognitive deficits and developmental delays (Wilmshurst et al., 2006, Crowell et al., 2014, Wilmshurst et al., 2014). WHO guidelines urge that all infected children be treated, with those under 3 years of age receiving a
Lopinavir/Ritonavir first-line regimen (WHO, 2013). Importantly, this PI-based therapy is advised during the critical period for myelination (Miller et al., 2012), yet an understanding of the effects of HIV and antiretroviral drugs on myelin formation and maintenance is lacking.

The myelin membrane is critical for axons to transmit rapid neuronal impulses as well as axonal maintenance (Lappe-Siefke et al., 2003, Edgar and Garbern, 2004). Conversely, impaired myelination can disrupt proper signaling, and hasten axonal degeneration, resulting in neurological dysfunctions (Criste et al., 2014, Mighdoll et al., 2015). Although sustained effects of continuing viral replication and treatment noncompliance are suggested to underlie continuing neurocognitive impairment, antiretrovirals themselves may contribute to HAND pathogenesis (Marra, 2009, Joska et al., 2010, Kahouadj et al., 2013). Crucially, while antiretroviral-mediated toxicity has been reported in primary rat cortical neurons (Robertson et al., 2012, Akay et al., 2014), to date there are no published studies addressing the effects of antiretrovirals on oligodendrocytes, the myelin forming cells of the CNS. Here we examine whether antiretrovirals affect the survival and maturation of developing oligodendrocytes using a well-established primary mouse cortical cell culture model (See et al., 2004) and the effects of cART compounds on myelin maintenance in a mouse model and in patients with HAND. Our results support a role for a subset of cART agents in the white matter changes seen in HIV(+) individuals with HAND.

4.3 MATERIALS AND METHODS

Chemicals and Reagents
Abcam (Cambridge, United Kingdom): rabbit polyclonal anti-inositol-requiring enzyme 1 antibody (IRE1α, ab37073), rabbit polyclonal anti-inositol-requiring enzyme 1 antibody (IRE1α, phospho S724, ab48187), rabbit polyclonal anti-NAD(P)H:quinone oxidoreductase antibody (NQO1, ab34173). Affymetrix (Santa Clara, CA): terminal deoxynucleotidyl transferase, recombinant (rTdT). AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: antiretroviral reagents. BD Transduction Laboratories: mouse monoclonal anti-BiP/Grp78 antibody (610978). Cell Signaling Technology (Beverly, MA): rabbit polyclonal anti-eukaryotic initiation factor 2 alpha antibody (eIF2α, 9722S), rabbit polyclonal anti-phospho-eukaryotic initiation factor 2 alpha (Ser51) antibody (p-eIF2α, 9721S). Chemicon

Additional antibodies: anti-A2B5 mouse hybridoma supernatant (ATCC (Eisenbarth et al., 1979)), anti-galactocerebroside mouse hybridoma supernatant (GalC H8H9, (Ranscht et al., 1982)), anti-myelin oligodendrocyte glycoprotein (MOG, (Piddlesden et al., 1991)), anti-myelin basic protein rat hybridoma supernatant (MBP, gift of Virginia Lee, University of Pennsylvania), anti-proteolipid protein rat hybridoma (AA3, gift of Dr. Alex Gow, Wayne State University).
Primary Cortical Oligodendrocyte Cultures

All experiments were performed in accordance with the guidelines set forth by The Children's Hospital of Philadelphia and The University of Pennsylvania Institutional Animal Care and Use Committees (IACUC). Primary mouse oligodendrocyte precursor cell (OPC) cultures were isolated from postnatal day 1 CD1 pups obtained from Charles River Laboratories, with modifications from previously described protocols (See et al., 2004). Briefly, cortical cell suspensions isolated from mouse pups using standard protocols were plated on poly-D-lysine coated T-75 flasks in Neurobasal medium with B27 supplement at 37°C with 5% CO₂. After 24 hours, cultures were switched to growth medium consisting of Neurobasal medium with B27 which contained 10 ng/ml bFGF, 2 ng/ml PDGF-AA, and 1 ng/ml NT-3. Within 7 days, confluent cells were purified to 90-95% OPCs and 5-15% astrocytes using a gentle wash-down procedure (Feigenson et al., 2009), and sub-cultured onto poly-lysine-coated flasks, coverslips, or Petri dishes. For differentiation experiments, growth medium was replaced with differentiation medium, consisting of 50% DMEM, 50% Ham’s F12, Pen/Strep and 2 mM glutamine with 50 µg/ml transferrin, 5 µg/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 µg/ml insulin, 0.4 µg/ml T4, 0.3% glucose, and 10 ng/ml biotin (Feigenson et al., 2009).

Immunofluorescence

Cells on coverslips were processed for detection of specific antigens as described previously (Grinspan and Franceschini, 1995, See et al., 2004). Prior to fixation, live cells were labeled for cell surface marker detection using anti-A2B5 (mouse hybridoma supernatant, undiluted), and anti-GalC (mouse hybridoma supernatant, undiluted). For detection of internal antigens following acid alcohol fixation and permeabilization, antibodies used were anti-MBP (rat hybridoma supernatant, 1:2 dilution) or anti-PLP (rat hybridoma supernatant, 1:2 dilution). Secondary antibodies used at a 1:100 dilution were Rhodamine or Fluorescein conjugated. Vectashield with DAPI was used to mount coverslips, and to stain all nuclei. Antigen-positive and DAPI-positive cells were counted in 10 fields in each of 3 to 4 coverslips from at least 3 biologically separate preparations of cells using a Leica DM6000B fluorescence microscope at 40x magnification. A total of approximately 2000 cells were counted per condition. The chosen and accepted standard method for quantification of differentiation determines the number of cells progressing through
various stages using well-defined stage-specific markers (Scherer et al., 1994, Miller, 2002, Feigenson et al., 2009). For phalloidin staining, cell surface markers were stained as above. Next, cells were fixed with 4% paraformaldehyde for 8 minutes, washed with PBS, solubilized with 0.5% Triton X-100 in PBS, and blocked for 30 minutes with a 1:1 dilution of 100 mg/ml BSA and CAS-block. Cells were then incubated with TRITC-conjugated phalloidin at a final concentration of 500 nM for 2 hours. Cells were mounted and visualized as described above. To assess cells committed to apoptotic cell death, a TUNEL staining protocol adapted from that of Gavrieli et al. was utilized (Gavrieli et al., 1992). Cells were fixed with ice-cold acetone for 10 minutes, washed with PBS, and solubilized with 0.5% Triton X-100 in PBS for 15 minutes. Positive control coverslips were generated during this period by incubating in DN buffer (30 mM Trizma base pH 7.2, 140 mM Na cacodylate, 4 mM MgCl, and 0.1 mM DTT) for 2 minutes, followed by DNase (1:200) in DN buffer for 10 minutes. All coverslips were washed with PBS then placed in TDT buffer for 2 minutes (30 mM Trizma base pH 7.2, 140 mM Na cacodylate, 1 mM CoCl). Cells were incubated for 1 hour with TdT and biotin-UTP in TDT buffer (8 µL of each in 1mL TDT Buffer). Following a subsequent PBS wash, cells were placed in TB buffer for 15 minutes (300 mM NaCl, 30 mM sodium citrate), and then a 2% BSA solution for 30 minutes. Finally cells were incubated with Rhodamine-conjugated streptavidin for 20 minutes prior to a final PBS wash and mounting on slides with Vectashield containing DAPI. Visualization and counting were performed as described above.

Jugular Vein Antiretroviral Administration Mouse Model

Surgical procedures were performed with the approval of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Adult male C57BL/6 mice were catheterized via jugular vein, as described previously (Thrivikraman et al., 2002, Briand et al., 2012). Briefly, mice were anesthetized prior to surgery with 80 mg/kg ketamine and 12 mg/kg xylazine. An indwelling silastic catheter (Strategic Applications, Inc.) was placed into the right jugular vein and sutured in place. The catheter was connected to a mesh platform which was subcutaneously placed on the animals back and sutured in place. Catheters were flushed daily with 0.1 ml of the antibiotic Timentin dissolved in heparinized saline (0.93 mg/ml). Treatment regimens were composed of Vehicle (DMSO) or Ritonavir (20 mg/kg), administered twice daily for 14 days by continuous intravenous injection (Vehicle n= 6, Ritonavir n= 7). This drug dose was based
on previously published pharmacokinetic studies (Kageyama et al., 2005, Pistell et al., 2010, du Plooy et al., 2011). At time of euthanasia, catheter patency was verified by response to pre-euthanasia sedation (80 mg/kg ketamine and 12 mg/kg xylazine). Brains were removed, the frontal cortex, cerebellum, and hippocampus were dissected on ice, and tissue samples were stored at -80 °C until immunoblot analyses were performed.

Human Subjects

For immunoblotting analyses, samples from a cohort of individuals were obtained from the National NeuroAIDS Tissue Consortium (NNTC) and the Texas NeuroAIDS Research Center. The cohort encompasses 20 HIV(-), 20 HIV(+) cART-naïve, and 20 HIV(+) cART-medicated for greater than 12 months (HIV(+)/ART) cases. Six HIV(+) cART-naïve and 14 HIV(+) cART-medicated subjects had clinically diagnosed HAND. The pathological diagnosis of HIV encephalitis was established in six HIV(+)
cART-naïve and one HIV(+) cART-medicated patients. Diagnosis of HAND by neuropsychological assessment was based on the results from the NNTC neurocognitive test panel guided by Frascati Criteria, which entails evaluation of seven domains of cognitive functioning at six-month intervals prior to death (Antinori, 2007). NNTC site pathologists rendered nosological diagnoses of HIVE according to established criteria (Budka et al., 1991). Evaluation of regimen history determined that 85% of cART-medicated patients were HIV protease inhibitor (PI) experienced, and 100% were nucleoside reverse transcriptase inhibitor (NRTI) experienced. Table 4.1 contains complete cohort demographics and clinical data.

The human studies were conducted in accordance with human subject protection protocols, with written consent obtained at four collections sites within the USA. The following institutions maintained the IRBs set forth to provide oversight for the protection of human subjects: 1) The University of Texas Medical Branch Office of Research Subject Protections (Galveston, TX), 2) Mount Sinai Medical Center Program for the Protection of Human Subjects (New York, NY), 3) University of California (San Diego, CA), 4) University of California Los Angeles, Office of the Human Research Protection Program (Los Angeles, CA).
**Immunoblotting**

Whole cell extracts of primary mouse oligodendrocyte cultures were prepared with ice-cold cell lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 10 mM EDTA, 0.5 mM Na$_3$VO$_4$, and 1:100 protease inhibitor cocktail), followed by centrifugation at 14,000 rpm at 4°C for 30 minutes. Whole cell extracts of mouse and human tissue samples were prepared by homogenization of 100 mg dissected frontal cortex tissue in ice-cold tissue extraction buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 1% NP-40, 1% SDS, 2 mM EDTA, 2 mM EGTA, 5 mM NaF, 0.4 mM Na$_3$VO$_4$, 1 mM DTT and 1:100 protease inhibitor cocktail), followed by centrifugation at 14,000 rpm at 4°C for 30 minutes. Protein concentrations of collected supernatants were determined by the Bradford Method. 10-25 µg of protein was loaded into each lane of 4-12% Bis-Tris gradient gels for separation. For detection of PLP, gels were run under non-reducing conditions due to antibody specificity. A broad-spectrum molecular weight ladder was run on each gel. Following separation, proteins were transferred onto Millipore Immobilon-FL membranes, and blocked in PBS with 0.1% Tween-20 (PBST) and 5% milk for 20 minutes at 4°C. Membranes were incubated overnight at 4°C with primary antibodies in PBST + 5% milk. The following primary antibodies were used: MBP (SMI-99, 1:1000 dilution), PLP (1:1000 dilution), MAG (1:500 dilution), CNPase (1:1000 dilution), HO-1 (1:1000 dilution), NQO1 (1:1000 dilution), BiP (1:1000 dilution), phospho-eIF2α (1:1000), total eIF2α (1:1000), phospho-IRE1α (1:1000), total IRE1α (1:1000), and ATF6β (1:1000). Membranes were washed with PBST, re-blocked for 20 minutes in PBST+ 5% milk, and incubated with corresponding antigen-specific fluorescent probe-conjugated secondary antibodies (1:10,000 dilution) in PBST + 5% milk. Membranes were visualized using an Odyssey Infrared Imaging System (LiCOR). Loading controls were obtained by reblotting for GAPDH (1:1000 dilution), α-tubulin (1:1000 dilution), or FastGreen FCF. To obtain ratios of phosphorylated to total protein levels for eIF2α and IRE1α, first phosphorylated proteins were detected. Following visualization, membranes were stripped using Restore Western Blot Stripping Buffer and re-examined using the Odyssey to ensure all detectable first-round antibodies were removed. Membranes were then re-blocked and incubated with the corresponding primary antibodies for total eIF2α or IRE1α. Bands of interest were specified to determine pixel intensities for each treatment using the NIH ImageJ program (V 1.36b, Bethesda, MD), and the band intensities were normalized to loading controls to ensure equal loading. Proteins of interest were quantified by analyzing bands corresponding to the
appropriate molecular weight reported by the manufacturers and observed in our previous studies. In mouse and human brain tissue in which myelin proteins are found in high abundance, MBP and MAG present as doublets, which were evaluated simultaneously and quantified. FastGreen staining was used for samples from in vivo tissue, to ensure protein integrity as well as equal loading. To quantify FastGreen densitometry, a horizontal section of the membrane spanning a broad molecular weight range was scanned together, and overall intensity was determined for each lane. This method allows for normalization across lanes within gels without bias towards a single representative protein loading control. Due to large sample size in the human studies, a standard combined sample was run on each gel, and values were then normalized across gels.

**Measurement of Reactive Oxygen Species**

The superoxide indicator dihydroethidium (DHE) was used to detect the presence of reactive oxygen species (ROS) in vitro. Fifteen minutes prior to conclusion of treatments, 3 µM DHE was added to the culture medium. Oxidation of DHE to ethidium allows for ethidium intercalation with nuclear DNA, emitting a quantifiable red fluorescence (Zhao, 2003). Cells were washed with F12 medium, and fixed with 4% paraformaldehyde at room temperature for 8 minutes. The coverslips were then washed with F12 medium, mounted on slides with DAPI-containing Vectashield, and visualized using fluorescent microscopy, as described above. The oxidant tert-Butyl hydroperoxide (tBHP, 2.5 µM) was used as a positive indicator of ROS in culture. Post-acquisition analysis was performed using MetaMorph 6.0 software (Molecular Devices, Sunnyvale, CA) to determine the fluorescence intensity of nuclear intercalated DHE normalized to the area of DAPI signal.

**Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

The expression of HO-1, NQO1, spliced X-box Binding Protein-1 (XBP-1), total XBP-1, Binding Immunoglobulin Protein (BiP), C/EBP homologous protein (CHOP), IRE1α, ATF6β and activating transcription factor 4 (ATF4) mRNA in oligodendrocyte cultures were quantified by qRT-PCR. OPC cultures were grown on 100-mm dishes and harvested after treatment with specified antiretrovirals for 6 hours. RNA was extracted with Trizol, and 5 µg of RNA was converted to cDNA by the Invitrogen
Superscript First-strand kit. q-PCR was performed using Power SYBR Green, as previously described (French et al., 2009, Feigenson et al., 2011). Samples were measured in triplicate for each experiment from three biological replicates (n=3). Data were normalized using Protein Kinase Gene 1 (PKG1), and analyzed according to the ΔΔCT method. Primer pairs obtained from Integrated DNA Technologies for each gene can be found in Table 4.2.

**Statistical Analysis**

All data were analyzed by Prism 5.0 software (GraphPad Software, San Diego, CA). Values are expressed as mean ± SEM. All statistical tests compared Zidovudine (AZT) to untreated as it was dissolved in water, and Lopinavir and Ritonavir to the vehicle (DMSO) in which they were resuspended. Following differentiation, untreated and vehicle do not statistically differ in the number of cells staining with GalC, which marks immature oligodendrocytes, or MBP and PLP, which mark mature oligodendrocytes (data not shown). One-way ANOVA with post-hoc Newman-Keuls multiple comparison test, One-way ANOVA with post-hoc Dunnett’s multiple comparison test or Student’s t-test were performed where indicated. For comparison of specific treatment groups at two time-points, One-way ANOVA with post-hoc Bonferroni correction was performed. Values of $p < 0.05$ were considered significant for all analyses.

### 4.4 RESULTS

**HIV Protease Inhibitors Prevent Oligodendrocyte Differentiation**

The potential impacts of antiretroviral compounds on oligodendrocyte survival, maturation, or myelination have not been determined to date. We chose to test three widely prescribed antiretroviral drugs: Zidovudine (AZT), a nucleoside/nucleotide reverse-transcriptase inhibitor (NRTI), and Ritonavir and Lopinavir, two protease inhibitors (PI). We based our range of antiretroviral doses on previously reported plasma and cerebrospinal fluid (CSF) levels in humans, as measurements from human brain parenchyma have not been performed (Wynn et al., 2002, Noor et al., 2006, Cusini et al., 2013, Tiraboschi et al., 2014, Decloedt et al., 2015). Many antiretrovirals are thought to have limited CNS penetration due to pharmacological properties which impair transport across the blood brain barrier (BBB) and promote active exclusion through efflux transporters (McGee et al., 2006, Cysique and Brew, 2009, Varatharajan, 2009). However, there is
extensive evidence of HIV-mediated disruption of the BBB resulting in increased entry of normally excluded small molecules and serum proteins (Toborek et al., 2005, Wang et al., 2008, Nakagawa et al., 2012). In addition to HIV-mediated effects, even in the absence of virus, CNS accessibility is not necessarily as restricted as anticipated, as brain entry can be facilitated if drugs are given in combination (Marzolini et al., 2013). Importantly, animal studies predict that parenchymal concentrations reach levels equal to, or greater than those found in the CSF, suggesting that concentrations attained in human brain may be higher than current clinical estimates (Anthonypillai et al., 2004, Anderson and Rower, 2010).

We first determined the dose-dependent effects of these compounds on differentiation utilizing our previously described paradigm (See et al., 2004). Primary mouse oligodendrocyte precursor cells (OPCs) were purified and grown in the presence of bFGF, PDGF-AA, and NT-3 to 75% confluence, at which time growth factors were removed and thyroid hormone was added to initiate differentiation, allowing OPCs to progress first to immature then mature oligodendrocytes. OPCs were allowed to differentiate for 72 hours in the presence of Vehicle (DMSO) or the antiretrovirals tested: AZT (1 µM, 10 µM, or 25 µM), Ritonavir (100 nM, 1 µM, or 3 µM), or Lopinavir (150 nM, 1.5 µM, or 15 µM). Oligodendrocyte maturation is well defined and regulated; therefore, cellular markers correlating to specific stages of differentiation are commonly used to illustrate lineage progression (Miller, 2002). A2B5 was used as a marker of OPCs, galactocerebroside (GalC) as a marker of immature oligodendrocytes, and myelin basic protein (MBP) or proteolipid protein (PLP) as markers of mature oligodendrocytes.

Immunofluorescent staining following treatments revealed that the PIs Ritonavir and Lopinavir but not NRTI AZT resulted in fewer cells expressing differentiation markers, compared with controls. Representative images of oligodendrocytes after 72 hours in differentiation medium demonstrate dose-dependent maturation decreases with PIs (Figure 4.1A), with the extent of maturation determined by comparing the percentage of GalC+ cells and MBP+ cells across treatments (Figure 4.1B-C). Following PI treatment, the number of cells expressing the immature marker GalC after three days of differentiation was reduced by 69.43% ± 6.07% for the highest dose of Ritonavir and by 95.63% ± 0.96% with the highest dose of Lopinavir, compared with vehicle controls (p < 0.001) (Figure 4.1B). Similarly, the number of
MBP+ cells was significantly reduced by 75.14% ± 4.94% with Ritonavir and by 97.62% ± 1.03% with Lopinavir treatments compared with vehicle (p < 0.001) (Figure 4.1C). In contrast, AZT treatment did not alter the number of GalC+ or MBP+ cells, compared with controls (Figures 4.1B-C). Quantification of PLP+ cells mirrored that of MBP+ cells for all conditions (data not shown).

Changes in the number of cells expressing markers of oligodendrocyte differentiation could be attributed to cell death, altered precursor proliferation, or inhibition of differentiation. To address changes in cell death or OPC proliferation, we determined the number of total cells (DAPI+ cells) (Figure 4.2A) and A2B5+ OPCs (Figure 4.2B) following treatments. AZT, which did not cause a loss of GalC+ or MBP+ oligodendrocytes compared to controls, did not lead to a loss of OPCs or total cells at any dose as expected. Interestingly, Ritonavir, at any dose, also did not lead to loss of OPCs or total cell number in culture, suggesting that Ritonavir treatment decreased GalC+ and MBP+ oligodendrocytes by inhibition of the differentiation process. In contrast, Lopinavir treatment trended towards a reduction in total cell number at the 1.5 µM dose (81.5% ± 5.52%) and significantly decreased total cell numbers at the 15 µM dose (73.9% ± 6.29% of vehicle total) (p < 0.05) (Figure 4.2A). A significant reduction between the 1.5 µM and 15 µM concentrations was not observed. Lopinavir did not alter A2B5 precursor cell number at any concentration (Figure 4.2B). These observed decreases, although statistically significant, are modest compared to the near total loss of GalC+ and MBP+ cells with 15 µM Lopinavir treatment. To verify that the dramatic reductions observed in expression of GalC and MBP following Lopinavir treatment were due to inhibition of differentiation and not apoptotic cell death, we assessed whether remaining cells were committed to the apoptotic cell death cascade using the TUNEL assay, which labels double-stranded DNA breaks in extensively damaged cells. Treatment with 15 µM and 1.5 µM concentrations of Lopinavir resulted in negligible TUNEL+ cells, similar to vehicle treatment (Figure 4.2C). Together, these results suggest that Lopinavir inhibits proper oligodendrocyte maturation in a dose-dependent manner. The modest reductions in GalC+ and MBP+ cells observed at 1.5 µM (57.0% ± 12.43% and 56.7% ± 12.72% of control differentiation levels) plummets to 4.37% ± 0.96% and 2.38% ± 1.03%, respectively, at 15 µM without activation of the apoptotic cascade or a significant reduction in cell number between these two concentrations.
Lopinavir Reduces the Expression of Myelin Proteins

Given that PI-treated cultures contained fewer GalC+, MBP+ and PLP+ cells, as compared with vehicle by immunofluorescence, next we determined whether this effect resulted from decreased protein levels. The cultures were treated as described above for 72 hours, and protein lysates were prepared for immunoblot analysis. The highest dose of Lopinavir (15 µM) led to a significant decrease in MBP levels when compared with vehicle (p < 0.01) (Figure 4.3C). In contrast, neither AZT nor Ritonavir significantly altered MBP expression levels (Figure 4.3A-B). Additionally, PLP and cyclic-nucleotide-3′phosphodiesterase (CNP), two other major myelin proteins, were also decreased in 15 µM Lopinavir-treated cells compared with controls, whereas no change in either protein was observed following high dose (25 µM) Azt or (3 µM) Ritonavir treatments (Figure 4.3D-E). This immunoblotting analysis of Lopinavir treated cells parallels that of the immunofluorescence results in Figure 1, with dramatic reductions in protein markers of maturation observed between the 1.5 µM and 15 µM concentrations of Lopinavir (Figure 4.2A). Intriguingly, all doses of Ritonavir and the intermediate dose of Lopinavir (1.5 µM) decreased the number of GalC+ and MBP+ cells by immunofluorescence, but not myelin protein levels by immunoblot.

Protease Inhibitor-Mediated Reduction of Differentiation is Reversible

Our observation that at low doses of PIs there were significantly fewer mature oligodendrocytes, despite unchanged levels of myelin proteins prompted us to determine whether oligodendrocyte processes were elaborated but myelin proteins were not being properly localized. Therefore, we utilized phalloidin staining to visualize actin microfilaments. Cells were treated with vehicle, 3 µM Ritonavir, or 15 µM Lopinavir and allowed to mature for 72 hours, followed by phalloidin staining. As shown in the representative images of Figure 4.4A, cells maintained normal differentiated geometry and process extension after treatment, however the immature oligodendrocyte stage marker GalC did not appear to correctly localize to the cell surface.

To determine if the effects of the PIs were permanent, or if cells were still capable of proper myelin protein production and localization, we differentiated OPCs in the presence of 3 µM Ritonavir or 15 µM Lopinavir and allowed 72 hours for maturation. At this time, a subset of cultures was switched into fresh
differentiation medium lacking further treatment for an additional 24 hours. Cultures that underwent a drug washout period were analyzed for markers of oligodendrocyte differentiation and compared with the cultures that did not undergo drug washout but were exposed to PI treatments for 72 hours. As we have shown in Figure 4.1, while 72 hour treatment with 3 µM Ritonavir or 15 µM Lopinavir resulted in fewer GalC+ cells compared with vehicle, this effect was completely reversed after the removal of both PIs (Figure 4.4B). Similarly, drug washout for 24 hours resulted in the reversal of the deficit in MBP levels following treatment with 15 µM Lopinavir (0.28 ± 0.12 fold of control levels, p < 0.05), as detected by immunoblotting (Figure 4.4C). In 15 µM Lopinavir treated cultures, the rapid restoration of maturation to vehicle-treated levels following drug washout further corroborates the findings from Figures 4.1-4.3, which together substantiate that cell death did not cause the original reduction in myelin proteins and cell surface markers of differentiation following Lopinavir treatment.

**Antiretroviral Drugs Induce Oxidative Stress**

Previous work from our laboratory has shown that oxidative stress is capable of halting oligodendrocyte differentiation (French et al., 2009, Reid et al., 2012). We have also demonstrated in primary neuroglial cultures that antiretroviral compounds (PIs and NRTIs) evoke the generation of reactive oxygen species (ROS) (Akay et al., 2014). In light of this, we postulated that oxidative stress could be the instigating factor for altered differentiation in the presence of Ritonavir and Lopinavir. OPCs were exposed to AZT, Ritonavir, and Lopinavir for 30 minutes, 1 hour, 2 hours, 6 hours, 12 hours, or 24 hours at the doses used in previous experiments, followed by staining with dihydroethidium (DHE) as an indicator of ROS. This method is a readout for cellular ROS levels, as oxidation of DHE to ethidium allows for ethidium intercalation with DNA, emitting a quantifiable red fluorescence which overlaps with DAPI area (Zhao, 2003). Tert-butyl hydroperoxide (tBHP, 2.5 µM) treatment was used as positive indicator of ROS generation for each timepoint. Following the timecourse outlined above, we found that Ritonavir produced a dose-dependent, rapid and robust accumulation of ROS by 1 hour (1756% ± 360.8% compared with vehicle, p < 0.001), while AZT produced a more modest effect, which was not observed until 2 hours post-treatment (505% ± 80.4% compared with control, p < 0.001) (Figure 4.5). Interestingly, Lopinavir did not produce detectable ROS levels at any time-point up to 24 hours (not shown).
The Endogenous Antioxidant Response is not Activated with Antiretroviral Drugs in Developing OPCs

Robust ROS were rapidly detectable following Ritonavir and AZT treatments. The typical cellular response to such an imbalance in redox homeostasis is activation of the endogenous antioxidant response (EAR) (Li, 2009). Therefore, we investigated whether exposure of antiretroviral compounds to developing oligodendrocytes activated this cellular stress signaling pathway. OPCs were treated with the highest dose of each antiretroviral at the time of differentiation. Then, mRNA or protein was extracted 6 or 16 hours later, respectively. The analysis of principle cellular targets of the EAR: Heme Oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase (NQO1), revealed no changes in mRNA levels after 6 hours of treatment with AZT. Surprisingly, Lopinavir led to a decrease in HO-1 mRNA levels to 0.654 fold compared with vehicle, with a range between 0.331 and 1.294 fold \((p < 0.001)\). In contrast, NQO1 levels were significantly increased with both Ritonavir to 1.716 fold \((1.474-2.00 \text{ fold range, } p = 0.015)\) and Lopinavir to 1.272 fold \((0.554-2.921 \text{ fold range, } p < 0.001)\) \(\text{Table } 4.3\). The incremental changes in NQO1 in our treatments, as compared with the robust increases normally seen with EAR activation with classical pathway inducers suggest that the response may not be functionally significant \(\text{Table } 4.3\). Consistent with this interpretation, immunoblotting for HO-1 and NQO1 showed no alterations in protein levels following 16 hours of treatment with any of the compounds tested \(\text{Figure } 4.6A-B\).

Effective Reduction of Reactive Oxygen Species Does not Rescue Maturation Defects

Robust oxidative stress was evident following treatment with Ritonavir, yet the EAR was not activated following treatment with any of the antiretroviral drugs tested. Therefore, we sought to determine if decreasing the accumulation of ROS would rescue the observed differentiation defects. To do this, we utilized the fumaric acid ester, monomethyl fumarate (MMF). MMF is the active metabolite of dimethyl fumarate (DMF), which is now approved for treatment of the demyelinating disorder Multiple Sclerosis \(\text{FDA, 2013, Limmroth, 2013}\). Importantly, we have previously shown that MMF successfully ameliorates ROS accumulation and antiretroviral-induced toxicity in neuroglial cell cultures \(\text{Akay et al., 2014}\). To determine whether MMF was effective at reducing ROS accumulation triggered by antiretrovirals in oligodendrocyte cultures, OPCs were pre-treated with 10 \(\mu\text{M}\) MMF for 6 hours, followed by
supplementation with MMF and/or treated with 25 µM AZT or 3 µM Ritonavir for 2 hours or 15 µM Lopinavir for 6 hours. DHE staining revealed that MMF pre-treatment significantly attenuated ROS accumulation after AZT and Ritonavir treatments (Figure 4.7A). As we previously established, Lopinavir did not induce ROS at 6 hours, and MMF had no effect on endogenously detectable ROS levels (Figure 4.7A). We next assessed if reducing oxidative stress by attenuating ROS accumulation by MMF would increase the number of mature oligodendrocytes in the presence of PIs. OPCs were pre-treated with MMF for 24 hours prior to switching to differentiation medium, and were treated with 3 µM Ritonavir or 15 µM Lopinavir. MMF was replenished at the time of PI treatment and again every 24 hours. After 72 hours in differentiation medium, cells were stained for GalC and compared with vehicle or cells incubated with MMF without PIs. There was no difference between the number of GalC+ cells in cultures treated with Ritonavir +MMF or Lopinavir +MMF (Ritonavir 35.2% ± 0.77%, Lopinavir 36.4% ± 18.2% of vehicle) and those exposed to Ritonavir or Lopinavir alone (Ritonavir 29.9% ± 5.90%, Lopinavir 19.9% ± 14.5%) (Figure 4.7B). Thus MMF decreased oxidative stress but did not improve oligodendrocyte differentiation.

The Unfolded Protein Response is Not Triggered by Antiretroviral Drugs in Maturing Oligodendrocytes

We and others have demonstrated that antiretroviral drugs induce the unfolded protein response (UPR) in neuroglial and somatic cell types (Gannon and Jordan-Sciutto, personal communication, Zhou, 2005, 2006). It has also been well documented that the UPR plays an important role in a variety of demyelinating disorders (Gow and Wrabetz, 2009). This complex signaling pathway is modulated by the actions of three branches, PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), all of which complex with ER-resident chaperone protein Binding Immunoglobulin Protein ((BiP)/GRP78) in the absence of ER stress (Reviewed in (Hetz et al., 2015)). Activation of these pathways leads to attenuation of global protein translational in conjunction with selective upregulation of pro-survival proteins (Bertolotti et al., 2000, Lee and Ozcan, 2014, Hetz et al., 2015).

Therefore, we analyzed whether the UPR was activated in OPCs treated with antiretroviral drugs for 6 or 16 hours by assessing mRNA and protein levels of markers of UPR activation. Upon disruption of
endoplasmic reticulum homeostasis and activation of the UPR, X-box binding protein 1 (XBP-1) mRNA is spliced to promote the production of the active XBP-1 protein (Yoshida et al., 2001, Calfon et al., 2002). The relative ratio between spliced and total XBP-1 mRNA is an accepted standard for UPR activation (Yoshida et al., 2001, Calfon et al., 2002). The analysis of this ratio revealed no increases in XBP-1 mRNA splicing in antiretroviral treated cells at 6 hours, suggesting that UPR activation did not occur upon treatment of OPCs with these drugs (Table 4.4). While Lopinavir treatment led to a significant decrease in XBP-1 mRNA splicing (0.398 fold as compared with vehicle, with a range of 0.297-0.534 fold ($p < 0.001$)) (Table 4.4), this effect is negligible compared with biologically relevant changes, and does not correspond to the robust increases in the ratio of spliced-to-total XBP-1 mRNA which would indicate UPR activation. Additional genes targeted by the UPR pathway were also examined by this method. No significant alterations in transcript levels were noted for CHOP, BiP, ATF6, or ATF4 with any antiretroviral tested (Table 4.4). IRE1α was not altered with AZT or Ritonavir, but was highly upregulated following Lopinavir treatment (8.664 fold compared with vehicle, with a range of 8.532-8.797 fold). Binding immunoglobulin protein (BiP) served as an indicator of UPR activation at the protein level. As the primary sensor of endoplasmic reticulum stress, its cellular levels are tightly regulated by transcription, translation, and selective stabilization during stress conditions (Brown and Naidoo, 2012). Additionally, BiP is known to be upregulated in oligodendrocytes under conditions promoting UPR activation (Cortopassi et al., 2006). Immunoblotting for BiP protein showed no difference between PI-treated and vehicle cultures (Figure 4.8A). To further examine the individual arms of the UPR, western blotting was performed for post-translational modifications of representative proteins. As eIF2α phosphorylation was not increased following 16 hours of treatment with any antiretroviral, the PERK pathway is likely not involved at this timepoint (Figure 4.8B). ATF6β cleavage was also not observed; suggesting these antiretrovirals do not modulate ATF6-mediated transcriptional activity (Figure 4.8C). Finally, despite a significant increase in IRE1α mRNA observed by RT-PCR following Lopinavir treatment, total protein IRE1α protein level was not increased in this condition compared to vehicle (Figure 4.8D). More importantly, activation of the IRE1α branch of the UPR is dependent on protein dimerization facilitated by phosphorylation status. IRE1α phosphorylation status was not increased with Lopinavir or any antiretroviral condition (Figure 4.8E). Functional activation of the IRE1α pathway arm leads to the splicing event of XBP1 (Bertolotti et al., 2000,
Lee and Ozcan, 2014, Hetz et al., 2015). Rather than increasing, we observed that XBP1 splicing was significantly decreased following Lopinavir treatment, suggesting potential IRE1 pathway suppression, complete with compensatory increase of regulator IRE1α transcription as was also observed by RT-PCR (Table 4.4). In summary, these findings suggest that neither the EAR nor the UPR are activated in OPCs by any of the antiretrovirals tested during the timeframe that was previously determined to induce responses within these pathways neuroglial culture. Thus we do not currently ascribe the observed negative maturation effects to actions of either of these pathways.

**Myelin Protein Expression In Vivo is Reduced after Treatment with a Ritonavir Regimen**

After investigating how antiretrovirals altered oligodendrocyte maturation in vitro, we asked whether these compounds also had detrimental effects to established myelin in vivo. To this end, we examined the effects of a repeated/chronic antiretroviral exposure. We administered Ritonavir or vehicle intravenously to adult male mice through implanted jugular vein catheters over a two-week period, according to the same protocol followed in our previous studies (Briand et al., 2012, Akay et al., 2014). Recommendations for treatment regimens are constantly changing to improve patient outcomes while reducing adverse effects, as well as incorporating new drug classes and next-generation therapeutics. For our purposes, Ritonavir was chosen as a representative antiretroviral compound for our in vivo study. Ritonavir is globally used clinically as a boost for other antiretrovirals to increase bioavailability, as it is a potent inhibitor of cytochrome P450 which metabolizes these compounds (Moyle, 2001). Increased bioavailability of co-administered compounds with high CNS penetrance likely leads to higher brain levels of these compounds (Marzolini et al., 2013). Additionally, Ritonavir-boosted regimens are still commonly prescribed in resource-limited settings as first-line treatment, making it globally among the most-prescribed treatment options (AIDSTAR-One, 2012). The dose of Ritonavir was based on previously published pharmacokinetic studies (Kageyama et al., 2005, Pistell et al., 2010, du Plooy et al., 2011). The animals showed no signs of distress or gross phenotypic alterations over the course of treatment. To determine the effects of Ritonavir on established myelin, myelin protein levels were assessed by immunoblotting cellular lysates prepared from frontal cortex. In addition to myelin basic protein (MBP), other components of mature myelin were also evaluated in vivo: myelin oligodendrocyte glycoprotein (MOG), cyclic-nucleotide-3’phosphodiesterase
(CNPase), myelin-associated glycoprotein (MAG), and proteolipid protein (PLP). We observed that protein levels of MOG and CNPase were significantly decreased with Ritonavir compared with vehicle treatment ($p < 0.05$), whereas MBP, MAG, and PLP levels were not significantly altered (Figure 4.9).

Myelin Protein Expression in Humans is Altered by HIV- and Antiretroviral Treatment-Status

To address potential translational relevancy of our observations, we examined prefrontal cortex autopsy specimens from cohort of HIV-infected patients who were exposed to antiretroviral agents. To date, diffusion tensor and magnetic resonance imaging studies and a single transcriptome study have been performed to examine white matter dysfunction in HIV and HAND patients (Borjabad et al., 2011, Tate et al., 2011, Kelly et al., 2014), but changes in post-mortem myelin protein levels have not been reported. The cohort contained age-matched HIV(-), HIV(+) patients who were cART-naïve (HIV(+)), and HIV(+) cART-medicated >12 month individuals (HIV(+)/ART) (n = 20 for each group). The HIV(+) cART-naïve group was comprised of six individuals with diagnosed HAND, one who was neurocognitively normal, five with neuropsychological impairment that was potentially due to factors other than HIV infection. Eight of the cases did not undergo a structured ante-mortem neurocognitive assessment. The HIV(+) cART-medicated >12 month group (HIV(+)/ART) contained 14 individuals with HAND, two who were neurocognitively normal, and four with neuropsychological impairment potentially due to factors other than HIV infection. The full details of their demographics and clinical data are in Table 4.1. The prefrontal cortex was selected for analysis for the following reasons: 1) This brain region is utilized in performing the functional modalities in which HAND patients display impairments (Antinori, 2007), 2) Synaptodendritic damage has been previously reported in this region in affected patients (Ellis et al., 2007), and 3) A region of mixed white and grey matter was desired for analysis as is seen in the cortex, so that the overall myelin contribution and relative protein amounts could be assessed. Previous studies have shown that the volume of the corpus callosum is reduced in HIV and with antiretroviral treatment, however as this is an extremely myelin-rich environment, to observe a quantitative change in immunoblot analysis would require a sizable loss of myelination. For all cases, the myelin proteins MBP, MOG, CNPase, and MAG were examined by immunoblot analysis. When subdivided according to HIV status alone, no statistically significant alterations were noted in the myelin protein levels between uninfected controls and the HIV(+) patients
(Figure 4.10). However, when the cohort was stratified into categories based on antiretroviral therapy status, CNPase protein was significantly increased in the HIV(+) cART > 12 month group (HIV(+)/ART) compared with the HIV(-) group ($p < 0.01$) (Figure 4.11A). Finally, individuals for whom neurocognitive data was not known, who were neurocognitively normal, or who had neuropsychological impairment due to factors other than HIV-infection were excluded from analysis to remove potential confounding factors, and to allow us to examine only those cases with known HAND diagnosis in relation to cART-medication status. This analysis revealed a statistically significant decrease in MBP protein levels in the HIV(+)/ART group, as compared with the HIV(-) ($p < 0.05$), and HIV(+) groups ($p < 0.05$) (Figure 4.11B). On the other hand, a significant increase in CNPase protein was observed in the HIV(+)/ART group, compared with the HIV(-) group ($p < 0.001$) (Figure 4.11B). These results indicate that HIV infection compounded with the effects of antiretroviral therapy can lead to alterations in myelin protein levels in patients.

4.5 DISCUSSION
Despite effective viral suppression through antiretroviral therapy, HIV-Associated Neurocognitive Disorders (HAND) persists in infected individuals and is frequently accompanied by white matter pathologies (Tate et al., 2010, Kelly et al., 2014, Sacktor and Robertson, 2014). Here, we investigate the effects of antiretrovirals *in vitro* on oligodendrocyte survival and maturation, and *in vivo* on myelin maintenance in both adult mice and HIV(+) individuals and provide novel, compelling evidence that the protease inhibitors Ritonavir and Lopinavir impair both the maturation of oligodendrocyte precursors into myelin-producing cells, and the maintenance of myelin proteins *in vivo*. Furthermore, human brain specimens demonstrated a reduction in MBP and an increase in CNPase in HAND patients who had been cART-medicated for greater than one year, suggesting a loss of myelin integrity yet failure of successful remyelination. These novel observations of the effects of a subset of antiretrovirals on oligodendrocyte development and maintenance may have critical clinical repercussions for both pediatric and adult HIV-patients.

Utilizing stage-specific markers, we have shown that PIs Ritonavir and Lopinavir prevent oligodendrocyte maturation *in vitro* while NRTI AZT does not. Interestingly, myelin protein levels as evaluated by
immunoblotting remained unchanged under most conditions, except at the highest dose of Lopinavir. During normal maturation, OPCs enter into differentiation and proceed with upregulation of myelin component mRNAs (Miller, 2002). During this progression, cells extend actin filaments to elaborate processes. Most myelin proteins are then translated in the perinuclear endoplasmic reticulum and trafficked through the secretory pathway to be inserted into the forming myelin membrane (Colman et al., 1982). An exception is MBP, in which mRNA is trafficked to sites proximal to membrane insertion where the protein is then translated on free-ribosomes (Colman et al., 1982, Ainger et al., 1997). In that context, our data suggest that treatment with PIs prevents proper membrane insertion of both myelin proteins traversing the secretory network and locally synthesized MBP as we have detected by immunofluorescence; however, in these conditions, protein levels remain unchanged, as determined by immunoblotting. Specifically, even at the highest Ritonavir dose where we observed substantial decreases in maturation by immunofluorescence, we did not detect parallel changes at the protein level. This finding, while unusual, is not unprecedented in oligodendrocytes. Maier et al. (2009) demonstrated that reduction in cellular cholesterol levels can result in decreased PLP surface expression with minimal reduction at the protein level. Along these lines, a recent study also suggested that myelin membrane lipid composition may be altered under two models of demyelination. In this work, MBP surface localization to myelinated processes by immunohistochemistry was diminished, while total MBP levels remained unchanged (Frid et al., 2015). Additionally, a study investigating the role of mammalian target of rapamycin in oligodendrocyte differentiation observed maintenance of normal myelin protein levels despite a significant reduction in the number of maturing oligodendrocytes (Wahl et al., 2014). Further, we observed that cells treated with either PI adopted properly elaborated mature oligodendrocyte morphology, as evidenced by normal actin cytoskeleton morphology despite reduction in myelin protein expression at the cell surface. The effect of PIs was reversible, as cells proceeded to differentiate rapidly following drug removal, with both cell surface marker expression and myelin protein levels indistinguishable from controls after 24 hours. The accelerated rescue of maturation deficits further suggests that required components are already present within the treated cells, and that these PIs are most probably suppressing maturation on a post-translational level. Drug removal alleviates this blockade, resulting in appropriate protein localization to the myelin membrane.
We have observed differences in the effects of Ritonavir and Lopinavir. While both are PIs, their differing chemical structures likely result in disparate cellular uptake, metabolism, and intracellular interactions. In agreement with this notion, other commonly prescribed PIs behave differently within cells of the same lineage. For example, inhibition of differentiation was observed with Nelfinavir and Indinavir, but not Amprenavir in adipocytes (Caron et al., 2003). Similarly, in CNS-derived astrocytes, Nelfinavir and Indinavir displayed striking differences in ROS production, glutathione export, and toxicity (Brandmann et al., 2012). Regardless of the differing subcellular effects that Lopinavir and Ritonavir invoke, it is clear based on our observations that properly functioning myelin would not arise following exposure to either PI even at low concentrations.

Several antiretroviral drugs induce ROS, mitochondrial depolarization and neurotoxicity in mixed neuroglial cultures (Robertson et al., 2012, Akay et al., 2014), and we have shown that oxidative stress can hinder the process of oligodendrocyte differentiation (French et al., 2009). While both AZT and Ritonavir induced robust ROS accumulation, Lopinavir did not, even at extended timepoints. When we assessed whether the EAR, which is triggered by cellular redox imbalance, was activated following antiretroviral exposure, no biologically relevant increases in target gene mRNAs or proteins, such as HO-1 or NQO1 were detected (Li, 2009). Furthermore, scavenging of ROS with the antioxidant compound MMF did not rescue PI-induced decreases in oligodendrocyte differentiation despite previously demonstrated neuroprotection (Akay et al., 2014). PIs have also been shown to activate the UPR in non-CNS cells, which potently suppresses protein translation while upregulating stress-response proteins (Zhou, 2005, 2006, Brown and Naidoo, 2012). However, we found no evidence of UPR activation in oligodendrocytes following antiretroviral treatments at two timepoints where such activation was evident in neuroglial cultures (Akay et al., 2014), although we acknowledge that there may be activation at earlier or later time points. Thus, our data suggest that PIs inhibit oligodendrocyte maturation through an alternate mechanism. An intriguing possibility is whether PIs disrupt the crucially important myelin-lipid balance, which may result in inappropriate organization of myelin components, as previously reported (Maier et al., 2009, Chrast et al., 2011, Lee et al., 2014, Frid et al., 2015). Since PIs have been shown to cause dyslipidemia in
patients and in non-CNS cell types (Carr, 1998, Haughey et al., 2004, Zhou, 2005), this could contribute to the white matter loss seen in HAND patients (Tate et al., 2010, Kelly et al., 2014).

The effects of antiretrovirals on myelin *in vivo* using both a mouse model and human autopsy cases also indicate alterations in myelin proteins. In mice, we detected reduced levels of less abundant myelin proteins in as little as 14 days of chronic exposure. Levels of the abundant structural myelin components MBP and PLP were not affected in this short-term administration model. Changes in these critical proteins may be unexpected in such a short time frame, as gross changes in myelin may be necessary to result in detectable alterations. In our human cohort, we did observe a reduction in MBP in cART-experienced individuals with HAND compared with cART-naïve individuals with HAND, and HIV(-) controls. This finding correlates with a transcriptome analysis of virally controlled patients with HAND, which showed reduction of MBP mRNA (Borjabad et al., 2011). The HIV(+)/ART group also displayed a significant increase in the amount of CNPase over both HIV(-) and HIV(+) cART-naïve individuals, regardless of their neurocognitive status. CNPase is the earliest myelin protein to be expressed during maturation (Scherer et al., 1994), and our observation may reflect a remyelination attempt that is prematurely halted, failing to form mature myelin.

Current WHO guidelines urge immediate treatment of all infected children under the age of 5 years, and adults with a CD4 count ≤ 350 cells/mm³. Guidelines suggest that first-line cART consist of two NRTIs and a non-nucleoside reverse-transcriptase inhibitor (nNRTI). When second-line cART is necessary due to acquired drug resistance, recommended regimens are composed of two NRTIs plus a Ritonavir-boosted PI, either Lopinavir or Atazanavir. All infected children under 3 years of age are recommended a Lopinavir/Ritonavir first-line regimen (WHO, 2013). As is clear from these guidelines, PI-based regimens are extensively used in HIV treatment, particularly in children, during a time of critical cortical myelination which primarily occurs from the postnatal period until late adolescence (Miller et al., 2012). Neurological complications are common in infected children, with documentation of encephalopathy, progressive multifocal leukoencephalopathy, cerebrovascular events, and emerging neurocognitive deficits leading to developmental delays, motor, cognitive, and behavioral deficits (Wilmshurst et al., 2006, Crowell et al., 2014, Wilmshurst et al., 2014). Our results showing an effective disruption in myelination following PI
exposure may underlie these clinical presentations, and therefore have important ramifications for the future treatment of pediatric HIV infection.

PI-supplemented regimens are generally prescribed to adults as second-line options. However, as lifelong cART adherence is necessary, over the course of regimen alterations, most patients will be exposed to a boosted PI. The maintenance and replacement of myelin in the brain is an active and ongoing process, which is critical for proper neuronal functioning and survival (McLaurin and Yong, 1995). Remyelination of neurons in the adult has been well documented, particularly through investigation into pathology and potential treatments for Multiple Sclerosis (Hartley et al., 2014, Kutzelnigg and Lassmann, 2014). Additionally, while traditionally viewed as an early life event, active myelin formation in adulthood is required for motor learning (Long and Corfas, 2014, McKenzie et al., 2014). We have shown through our mouse model that prolonged PI exposure may lead to reductions in adult cortical myelin proteins. More importantly, human patients with HAND who had been treated with antiretrovirals for at least a year demonstrated a significant reduction in MBP compared with uninfected individuals and HAND patients who were cART-naïve. Therefore, therapeutics which will preserve the capacity for myelin formation and the integrity of existing structures are of vital importance.

In summary, this study implicates antiretrovirals as a contributor to HAND pathology and its persistence in virologically suppressed individuals. In human patients, the underlying viral infection complicates analysis of consequences solely due to antiretrovirals. Our observed effects of antiretrovirals in conjunction with the potential contribution of HIV-induced pathogenesis on oligodendrocytes highlight the necessity for strategic drug development for new therapeutics with fewer deleterious side effects. While this study has highlighted the effects of antiretroviral compounds, future work investigating the consequences of HIV infection in the CNS on the oligodendrocyte population are also essential for a more complete understanding of the changes to white matter in HIV-positive patients.

Furthermore, we must consider adjunctive therapies designed not only to alleviate neuronal dysfunction, but also to preserve myelin formation and maintenance by oligodendrocytes. Persistent ROS elevation and
oxidative damage are seen in treated HIV(+) individuals (Roc et al., 2007, Blas-Garcia et al., 2011). HO-1 deficiency has recently been reported in HIV(+) and HAND patients, suggesting inactivity of the EAR pathway (Gill et al., 2014). Supporting this notion, we have reported that EAR and HO-1 upregulation by the antioxidant MMF was successful in preventing ARV-induced oxidative stress and neuronal toxicity in neuroglial cultures (Akay et al., 2014). Unfortunately, based on our current findings, while neurons may be initially protected by adjunctive antioxidant therapy, oligodendrocytes would remain vulnerable and neuronal pathology caused by myelin disruption would still persist. Thus, the effects of antiretrovirals on oligodendrocyte maturation and myelin unveiled in this study illuminate the need for greater understanding of viral- and therapy-mediated repercussions to the critically important oligodendroglial cell population in order to attenuate neurocognitive deficits in HIV(+) individuals in the cART era.

4.6 ACKNOWLEDGEMENTS

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### Table 1: Demographics of Human Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV (-)</th>
<th>HIV (+) ART-naive</th>
<th>HIV (+) ART-mediated &gt;12mo</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Subjects</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Age at death, mean ± SD</td>
<td>42.8 ± 5.6</td>
<td>39.7 ± 7.3</td>
<td>45.3 ± 6.7</td>
<td>0.032a</td>
</tr>
<tr>
<td>Hours post-mortem, mean ± SD</td>
<td>9.9 ± 5.7</td>
<td>10.0 ± 6.4</td>
<td>12.4 ± 7.9</td>
<td>0.455a</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>80%</td>
<td>90%</td>
<td>80%</td>
<td>0.619b</td>
</tr>
<tr>
<td>Female (%)</td>
<td>20%</td>
<td>10%</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>11 (55%)</td>
<td>12 (60%)</td>
<td>15 (75%)</td>
<td></td>
</tr>
<tr>
<td>Black (%)</td>
<td>7 (35%)</td>
<td>8 (40%)</td>
<td>5 (25%)</td>
<td>0.498b</td>
</tr>
<tr>
<td>Other/Unknown (%)</td>
<td>2 (10%)</td>
<td>0 (0%)</td>
<td>1 (5%)</td>
<td></td>
</tr>
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<td>Ethnicity</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Hispanic (%)</td>
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<td>3 (15%)</td>
<td>3 (15%)</td>
<td>0.641b</td>
</tr>
<tr>
<td>Non-Hispanic (%)</td>
<td>15 (75%)</td>
<td>17 (85%)</td>
<td>17 (85%)</td>
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</tr>
<tr>
<td>Neurocognitive Impairment Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAND (%)</td>
<td>-</td>
<td>6 (30%)</td>
<td>14 (70%)</td>
<td></td>
</tr>
<tr>
<td>Neuropsych. Impair. Other Origin (%)</td>
<td>-</td>
<td>5 (25%)</td>
<td>4 (20%)</td>
<td>0.009b</td>
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<tr>
<td>Neurocognitively Normal (%)</td>
<td>-</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
<td></td>
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<tr>
<td>No Neurocognitive Data (%)</td>
<td>-</td>
<td>8 (40%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>ARV Treatment Status</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI-Experienced (%)</td>
<td>-</td>
<td>-</td>
<td>17 (85%)</td>
<td></td>
</tr>
<tr>
<td>PI-Naive (%)</td>
<td>-</td>
<td>-</td>
<td>3 (15%)</td>
<td></td>
</tr>
<tr>
<td>NRTI-Experienced (%)</td>
<td>-</td>
<td>-</td>
<td>20 (100%)</td>
<td></td>
</tr>
<tr>
<td>NRTI-Naive (%)</td>
<td>-</td>
<td>-</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Disease Parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIVE (%)</td>
<td>-</td>
<td>6 (30%)</td>
<td>1 (5%)</td>
<td></td>
</tr>
<tr>
<td>Log Plasma HIV c/mL, mean ± SD</td>
<td>-</td>
<td>4.1 ± 1.4</td>
<td>4.7 ± 1.1</td>
<td>0.196c</td>
</tr>
<tr>
<td>Log Brain HIV g/mL, mean ± SD</td>
<td>-</td>
<td>3.4 ± 1.9</td>
<td>4.1 ± 0.9</td>
<td>0.358c</td>
</tr>
<tr>
<td>Log CSF HIV c/mL, mean ± SD</td>
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<td>3.4 ± 1.8</td>
<td>2.8 ± 1.1</td>
<td>0.261c</td>
</tr>
<tr>
<td>CD4+lymphocytes/mm³, mean ± SD</td>
<td>-</td>
<td>108 ± 118</td>
<td>85 ± 118</td>
<td>0.624c</td>
</tr>
</tbody>
</table>

Table 4.1: Demographics of Human Subjects

Summary of human cohort demographics, antiretroviral therapy history, and clinical data utilized for post-mortem analysis. Abbreviations: Antiretroviral Therapy (ART), standard deviation (SD), HIV-Associated Neurocognitive Disorders (HAND), Antiretroviral (ARV), HIV protease inhibitor-experienced (PI-experienced), nucleoside reverse transcriptase inhibitor-experienced (NRTI-experienced), HIV encephalitis (HIVE). aAnalysis of Variance (ANOVA) p-value, bChi-square test p-value, c Student’s t-test p-value.
Table 4.2 Primers Used for q-RT PCR

Primer pairs used for qRT-PCR are shown, which were obtained from IDT. Abbreviations are: PKG1, Protein Kinase Gene 1; HO-1, Heme Oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase; XBP-1, X-box Binding Protein 1; BiP, Binding Immunoglobulin Protein; CHOP, C/EBP homologous protein; Ire1α, inositol-requiring enzyme 1 alpha; ATF6, activating transcription factor 6; ATF4, activating transcription factor 4.

<table>
<thead>
<tr>
<th>Gene:</th>
<th>Primer Sequences:</th>
</tr>
</thead>
</table>
| PKG1          | Forward: 5’ ATG CAA AGA CTG GCC AAG CTA C 3’  
              | Reverse: 5’ AGC CAC AGC CTC AGC ATA TTT C 3’          |
| HO-1          | Forward: 5’ TGT AAG GGA GAA TCT TGC CTG GCT 3’  
              | Reverse: 5’ TGC TGG TTT CAA AGT TCA GGC CAC 3’        |
| NQO1          | Forward: 5’ AAG AGC TTT AGG GTC GTC TTG GCA 3’  
              | Reverse: 5’ AGC CTC CTT CAT GGC GTA GTC GAA 3’        |
| XBP-1 Spliced | Forward: 5’ GAG TCC GCA GCA GGT G 3’  
              | Reverse: 5’ GCT TAG AGG TGC TCC CTC AAT 3’            |
| XBP-1 Total   | Forward: 5’ CAC CTC TGC AGC AGG TG 3’  
              | Reverse: 5’ GCT TAG AGG TGC TCC CTC AAT 3’            |
| BiP           | Forward: 5’ TGG ATA AGA GAG AGG AGA AGA 3’  
              | Reverse: 5’ GTG AGA AGA GAC ACA TCG AAG G 3’          |
| CHOP          | Forward: 5’ CAG CGA CAG AGC CAG AAT AA 3’  
              | Reverse: 5’ CAG GTG TGG TGG TGT ATG AA 3’             |
| Ire1α         | Forward: 5’ TCC TAA CAA CCT GCC CAA AC 3’  
              | Reverse: 5’ TCT CCT CCA CAT CCT GAG ATA C 3’          |
| ATF6          | Forward: 5’ CTC GCC TCG GTA GTT TGT ATC 3’  
              | Reverse: 5’ AGA CCT GAA TGG CTG CTT AC 3’             |
| ATF4          | Forward: 5’ CCA CTC CAG AGC ATT CCT TTA G 3’  
              | Reverse: 5’ CTC CTT TAC ACA TGG AGG GAT TAG 3’
Table 4.3 mRNA alterations in Endogenous Antioxidant Response Genes Following Antiretroviral Application in Primary OPCs

Extracted RNA (5 µg) was converted to cDNA using the Superscript First-strand kit and q-PCR was performed using Power SYBR Green. All measurements were normalized first to the oligodendrocyte housekeeping gene PKG1, and then to controls. Analysis was performed by the ΔΔCT method. Endogenous Antioxidant Response genes: HO-1 and NQO1 were probed. ΔΔCT ±SEM, fold change, and fold change range are presented from 3 independent biological replicates (n = 3). P-values are indicated, (* = p< 0.05).
Table 4.4 mRNA alterations in Unfolded Protein Response Genes Following Antiretroviral Application in Primary OPCs

Extracted RNA (5 µg) was converted to cDNA using the Superscript First-strand kit and q-PCR was performed using Power SYBR Green. All measurements were normalized first to the oligodendrocyte housekeeping gene PKG1, and then to controls. Analysis was performed by the ΔΔCT method. Unfolded Protein Response genes: XBP-1 spliced, XBP-1 total, BiP, CHOP, Ire1α, ATF6, and ATF4 were probed. ΔΔCT ±SEM, fold change, and fold change range are presented from 3 independent biological replicates (n = 3). *P-values are indicated, (*= p< 0.05).

<table>
<thead>
<tr>
<th>Gene:</th>
<th>Treatment:</th>
<th>ΔΔCT ±SEM:</th>
<th>Fold Change Relative to Control</th>
<th>P value:</th>
</tr>
</thead>
<tbody>
<tr>
<td>XBP-1</td>
<td>AZT</td>
<td>2.991 ± 0.841</td>
<td>0.126 (0.070-0.225)</td>
<td>0.1414</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>0.7938 ± 0.4721</td>
<td>0.577 (0.416-0.900)</td>
<td>0.705</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>1.328 ± 0.4233</td>
<td>0.398 (0.297-0.534)</td>
<td>&lt;0.0001 *</td>
</tr>
<tr>
<td></td>
<td><strong>BIP</strong></td>
<td>-0.662 ± 0.736</td>
<td>1.582 (0.695-2.635)</td>
<td>0.4630</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>-0.4063 ± 0.482</td>
<td>1.327 (0.950-1.853)</td>
<td>0.5523</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>0.7886 ± 1.029</td>
<td>1.727 (0.847-3.525)</td>
<td>0.5236</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>0.2650 ± 0.868</td>
<td>0.8832 (0.456-0.658)</td>
<td>0.8114</td>
</tr>
<tr>
<td></td>
<td><strong>CHOP</strong></td>
<td>2.214 ± 2.021</td>
<td>0.216 (0.063-0.875)</td>
<td>0.4710</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>-0.0517 ± 1.572</td>
<td>1.036 (0.349-3.062)</td>
<td>0.9791</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>-3.115 ± 0.022</td>
<td>8.864 (8.532-8.797)</td>
<td>0.004 *</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>0.5700 ± 1.047</td>
<td>1.760 (0.440-7.040)</td>
<td>0.7225</td>
</tr>
<tr>
<td></td>
<td><strong>ATF6</strong></td>
<td>-0.8156 ± 2.000</td>
<td>0.830 (0.524-1.315)</td>
<td>0.7242</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>0.2694 ± 0.864</td>
<td>0.874 (0.328-1.392)</td>
<td>0.6025</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>0.5700 ± 1.047</td>
<td>1.760 (0.440-7.040)</td>
<td>0.7225</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>-1.307 ± 0.435</td>
<td>2.474 (1.830-3.345)</td>
<td>0.0053</td>
</tr>
<tr>
<td></td>
<td><strong>ATF4</strong></td>
<td>0.2317 ± 0.753</td>
<td>0.852 (0.505-1.435)</td>
<td>0.7875</td>
</tr>
<tr>
<td></td>
<td>AZT</td>
<td>-0.0650 ± 0.892</td>
<td>1.046 (0.981-1.115)</td>
<td>0.9537</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>0.2317 ± 0.753</td>
<td>0.852 (0.505-1.435)</td>
<td>0.7875</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>-0.0650 ± 0.892</td>
<td>1.046 (0.981-1.115)</td>
<td>0.9537</td>
</tr>
</tbody>
</table>
Figure 4.1: Antiretrovirals Inhibit Oligodendrocyte Differentiation

Primary mouse OPCs plated on coverslips were put into differentiation medium and treated with vehicle (DMSO), doses of AZT (1 µM, 10 µM, or 25 µM), Ritonavir (100 nM, 1 µM, or 3 µM), or Lopinavir (150 nM, 1.5 µM, or 15 µM). After 72 hrs, cells were fixed and stained with antibody to MBP (green) with DAPI (blue) for total cell nuclei. Epifluorescent images were captured, with 10 fields on three coverslips per condition. Results from n= 4 independently prepared cultures.

A) Representative images from immunofluorescent staining, scale bar indicates 25 µm.

B-C) Immature oligodendrocytes (GalC+ cells) (B) and mature oligodendrocytes (MBP+ cells) (C) were counted using ImageJ software, represented as percentage normalized to untreated. Results from AZT are in the top row, Ritonavir in the center, and Lopinavir in the bottom row respectively. Data are presented as mean ±SEM. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance. Significance compared with control (###= p< 0.001, ##= p< 0.01).
Figure 4.2: Antiretrovirals Do Not Alter Oligodendrocyte Precursor Number or Induce Apoptotic Cell Death

A-B) Primary mouse OPCs plated on coverslips were put into differentiation medium and treated with vehicle (DMSO), doses of AZT (1 µM, 10 µM, or 25 µM), Ritonavir (100 nM, 1 µM, or 3 µM), or Lopinavir (150 nM, 1.5 µM, or 15 µM). After 72 hrs, cells were fixed and stained with antibody to A2B5 with DAPI (blue) for total cell nuclei. Results from AZT are in the top row, Ritonavir in the center, and Lopinavir in the bottom row respectively. Epifluorescent images were captured, with 10 fields on three coverslips per condition.

A) Total cell number (DAPI+ nuclei) were counted using ImageJ software from the biological replicates utilized for the dose curves in Figures 1 and 2 (n=8). Results are represented as percentage normalized to untreated. Significance compared with control (#= p< 0.05). Data are presented as mean ±SEM. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance.

B) Oligodendrocyte precursor cells (A2B5+ cells) were counted using ImageJ software and are represented as percentage normalized to untreated. Data are presented as mean ±SEM. Results from n= 3 independently prepared cultures. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance.

C) Primary mouse OPCs plated on coverslips were put into differentiation medium and treated with vehicle (DMSO), or doses of Lopinavir (1.5 µM, or 15 µM). After 72 hrs, cells were fixed and stained using a TUNEL assay to label cells with double-stranded DNA breaks indicative of activation of the apoptotic cascade. DNase was used as a positive control. Epifluorescent images were captured at 40x magnification, with 10 fields on three coverslips per condition. Results from n= 2 independently prepared cultures. Data are presented as mean ± SEM. One-way ANOVA followed by post-hoc Dunnett’s comparison determined statistical significance. Significance compared with control (###= p< 0.001).
Figure 4.3: Lopinavir Reduces Myelin Proteins but AZT and Ritonavir Do Not

OPCs were treated with vehicle, AZT (1 µM, 10 µM, or 25 µM), Ritonavir (100 nM, 1 µM, or 3 µM) or Lopinavir (1.5 µM or 15 µM) at the time of differentiation. After 72 hrs cells were harvested for protein, undifferentiated OPCs were harvested at time 0. Cell lysates were immunoblotted for MBP protein (A-C), CNP protein (D), or PLP protein (E).

A) Representative Western blot image and quantification of band intensities normalized to loading control GAPDH from 5 separate culture groups (n= 5) reveals the typical increase in MBP expression over 72 hours between OPC and untreated, however no significant change with any dose of AZT.

B) Analysis as in A) from n= 5 reveals the typical increase in MBP expression over 72 hours between OPC and vehicle treatment, however no change with any dose of Ritonavir.

C) Representative Western blot image and quantification of band intensities normalized to loading control GAPDH from n= 4 reveals treatment with 15 µM Lopinavir resulted in significantly less MBP expression over 72 hours than with vehicle alone or with the lower 1.5 µM dose of Lopinavir.

D) Representative Western blot images with loading control GAPDH reveals less CNP expressed at 72 hours following treatment with 15 µM Lopinavir than with the vehicle alone, 25 µM AZT, or 3 µM Ritonavir.

E) Representative Western blot images with loading control GAPDH reveals less PLP expressed at 72 hours following treatment with 15 µM Lopinavir than with the vehicle alone, 25 µM AZT, or 3 µM Ritonavir.

Data are presented as mean ± SEM. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance across concentrations. Undifferentiated and controls (untreated or vehicle) were compared by Student’s t-test (##= p< 0.01, #= p< 0.05).
A) DAPI / GalC / Phalloidin

B) Graph showing the number of GalC positive cells normalized to vehicle. Graphs show the effect of 3 μM Ritonavir and 15 μM Lopinavir on GalC positive cells compared to vehicle. Vehicle: +, Ritonavir: -, Lopinavir: +, Washout: +.

C) Graph showing the fold change in MBP protein normalized to vehicle. Graphs show the effect of Lopinavir on MBP protein levels compared to vehicle. Vehicle: +, Lopinavir: -.

Legend: 

- = no treatment
+ = treatment

Statistical significance: 

### = p < 0.001
## = p < 0.01
# = p < 0.05
ns = not significant
Figure 4.4: Antiretroviral Inhibition of Differentiation is Reversible

A) OPCs were treated with vehicle, 3 μM Ritonavir, or 15 μM Lopinavir at the time of differentiation. After 72 hrs, cells were fixed and stained for GalC (green) with DAPI (blue) and Phalloidin (red) for actin. Sample images reveal that cells in Ritonavir and Lopinavir treatments have elaborated morphology but lack the appropriate maturation marker GalC co-staining. Scale bar indicates 25 μm.

B) OPCs were treated as in A. After 72 hrs, the washout group received new differentiation medium without antiretrovirals and was allowed to further mature for 24 hrs. Cells were fixed and stained for GalC with DAPI. Cells were counted as in Figure 1 for 15 fields in 3 coverslips per condition at 40x magnification. Data are presented as mean ± SEM, n= 4. One-way ANOVA followed by post-hoc Bonferroni correction compared vehicle and treatment conditions at the two time-points (#= p< 0.05, ##= p< 0.01, ###= p< 0.001, ns= not significant).

C) OPCs were treated with Vehicle or 15 μM Lopinavir at the time of differentiation. After 72 hrs, the washout group received new differentiation medium without treatment and was allowed to further mature for 24 hrs. Cell lysates were immunoblotted for MBP protein. Representative Western blot image shown. Quantification of band intensities normalized to a loading control reveals that Lopinavir-treated cells expressed less MBP protein compared with vehicle; however this level rose to be comparable to vehicle 24 hours after washout. Data are presented as mean ± SEM, n= 3. One-way ANOVA followed by post-hoc Bonferroni correction compared vehicle and treatment conditions at the two time-points (#= p< 0.05).
Figure 4.5: Antiretrovirals Induce Oxidative Stress

OPCs were treated with vehicle, AZT (1 µM, 10 µM, or 25 µM), Ritonavir (100 nM, 1 µM or 3 µM), Lopinavir (150 nM, 1.5 µM, or 15 µM), or positive control tBHP (2.5 µM) for 30 min, 1 hr, 2 hrs, 6 hrs, 12 hrs, and 24 hrs to determine if reactive oxygen species (ROS) were generated. DHE staining was used as an indicator of ROS, with DAPI staining for cell nuclei. Epifluorescent images were captured at 40× magnification, with 10 fields on three coverslips per condition at the time-point where ROS began to accumulate for each antiretroviral compound. Scale bar indicates 25 µm. In treatments up to 24 hrs, Lopinavir produced no accumulation. Analysis using Metamorph determined fluorescence intensity of DHE normalized to nuclear DAPI area. Representative experiments shown, values are presented as mean ±SEM, n= 2 for each time-point tested. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance (##= p < 0.01, ###= p < 0.001).

A) Representative images of 1 hr tBHP- and Ritonavir-treated and 2 hr AZT-treated OPCs, with DHE fluorescence (red) and cell nuclei stained with DAPI (blue).

B) Quantification of these experiments.
OPCs were treated with vehicle, 25 μM AZT, 3 μM Ritonavir, or 15 μM Lopinavir at the time of differentiation. After 16 hrs cells were harvested for protein. Cell lysates were immunoblotted for Heme Oxygenase-1 (HO-1) (A) and NAD(P)H:quinone oxidoreductase (NQO1) (B), target proteins upregulated following activation of the endogenous antioxidant response. Representative Western blot images shown, with quantification of band intensities normalized to GAPDH loading controls revealing no change with any treatment condition. Data are presented as mean ± SEM from 3 separate cultures (n= 3). One-way ANOVA followed by post-hoc Dunnett’s multiple comparison determined statistical significance.
Figure 4.7: Effective Reduction of Reactive Oxygen Species Does not Rescue Maturation Defects

A) OPCs were pre-treated with 10 μM monomethyl fumarate (MMF) for 6 hrs. Cultures were then supplemented with MMF and/or treated with vehicle, 25 μM AZT or 3 μM Ritonavir for 2 hrs or 15 μM Lopinavir for 6 hrs. DHE staining, imaging and analysis was performed as in Figure 4. Representative experiment shown, values are presented as mean ± SEM, n= 2. Student’s t-test determined significance for direct comparisons (#= p< 0.05, ##= p< 0.001).

B) OPCs plated on coverslips were pre-treated with 10 μM MMF for 24 hrs. Cells were then switched into DM, supplemented with MMF and/or treated with vehicle, 3 μM Ritonavir or 15 μM Lopinavir. MMF was replenished at 24 and 48 hrs. After 72 hrs, cells were fixed and stained for GalC and DAPI, and were imaged and analyzed as in Figure 1. Data are presented as mean ± SEM, from 3 separate cultures (n= 3). Student’s t-test determined significance for direct comparisons (##= p< 0.01, ####= p< 0.001).
Figure 4.8: The Unfolded Protein Response is Not Triggered by Antiretrovirals in Developing OPCs

OPCs were treated with vehicle, 25 μM AZT, 3 μM Ritonavir, or 15 μM Lopinavir at the time of differentiation. After 16 hrs cells were harvested for protein. Cell lysates were immunoblotted for protein alterations indicating activation of the unfolded protein response.

A) Representative Western blot images shown of BiP, with quantification of band intensities normalized to GAPDH loading controls from 3 independent biological replicates graphed.

B-E) Levels of phosphorylated compared to total eIF2α protein B), cleaved compared to total ATF6β protein C), total IRE1α protein D), and phosphorylated compared to total IRE1α protein E), were each analyzed from three separate biological replicates (n=3). Quantification of band intensities normalized to GAPDH loading controls revealed no changes in any of these proteins following treatment with the antiretrovirals tested. Data are presented as mean ± SEM. One-way ANOVA followed by post-hoc Dunnett’s multiple comparison test determined statistical significance.
Figure 4.9: *In Vivo* Ritonavir Administration Leads to Reduction in Myelin Proteins

Brain lysates derived from our jugular vein administration model were immunoblotted for various myelin proteins: MBP, Myelin Oligodendrocyte Glycoprotein (MOG), CNP, Myelin-Associated Glycoprotein (MAG), and PLP. The drug regimen was composed of vehicle (DMSO) or 20 mg/kg/day of Ritonavir for 14 days.

A) Western Blot images from frontal cortex lysates are shown (vehicle n= 6, Ritonavir n= 7).

B) Quantification of band intensities normalized to FastGreen FCF reveals statistically significant decreases in MOG and CNP protein levels. Data are presented as mean ± SEM. Student’s *t*-test determined statistical significance for direct comparisons (#= *p* < 0.05).
Figure 4.10: Myelin Protein Expression in Humans is Not Affected by HIV Status

Lysates from prefrontal cortex specimens of HIV(-) controls, HIV(+) cART-naïve, and HIV(+) cART-mediated >12mo (HIV(+)/ART), were immunoblotted for myelin component proteins MBP, MOG, CNP, and MAG (n=20 for each group). Protein expression levels were determined by quantification of band intensities normalized to a FastGreen total protein loading control. Patient samples were stratified by HIV status with HIV(-) individuals compared with the HIV(+) cases, both cART-naïve and cART-mediated combined, with red data points indicating patients diagnosed with HAND. Black lines indicate mean ± SEM. Student’s t-test determined statistical significance for direct comparisons, result was not statistically significant.
Figure 4.11: Myelin Protein Expression in Humans is Affected by cART-Medication Status

Lysates from prefrontal cortex specimens of HIV(-) controls (n= 20), HIV(+) cART-naïve (n= 20), and HIV(+) cART-medicated >12mo (HIV(+)/ART) (n= 20), were immunoblotted for myelin component proteins MBP, MOG, CNP, and MAG. Protein expression levels were determined by quantification of band intensities normalized to a FastGreen total protein loading control.

A) Patient samples were stratified by HIV-treatment status, with red data points indicating patients diagnosed with HAND. Black lines indicate mean ± SEM. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance (##= p< 0.01).

B) Individuals satisfying criteria for HAND diagnosis were stratified by HIV-treatment status, Black lines indicate mean ± SEM. One-way ANOVA followed by post-hoc Newman-Keuls determined statistical significance (#= p< 0.05, ###= p< 0.001).
4.7 REFERENCES


CHAPTER 5

Monomethyl Fumarate (MMF), the Active Metabolite of an MS Therapeutic, Fails to Rescue Oligodendrocyte Maturation Deficits Induced by Oxidative Stress

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5.1 ABSTRACT

Events leading to autoimmune targeting of myelin in Multiple Sclerosis (MS) patients remain unknown, yet chronic oxidative stress is a pervasive disease feature. Tecfidera, (dimethyl fumarate), a recently released drug that reduces MS relapse rates is thought to target oxidative stress. Tecfidera is rapidly metabolized to monomethyl fumarate (MMF), which effectively promotes transcription factor Nrf2-mediated activation of the endogenous antioxidant response (EAR) in neurons and macrophages. Effects of MMF have not been examined in oligodendrocytes. Effective therapy will require both prevention of myelin loss and promotion of re-myelination through maturation of oligodendrocyte precursors. We have previously demonstrated that oxidative stress can halt oligodendrocyte differentiation. Also, we have shown that MMF can attenuate oxidative stress-induced neurotoxicity through EAR upregulation. We hypothesized that MMF may reverse stress-induced differentiation deficits in oligodendrocytes by EAR upregulation and subsequent attenuation of oxidative stress. Treatment of mouse oligodendrocyte precursors and mature oligodendrocytes with an oxidant resulted in accumulation of reactive oxygen species (ROS) and significant reduction in differentiation; however, EAR target genes were not upregulated. MMF significantly reduced ROS accumulation induced by multiple oxidants, but did not coincide with EAR activation. MMF also did not rescue maturation defects, as comparable significant decreases were evident in oxidant alone and oxidant+ MMF conditions. Our results suggest that oligodendrocytes do not properly activate the EAR to restore redox homeostasis, even after treatment with antioxidants such as MMF. As such, the therapeutic effect of MMF in patients is likely due a mechanism other than direct antioxidant effects on oligodendrocytes.

5.2 SUMMARY STATEMENT

The biological metabolite of Multiple Sclerosis drug Tecfidera neither activates the endogenous antioxidant response in oligodendrocytes, nor rescues oxidative stress-mediated differentiation deficits. These findings suggest that Tecfidera reduces relapse rates in patients via a mechanism other than direct antioxidant activity in oligodendrocytes, or that antioxidant effects are mediated through indirect actions of other cell populations.
5.3 INTRODUCTION

Multiple Sclerosis (MS) affects 2.3 million people worldwide, yet precipitating events leading to the autoimmune targeting of myelin in patients are not well understood. Symptoms arise from disruption of myelin integrity within white matter tracts in the brain, as the myelin membrane synthesized by CNS oligodendrocytes is vital for proper transmission of rapid neuronal impulses through their axons (For recent reviews see (Markowitz, 2013) and (Mallucci et al., 2015)). Cellular pathology includes both destruction of myelin sheaths and death of the associated oligodendrocytes, as well as axonal damage and neuronal cell death (Criste et al., 2014, Mallucci et al., 2015). In addition to disruption of proper action potential signal facilitation, impaired myelination can alter the trophic support provided to neurons from oligodendrocytes, hastening axonal degeneration (Lappe-Siefke et al., 2003, Edgar and Garbern, 2004, Criste et al., 2014, Mighdoll et al., 2015).

Despite the unknown etiology of MS, two well-documented pathological observations are chronic oxidative stress and inflammation (reviewed in (Friese et al., 2014, Mahad et al., 2015)). Studies have also identified oxidative damage as one of the key driving elements in the aberrant loss of myelination observed in children who suffer from periventricular white matter injury (PWMI) (Back and Rosenberg, 2014). This brain damage occurring in pre-term infants targets white matter in particular, and is a major contributor to subsequent neurological dysfunctions, which commonly includes cerebral palsy (Volpe, 1981, 2001). While PWMI can be triggered by several independent events, one of the principal causes is lack of cerebral oxygenation, which leads to the generation of oxidative stress and cell death of the particularly vulnerable oligodendrocyte precursor cells (OPCs) (Back and Rosenberg, 2014). In several perinatal rat hypoxia/ischemia models, reactive oxygen species generated by the insult resulted in injury and death of OPCs, which led to subsequent decreased numbers of mature oligodendrocytes and hypomyelination of white matter (Levison et al., 2001, Reid et al., 2012). Additionally, in vitro studies have demonstrated maturation-stage specific vulnerability to oxidative stress, with OPCs and developing immature oligodendrocytes displaying significantly more sensitivity than mature oligodendrocytes due to lower levels of glutathione and antioxidant enzymes, as well as higher rates of oxygen consumption (Halliwell, 1992, Back et al., 1998, Fern and Moller, 2000, Baud et al., 2004).
In the developing brain, loss of this critical OPC population profoundly impacts the total capacity of initial myelination. Potential depletion of the pool of OPCs in the adult from chronic oxidative stress has similar repercussions for the capability for effective re-myelination of affected areas in MS. In addition to OPC dropout due to oxidative stress-induced toxicity, we have previously demonstrated that at sub-toxic levels, oxidative stress generated by multiple compounds halts oligodendrocyte differentiation (French et al., 2009). Together, these reports highlight the necessity for reduction of oxidative stress to facilitate recovery of function in demyelinating conditions, as the pool of cells responsible for re-myelination are negatively affected both by functional and toxicity-based mechanisms.

The recently FDA-approved drug Tecfidera (BG-12, (dimethyl fumarate)) has been demonstrated to reduce relapse rates in MS patients (FDA, 2013, Limmroth, 2013, Fox et al., 2014, Dubey et al., 2015, English and Alo, 2015). Intriguingly, while suspected to act as an antioxidant, the mechanism of action by which this compound is effective has not been elucidated. The chemical formulation of this drug is dimethyl fumarate (DMF), a fumaric acid ester, which is rapidly metabolized to monomethyl fumarate (MMF) (Litjens et al., 2004, Schmidt et al., 2007, Limmroth, 2013, Fox et al., 2014). Within minutes of administration, MMF is the primary metabolite found in blood plasma, with DMF no longer detectable (Schmidt et al., 2007). Critically, marked reduction of macrophage recruitment into actively demyelinating brain regions was observed following oral DMF administration in a rodent experimental autoimmune encephalomyelitis model of MS, demonstrating that therapeutic levels of MMF cross the blood brain barrier (Schilling et al., 2006). In neurons and macrophages, antioxidant effects of MMF have been reported, through NF-E2 (nuclear factor (erythroid-derived 2))-related factor-2 (Nrf2) mediated activation of the endogenous antioxidant response (EAR) and upregulation of target antioxidant and detoxifying genes (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012, Akay et al., 2014). We have demonstrated successful MMF amelioration of oxidative stress-mediated neuronal toxicity through such activation (Akay et al., 2014). Surprisingly, the direct effects of MMF on signaling pathways of primary oligodendrocytes, the initially affected cell population in MS pathology, have not been explored.
Here, we investigated whether MMF was capable of reducing oxidative stress and upregulating the EAR in oligodendrocytes, and if this led to a reversal of stress-induced deficits in differentiation. Our results indicate that oligodendrocytes do not activate the EAR under conditions of oxidative stress to restore redox homeostasis. Furthermore, in contrast to other CNS-resident cell populations, MMF does not upregulate this pathway in oligodendrocytes through Nrf2-mediated transcription of canonical EAR target genes. These findings suggest that the beneficial effect of Tecfidera in MS patients is likely due to a mechanism other than direct antioxidant effects on oligodendrocytes, either through alternative anti-inflammatory pathway actions, or through upregulation of EAR proteins in other cell types which act cell non-autonomously to provide protection to mature oligodendrocytes and OPCs differentiating to restore insufficient myelination.

5.4 MATERIALS AND METHODS

Chemicals and Reagents

selenium, tert-Butyl hydroperoxide (tBHP), thyroxine (T4), transferrin. Vector Laboratories (Burlingame, CA): Vectashield with DAPI.

Additional antibodies: anti-galactocerebroside mouse hybridoma supernatant (GalC H8H9, (Ranscht et al., 1982)), anti-myelin basic protein rat hybridoma supernatant (MBP, gift of Virginia Lee, University of Pennsylvania).

**Primary Mouse Oligodendrocyte Cultures**

All experiments were performed following the guidelines set forth by The Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee (IACUC). Primary oligodendrocyte precursor cell cultures were generated from postnatal day 1 CD1 mouse pups with modifications from previously described methods (See et al., 2004). Briefly, cortical cell suspensions isolated from pups obtained from Charles River Laboratories were plated on poly-D-lysine (PDL) coated flasks in Neurobasal Medium with B27 supplement at 37°C with 5% CO2. Cells were switched into growth medium consisting of Neurobasal Medium +B27 supplemented with 10 ng/ml bFGF, 2 ng/ml PDGF-AA, and 1 ng/ml NT-3 24 hours after isolation. After growing to confluence, cultures were purified to 90-95% oligodendrocyte precursor cells (OPCs) and 5-15% astrocytes using a wash-down procedure (Feigenson et al., 2009), after which cells were sub-cultured onto PDL-coated coverslips or Petri dishes. To facilitate transition of OPCs into maturing oligodendrocytes, growth medium was replaced with differentiation medium, consisting of 50% DMEM, 50% Ham’s F12, Pen/Strep, 2 mM glutamine, 50 µg/ml transferrin, 5 µg/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 µg/ml insulin, 0.4 µg/ml T4, 0.3% glucose, and 10 ng/ml biotin (Feigenson et al., 2009).

**Detection of Reactive Oxygen Species**

Dihydroethidium (DHE) was used to detect the presence of reactive oxygen species *in vitro* for cells grown on coverslips, as oxidation of DHE by superoxide allows for intercalation with nuclear DNA, and emission of a quantifiable red fluorescence (Zhao, 2003). 3 µM DHE was added to the culture medium 15 minutes prior to the end of treatments. Cells were then washed with F12 medium, fixed with ice-cold acid alcohol
for 8 minutes, re-washed, and then mounted on slides with Vectashield containing DAPI to stain nuclei. Ten non-overlapping fields per coverslip were imaged from 3 independent wells for each treatment from at least 3 biologically independent primary cell preparations. The Advanced Fluorescence Leica Application Suite was used for image acquisition, coupled with a Leica DM6000B fluorescence microscope equipped with a DFC240 camera at 40x magnification (HCX PL APO 40x/0.85 CORR objective). MetaMorph 6.0 software (Molecular Devices, Sunnyvale, CA) was used for post-acquisition analysis, which determined the fluorescence intensity of nuclear intercalated DHE normalized to the total DAPI area.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The expression of Heme Oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase (NQO1), NF-E2 (nuclear factor (erythroid-derived 2))-related factor-2 (Nrf2), and Myelin Basic Protein (MBP) mRNA were quantified by qRT-PCR. For endogenous antioxidant response targets HO-1, NQO1, and Nrf2, OPC cultures or 72 hour matured oligodendrocytes grown on 100-mm dishes were harvested 6 hours following treatment. To isolate RNA for myelin component protein MBP, cells were treated at the time of differentiation and harvested 72 hours later. Following Trizol extraction, 5 µg of RNA was converted to cDNA (Invitrogen Superscript First-strand kit). q-PCR was performed as previously described using Power SYBR Green, with samples measured in triplicate from three independent biological replicates (n=3) (French et al., 2009, Feigenson et al., 2011). Data was analyzed according to the ΔΔCT method, with normalization to Protein Kinase Gene 1 (PKG1). Table 5.1 details the primer pairs for each gene, obtained from Integrated DNA Technologies (IDT).

Immunoblotting

Whole-cell extracts were prepared from primary mouse oligodendrocyte cultures using ice-cold lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 10 mM EDTA, 0.5 mM Na₃VO₄, and 1:100 protease inhibitor cocktail), followed by centrifugation at 14,000 rpm at 4°C for 30 minutes. Protein concentrations were determined using a NanoDrop 1000 Spectrophotometer A280 absorbance assay. A broad spectrum molecular weight ladder was run alongside treatment samples containing 25-50 µg of protein per lane of 4-12% Bis-Tris gradient gels for separation. Following electrophoresis, proteins were transferred onto
Immobilon-FL membranes (Millipore), and blocked in PBS with 0.1% Tween-20 (PBST) and 5% milk for 20 minutes at room temperature (RT). Membranes were then incubated overnight with primary antibodies in PBST+ milk at 4°C. The following primary antibodies were utilized: HO-1 (1:1000 dilution), NQO1 (1:1000 dilution), and MBP (SMI-99, 1:500 dilution). Membranes were rinsed with PSBT, re-blocked for 20 minutes at RT in PBST+ milk, then incubated with secondary antibodies with corresponding antigen-specific fluorescent probes (1:10,000 in PBST+ milk). An Odyssey Infrared Imaging System (LiCOR) was used to visualize membranes. Re-blotting for GAPDH (1:1000 dilution) obtained loading controls for each membrane. Pixel intensities for each treatment were determined by quantifying specified bands of interest using the NIH ImageJ program (V1.36b, Bethesda, MD). These values were normalized to those obtained from analysis of loading control GAPDH.

**Immunofluorescence**

Detection of specific antigens for primary oligodendrocyte lineage cells grown on coverslips was performed as previously described (Grinspan and Franceschini, 1995, See et al., 2004). Live cells were labeled for cell surface marker detection prior to fixation using anti-GalC (mouse hybridoma supernatant, undiluted) followed by FITC-conjugated goat anti-mouse IgG3 (1:100 dilution). Following acid alcohol fixation and permeabilization, internal antigens were detected using anti-MBP (rat hybridoma supernatant, 1:2 dilution) followed by Rhodamine-conjugated goat anti-rat IgG (1:100 dilution). Vectashield with DAPI was used to mount slides while also staining all cell nuclei. Images were acquired as previously described for DHE staining, with 10 non-overlapping fields in each of 3 coverslips per condition from at least 3 independent primary cell isolations. Antigen-positive and DAPI-positive cells were counted from each sampled field, with approximately 2000 cells counted per treatment condition.

**Statistical Analysis**

Prism 5.0 software (GraphPad Software, San Diego, CA) was used to analyze all data. Values are expressed as mean ± SEM, with \( p < 0.05 \) considered significant. Student’s \( t \)-test or One-way ANOVA with post-hoc Dunnett’s multiple comparison test were performed where indicated.
5.5 RESULTS

Reactive oxygen species accumulate in oligodendrocytes following oxidant treatment

There is a large body of evidence gathered from in vitro, animal, and human patient studies to suggest that oligodendrocyte precursor cells (OPCs) and immature oligodendrocytes are particularly vulnerable to oxidative stress (Halliwell, 1992, Back et al., 1998, Fern and Moller, 2000, Levison et al., 2001, Baud et al., 2004, Back and Rosenberg, 2014). It critical to understand how oxidative stress affects the processes of differentiation and myelination of these susceptible precursor cells. The process of oligodendrocyte maturation is well defined, with tightly regulated transcription factor activity and characteristic cellular markers corresponding to specific stages of differentiation utilized to track lineage progression (Miller, 2002). Previous work within our laboratory determined that oligodendrocyte differentiation is halted following oxidant treatment using these established methods (French et al., 2009, Reid et al., 2012).

Surprisingly, the mechanism by which oligodendrocytes respond to oxidative stress is not understood. To model oxidative stress in an in vitro model of oligodendrocyte differentiation, we used the oxidant tert-butyl hydroperoxide (tBHP), a cell-permeable compound that generates tert-butoxyl radicals, resulting in depletion of intracellular glutathione and lipid peroxidation (Back et al., 1998, Zhao et al., 2005, French et al., 2009). Oligodendrocyte lineage cells were isolated as OPCs and induced to differentiate over a 72 hour period into maturing oligodendrocytes (OLs) following our previously described maturation paradigm (See et al., 2004). OPCs and 72hr differentiated OLs were exposed to 2.5 µM tBHP for 30 minutes, 1 hour, 2 hours, 6 hours, 12 hours, or 24 hours. Cells were then stained with the ROS indicator dihydroethidium, a cell-permeable fluor that intercalates into DNA when oxidized to ethidium (Zhao, 2003). Following the timecourse outlined above, we found that tBHP treatment produced robust ROS accumulation by 1 hour in both OPCs (677% ± 76.2%, p < 0.01) (Figure 5.1A), and in OLs (3451% ± 1454%, p < 0.01) when compared with untreated (Figure 5.1B).

Oxidative insult does not upregulate the EAR in oligodendrocytes

High levels of ROS were evident following application of the oxidant tBHP in both OPCs and mature OLs. In many cell types including neurons and astrocytes, when such a cellular imbalance in redox homeostasis
occurs, there is activation of the endogenous antioxidant response (EAR) (Chen et al., 2009, Li, 2009). We next examined whether oligodendrocyte lineage cells activated this cellular signaling pathway following exposure to oxidants and subsequent ROS accumulation. OPCs or OLs were treated with tBHP for 6 or 16 hours and assayed for EAR targets by qPCR and immunoblot analysis. Quantification of mRNA following tBHP application to OPCs revealed no significant changes after 6 hours in the principle targets of the EAR: Nrf2, Heme Oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase (NQO1) (Table 5.2). Consistent with these results, immunoblotting for HO-1 and NQO1 following 16 hours of treatment also revealed no significant changes in protein levels (Figure 5.2A-B). In mature OLs, analysis of the EAR targets also revealed no changes in mRNA levels after 6 hours of tBHP treatment (Table 5.2). Similarly, following 16 hours of treatment, no significant alterations in HO-1 or NQO1 protein levels were observed (Figure 5.2C-D). Therefore, in contrast to many other cell types, both oligodendrocyte precursors and mature OLs do not appear to activate the EAR despite robust accumulation of ROS.

Oxidative Insult Inhibits Oligodendrocyte Maturation

In our previous work we described alterations in levels of transcription factors responsible for induction of oligodendrocyte differentiation, and a consequent reduction in the number of cells expressing immature oligodendrocyte cell surface marker Galactocerebroside (GalC) following treatment with 5 µM tBHP (French et al., 2009). Here we have expanded on those findings to encompass markers for mature oligodendrocytes by immunofluorescent staining, mRNA, and protein evidence using our differentiation paradigm. Following tBHP treatment for 72 hours in differentiation medium, immunofluorescent staining reveals that the lower dose of 2.5 µM tBHP used in the present study significantly reduced the number of cells expressing differentiation markers, while not reducing total cell number. Representative images of untreated and tBHP treated oligodendrocytes 72 hours after differentiation illustrate differentiation deficits, with extent of maturation determined by quantifying the percentage of GalC+ and MBP+ cells across treatments (Figure 5.3A). Following tBHP treatment, the number of cells expressing the immature marker GalC was significantly reduced by 23.11% ± 5.4% (p < .01). Similarly, the number of MBP+ mature oligodendrocytes was significantly reduced by 22.42% ± 6.71% (p < .05). The number of total cells in the tBHP treated condition was not significantly affected (Figure 5.3A).
We next examined whether this reduction in maturation at the cell surface was reflected in a reduction in myelin basic protein (MBP) mRNA and protein. OPCs were treated with tBHP at the time of differentiation, with mRNA and protein harvested 72 hours later. Analysis determined that while a significant upregulation of MBP mRNA was observed between undifferentiated OPCs and 72 hour mature OLs \( (p < .05) \), the levels of MBP mRNA were comparable between the untreated OLs and the tBHP treated condition (Figure 5.3B). At this same point in differentiation, the amount of MBP protein in the untreated oligodendrocytes had significantly increased by approximately 2 fold over OPCs \( (p < 0.05) \). Notably, tBHP treatment resulted in a dramatic reduction in MBP protein expression levels when compared with untreated \( (p < 0.05) \) and no significant increase observed when compared with OPCs (Figure 5.3C). Taking these results together, we conclude that oxidative stress induced by tBHP halts oligodendrocyte maturation by altering expression of myelin component proteins levels.

**MMF reduces ROS accumulation induced by various oxidant compounds**

Oxidative stress generated by tBHP caused the accumulation of ROS and differentiation deficits in maturing OPCs without activation of the EAR. Therefore, we next determined if attenuating ROS would prevent the oxidant-induced maturation blockade. We utilized the fumaric acid ester monomethyl fumarate (MMF), which is the active metabolite of the FDA approved MS therapeutic compound dimethyl fumarate (FDA, 2013, Limmroth, 2013). In neurons and macrophages, MMF behaves as an antioxidant through Nrf2-mediated EAR activation (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012, Akay et al., 2014). We have previously demonstrated MMF-mediated protection from oxidative stress-induced toxicity in neurons through EAR pathway activity (Akay et al., 2014). In order to determine whether MMF is effective at reducing oxidant-induced ROS accumulation, OPCs were pre-treated with 10 \( \mu \)M MMF for 6 hours to allow for potential upregulation of EAR target proteins. Cells were then treated with oxidants for an additional 2 hours. In addition to 2.5 \( \mu \)M tBHP, 100 \( \mu \)M hydrogen peroxide, and 100 \( \mu \)M L-buthionine sulfoximine (BSO) were utilized. Hydrogen peroxide is a strong, oxidizing agent which will generate abundant highly reactive hydroxyl radicals within the cell (Sies, 1993). BSO was selected as it is a selective inhibitor of \( \gamma \)-glutamylcysteine synthetase, the enzyme which catalyzes the rate-limiting step of glutathione synthesis (Meister, 1992, French et al., 2009). This set of compounds allows for analysis
of oxidants with various properties, and can potentially differentiate between effects caused by tBHP and BSO-mediated glutathione depletion versus whole-cell effects caused by hydroxyl radical-based modifications. Following treatment, DHE staining revealed that MMF pre-treatment significantly reduced the amount of ROS accumulation induced by tBHP (Figure 5.4A), hydrogen peroxide (B), and BSO (C).

**MMF fails to rescue differentiation defect caused by oxidative insult**

MMF was successful at reducing the accumulation of ROS in OPCs induced by multiple oxidant compounds. We next assessed whether this reduction of oxidative stress would increase the number of mature oligodendrocytes following oxidant treatment. OPCs were pre-treated with MMF for 24 hours, after which they were switched into differentiation medium and treated with the oxidants as previously described. MMF was replenished at the time of differentiation, and at 24 and 48 hours. After 72 hours of differentiation, cells were stained for GalC and MBP to assess maturation. Significantly fewer cells expressed the immature marker GalC following treatment with each oxidant, with significantly fewer cells expressing the mature marker MBP with tBHP and hydrogen peroxide treatment (Figure 5.5A-B). Notably, concomitant treatment with MMF did not rescue the observed maturation deficits caused by any of the oxidants. The effects of BSO were milder than that of the other two oxidants, with modest though not significant reduction in percentage of cells maturing to the MBP+ stage (Figure 5.5B). At this dose, hydrogen peroxide appears to cause both a significant loss of cells due to toxicity (Figure 5.5C) as well as an oxidant-mediated inhibition of differentiation, as a negligible number of the remaining cells progressed to either the GalC+ or MBP+ maturation stages (Figure 5.5A-B). Together these results suggest that despite effectively reducing ROS accumulation and oxidative stress, MMF failed to rescue the oligodendrocyte differentiation deficits caused by several oxidant compounds.

**MMF does not upregulate components of the EAR in developing oligodendrocytes**

While MMF has been shown to upregulate components of the EAR in other CNS cell populations (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012, Akay et al., 2014), the direct effects of this compound on the EAR pathway of primary oligodendrocytes have not been examined. In order to investigate if MMF acts to stimulate the EAR pathway in oligodendrocytes, we examined
expression of downstream EAR targets in maturing oligodendrocytes following MMF application over time. OPCs were treated with 10 µM MMF at the time of differentiation. Lysates were harvested for protein analysis at 0, 2, 6, 12, and 24 hours. Surprisingly, neither HO-1 (Figure 5.6A) nor NQO1 (Figure 5.6B) protein levels were increased in MMF treated cells at any time point tested. We do not discount that EAR upregulation may occur at a later time interval. We feel that this is unlikely however, as in our neuroglial culture studies we saw effects within 6 hours, and MMF replenishment was required every 24 hours to maintain efficacy (Akay et al., 2014). Therefore, in contrast to other CNS cell types, MMF does not upregulate the EAR in oligodendrocytes.

5.6 DISCUSSION

Multiple Sclerosis is a devastating demyelinating condition for which the etiology is still not understood. At present, therapies which reduce chronic levels of oxidative stress and inflammation have been aimed at restoring quality of life and reducing relapse rates in patients (Friese et al., 2014, Mahad et al., 2015). Here, we investigate the consequences of oxidative stress on oligodendrocyte maturation, and the potential actions of an FDA approved MS therapeutic compound, which has been proposed to act through antioxidant means. This compound, Tecfidera, potentially works by boosting activation of Nrf2 transcription factor-mediated induction of the endogenous antioxidant response (EAR) element in the promoters of a specific cohort of genes which clear excess reactive oxygen species (ROS) from cells. The regulation of this pathway in oligodendrocytes and its role in directly decreasing oxidative damage in oligodendrocytes has not previously been tested. We provide compelling evidence that exposure to oxidants produces ROS accumulation in both oligodendrocyte precursors and mature oligodendrocytes, yet unlike other neural cell types fails to elicit a consequential activation of the endogenous antioxidant response element. As a possible result of this failure, when OPCs are exposed to an oxidant at the time of differentiation, they are unable to differentiate into immature and then mature oligodendrocytes and do not express myelin proteins nor traffic them appropriately for insertion into their cell membranes. We have shown that MMF, the active metabolite of Tecfidera (BG-12, dimethyl fumarate), successfully attenuated ROS accumulation in OPCs and mature oligodendrocytes caused by various oxidant compounds as anticipated. However, MMF failed to rescue oxidative-stress mediated differentiation deficits induced by
these compounds. Contrary to expectations, based on previously reported evidence in cells of a wide variety of neuroglial cell lineages (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012, Akay et al., 2014), MMF did not upregulate EAR target genes at any time during an extended timecourse. These observations may suggest that the antioxidant effects of Tecfidera in reducing MS relapse rates by lowering oxidative stress levels to allow for oligodendrocyte survival and maturation are indirect and mediated through other neural cell types.

The mechanism by which myelin is initially targeted in MS is not understood, however it is clear that once initiated, chronic inflammation and oxidative stress drive much of the pathology. Activated immune cells secrete a variety of pro-inflammatory cytokines and reactive oxygen species (Haider, 2015). Immature oligodendrocytes and oligodendrocyte precursors in particular are exceptionally vulnerable to oxidative insults due to high energy demands and low glutathione and antioxidant enzyme levels (Halliwell, 1992, Back et al., 1998, Fern and Moller, 2000, Baud et al., 2004). Disruption of myelin caused by the effects of oxidative stress on oligodendrocytes can then lead to degeneration, both of affected oligodendrocytes and the neurons they no longer effectively insulate. Concomitantly, oxidative stress stops OPCs from differentiating and replacing the damaged mature oligodendrocytes. The breakup of myelin and cellular loss can then lead to further macrophage recruitment to clear cellular debris, thus perpetuating the cycle (Friese et al., 2014, Mahad et al., 2015). The primary cellular response leading to attenuation of oxidative stress is the EAR pathway, through targeted Nrf2-mediated transcriptional upregulation of genes with an antioxidant response element in their promoter regions (Li, 2009). Activation of this pathway increases the expression of Phase II detoxifying enzymes and antioxidants, including NADP(H) quinone oxidoreductase (NQO1) and Heme oxygenase-1 (HO-1) (Li, 2009).

The consistent observation of oxidative stress in active demyelinating MS lesions prompted investigation into whether cells within these regions upregulate their EAR in an effort to combat the persistently high levels of ROS. The Nrf2 target protein, NQO1, was found to be highly abundant in chronic and active lesions, though this was restricted to the astrocyte and macrophage cell populations (van Horssen et al., 2006). In animal models of MS, HO-1 was found in high abundance in macrophages, microglia, and
astrocytes during the acute phase of injury, and in oligodendrocytes in early lesions (Stahnke et al., 2007). Recently, the presence of Nrf2 and its targets was identified in oligodendrocytes in MS lesions. Licht-Mayer et al examined active demyelinating regions from MS patients and found strong nuclear expression of the transcription factor Nrf2 and its downstream target HO-1 in oligodendrocytes, though the highest enrichment of these proteins is found in degenerating cells (Licht-Mayer et al., 2015). The authors comment that because Nrf2 is already highly expressed in oligodendrocytes in active lesions, it is doubtful that further stimulating the ARE by fumarates would protect oligodendrocytes in any way. Additionally, it can be difficult to draw conclusions regarding stress response proteins in diseased tissue, as cells viewed from a single point in time can either be effectively combatting the unfavorable stressful situation, or fated for cell death after an unsuccessful attempt at overcoming such conditions.

In contrast to other cell types examined, little is known about the protective effects of the EAR in oligodendrocytes. Studies using OLN-98 cells and primary rat oligodendrocytes exposed to hydrogen peroxide have demonstrated HO-1 upregulation by immunoblotting (Goldbaum and Richter-Landsberg, 2001, Stahnke et al., 2007). However, the oxidative insult applied also culminated in loss of mitochondrial function and apoptosis, suggesting this upregulation was not sufficient to protect the cells (Goldbaum and Richter-Landsberg, 2001). In our current work, we have examined the role of the EAR in protecting oligodendrocytes from oxidative stress in more detail. We demonstrated that ROS accumulate in OPCs and mature OLs following oxidant exposure, yet this failed to promote upregulation of the EAR at the mRNA or protein level in either stage of the oligodendrocyte lineage. Furthermore, exposure of OPCs to an oxidant at the time of differentiation resulted in reduced maturation evaluated both by myelin protein expression, as well as characteristic maturation-stage specific surface marker levels. Within this background, we attempted to exogenously activate the EAR by directly treating oligodendrocyte lineage cells with a newly approved drug aimed at ameliorating the chronic inflammation and oxidative stress seen in MS.

Tecfidera effectively reduces MS relapse rates (FDA, 2013, Limmroth, 2013, Fox et al., 2014, Dubey et al., 2015, English and Aloï, 2015). This drug is composed of dimethyl fumarate (DMF), yet despite much investigation, the cellular mechanism by which this compound is effective remains elusive. Antioxidant
effects of DMF have been reported from in vitro studies in neurons, with Nrf2-mediated upregulation of EAR target genes such as HO-1 conferring effective protection from oxidant challenge (Lehmann et al., 2007, Albrecht et al., 2012, Wang et al., 2015). It is also suggested through these studies that DMF culminates in antioxidant effects by directly interacting with glutathione via Michael-addition and affecting glutathione recycling. Reduced to oxidized glutathione ratio serves as a critical redox homeostasis sensor within the cell, and modulates amounts of xenobiotic and antioxidant enzymes accordingly (Ishii and Mann, 2014, Espinosa-Diez et al., 2015). Normally, following electron donation oxidized glutathione is reduced back to its original form by glutathione reductase (Ishii and Mann, 2014, Espinosa-Diez et al., 2015). In the process of Michael-addition however, such as that which occurs between the Michael-donor glutathione and Michael-acceptor dimethyl fumarate, a new carbon bond is formed between the nucleophile glutathione the α,β-unsaturated carbon bond present within the DMF molecule, preventing subsequent glutathione recycling (Lehmann et al., 2007, Albrecht et al., 2012, Wang et al., 2015). By depleting the cellular store of this critical redox regulator, DMF causes a transient oxidative stress within the cell and consequently an upregulation of EAR pathway activity (Lehmann et al., 2007, Albrecht et al., 2012).

Within our present study, we chose to evaluate the effects of the primary metabolite of DMF, monomethyl fumarate (MMF). A critical and previously overlooked element of many studies aiming to determine the mechanism of action of Tecfidera, is that DMF is extremely rapidly metabolized when administered orally, so that a vast majority of the effects seen in animal models and in human patients are likely caused by metabolic byproducts and not DMF directly. In vivo animal studies have shown that DMF is rapidly hydrolyzed to MMF or adducted to glutathione via Michael-addition (Schmidt et al., 2007). In rodents, when given orally no detectable levels of free DMF are found in blood plasma (Schmidt et al., 2007), and similarly when administered directly to the small intestine none can be detected in the portal vein (Dibbert et al., 2013). MMF is the dominant metabolite generated by DMF breakdown (Litjens et al., 2004, Dibbert et al., 2013). Similar to the findings from DMF in vitro studies, MMF has also been demonstrated to upregulate Nrf2-mediated EAR activity in neurons and macrophages (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012, Akay et al., 2014). Contrary to expectations, MMF did not upregulate Nrf2-pathway antioxidant genes HO-1 or NQO1 at any point during a 24-hour timecourse in
oligodendrocytes, and was unsuccessful at rescuing cells from an oxidant-induced maturation deficit. In an experimental model of MS, in which animals ingesting the copper chelator cuprizone develop a specific and reversible demyelinating lesion in the corpus callosum, boosting the Nrf2 pathway with either DMF or MMF had only minor effects on remyelination and were not protective against cell death (Moharregh-Khiabani et al, 2010). While in vitro differentiation was not examined by this group, they also demonstrated that pre-application of DMF or MMF to an oligodendrocyte–like cell line that was then treated with hydrogen peroxide showed no rescue from oxidative stress-induced toxicity (Moharregh-Khiabani et al, 2010). The lack of activation for this supposedly ubiquitous cellular stress response pathway to a variety of oxidant and antioxidant compounds which cause target gene upregulation in other cell types may help to explain the selective vulnerability of oligodendrocytes to oxidative stress.

One possible explanation for the lack of EAR pathway activation observed in the current work regards the regulation of glutathione synthesis and recycling in oligodendrocytes. The oxidant utilized in the initial component of this study to investigate the effects of oxidative stress was tBHP, which has been demonstrated to deplete cellular glutathione (Back et al., 1998, Zhao et al., 2005, French et al., 2009). In later experiments, the oxidant BSO was used, which selectively inhibits glutathione synthesis (Meister, 1992, French et al., 2009). Hydrogen peroxide was also used, which does not have as targeted effects with the cell, but rather generates highly reactive hydroxyl radicals which interact with proteins, lipids, as well as reducing agents such as glutathione (Sies, 1993). MMF was unable to rescue differentiation deficits caused by the cellular effects induced by any of these oxidants. While higher concentrations than DMF were required to attain comparable cellular effects, MMF itself has also been shown to reduce cellular glutathione levels in astrocytes (Schmidt and Dringen, 2010). The isoform of the enzyme responsible for glutathione conjugation to electrophilic compounds, glutathione s-transferase (GST), expressed in oligodendrocytes is GST-pi. Within the brain, this form of GST is uniquely found in oligodendrocytes (Cammer et al., 1989, Cammer and Zhang, 1992), and has been widely used as a marker for mature oligodendrocytes in vivo (Girolamo et al., 2011, Li et al., 2013, Yatomi et al., 2015). Furthermore, immunofluorescent studies have demonstrated that GST-pi is found in the nucleus of OPCs, and during differentiation attains a cytoplasmic distribution, which may account for some of the sensitivity of
oligodendrocytes to oxidative stressors during early lineage stages (Tamura et al., 2007). It may be that the glutathione system for monitoring redox homeostasis operates differently in oligodendrocytes, such that if levels are depleted the EAR is not effectively activated, particularly in OPCs and maturing cells. Interestingly, a recent study has demonstrated that DMF treatment can increase the glutathione level in a human oligodendrocyte cell line, suggesting that fumarate compounds can potentially impact cells of the oligodendrocyte cell lineage, although how metabolism in this cell line compares to primary cells is unknown (Huang et al., 2015). In our study, neither tBHP nor MMF caused EAR pathway upregulation. It will be intriguing to discover if alternative oxidants, which impact redox sensors other than glutathione, are capable of EAR upregulation in primary oligodendrocytes, or if the canonical pathway is not present in this cell lineage.

With this in mind, the mechanism of action by which Tecfidera reduces MS relapse rates is likely not due to direct effects on oligodendrocytes. Alternatively, secondary effects from upregulation of the EAR in neurons and macrophages may play a role in oligodendrocyte protection (Cross et al., 2011, Linker et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012, Akay et al., 2014). Furthermore, while not examined in the present work, the effects of Tecfidera may also be modulated by a DMF-mediated reduction of secreted inflammatory cytokines (Lehmann et al., 2007, Albrecht et al., 2012) or inhibition of the NF-κB pathway (Gillard et al., 2015).

Reducing levels of chronic oxidative stress is key to enabling functional recovery for affected patients, as remyelination will not proceed unless conditions are favorable. Our study suggests that reduction in oxidative stress will most likely require the concerted efforts of multiple CNS cell populations, with neurons, macrophages, and/or astrocytes together providing an environment free of oxidative stress with restored redox homeostasis for the susceptible oligodendrocyte cell population to survive, mature, and remyelinate affected areas.
5.7 ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
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| PKG1     | Forward: 5’ ATG CAA AGA CTG GCC AAG CTA C 3’  
          | Reverse: 5’ AGC CAC AGC CTC AGC ATA TTT C 3’   |
| HO-1     | Forward: 5’ TGT AAG GGA GAA TCT TGC CTG GCT 3’  
          | Reverse: 5’ TGC TGG TTTCAA AGT TCA GGC CAC 3’   |
| NQO1     | Forward: 5’ AAG AGC TTT AGG GTC GTC TTG GCA 3’  
          | Reverse: 5’ AGC CTC CTT CAT GGC GTA GTT GAA 3’   |
| Nrf2     | Forward: 5’ TCA CAC GAG ATG ATG AGC TTA GGG CAA 3’  
          | Reverse: 5’ TAC AGT TCT GGG CGG CGA CTT TAT 3’   |
| MBP      | Forward: 5’ TGG TAC ACA CTA ACC TCG GTG GAA 3’  
          | Reverse: 5’ AGC GAC TCG ATT CAG TGA CAG GAA 3’   |

Table 5.1: q-RT PCR Primers

Primer pairs used for qRT-PCR are shown. Abbreviations are: PKG1, Protein Kinase Gene 1; HO-1, Heme Oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, NF-E2 (nuclear factor (erythroid-derived 2))-related factor-2; MBP, Myelin Basic Protein. Primers were obtained from IDT.
### Table 5.2: mRNA alterations in Endogenous Antioxidant Response Genes Following 6hr tBHP Insult

Extracted RNA (5 µg) was converted to cDNA using the Superscript First-strand kit and q-PCR was performed using Power SYBR Green. All measurements were normalized first to the oligodendrocyte housekeeping gene PKG1, and then to controls. Analysis was performed by the ΔΔCT method. Endogenous Antioxidant Response genes: Nrf2, HO-1, and NQO1 were probed. ΔΔCT ±SEM are presented from 3 independent biological replicates (n=3), with p-values indicated.
Figure 5.1: Oxidant tBHP Induces Reactive Oxygen Species in OPCs and Mature Oligodendrocytes

Cells were treated with 2.5 µM tBHP for 1 hour to determine if reactive oxygen species (ROS) were produced. ROS accumulation was assayed by DHE staining, with cell nuclei stained by DAPI. Images were captured at 40x magnification, with 10 fields on 3 coverslips per condition. Scale bar indicates 25 µm. Metamorph image analysis determined DHE fluorescence intensity normalized to nuclear DAPI area.

A) Representative images of 1 hr treated OPCs, with DHE fluorescence (red) and cell nuclei stained with DAPI (blue), and quantification of this experiment.

B) Representative images of 1 hr treated mature oligodendrocytes, with DHE fluorescence (red) and cell nuclei stained with DAPI (blue), and quantification of this experiment.

Representative experiments shown from n=2 biological replicates, values are presented as mean ±SEM. Student’s t-test determined statistical significance (##= p< 0.01).
**Figure 5.2: Oxidative Insult does not Upregulate the EAR in Oligodendrocytes**

Protein was harvested from OPCs (A-B) or Mature Oligodendrocytes (C-D) 16 hours following 2.5 µM tBHP treatment. Cell lysates were immunoblotted for target proteins upregulated upon activation of the endogenous antioxidant response: HO-1 (A and C) and NQO1 (B and D). Representative Western blot images are shown, with quantification of band intensities normalized to GAPDH loading controls revealing no change between untreated and tBHP treated conditions. Data are presented as mean ± SEM from 4 separate cultures (n= 4). Student’s *t*-test determined statistical significance.
Primary mouse OPCs were put into differentiation medium (DM) and treated with 2.5 µM tBHP. After 72 hours, cells were stained for stage-specific markers or RNA or protein was extracted to evaluate levels of myelin components.

A) Cells on coverslips were stained with DAPI (blue) for total cell nuclei and with antibody to GalC (green) and MBP (red). Representative images are shown for untreated and tBHP treated conditions, with merged images in the rightmost column. Scale bar indicates 25 µm. Epifluorescent images were captured, with 10 fields on three coverslips per condition, from n=4 independently prepared cultures. Immature oligodendrocytes (GalC+ cells), mature oligodendrocytes (MBP+ cells), and total cell number (DAPI+ nuclei) were counted using ImageJ software, represented as percentage normalized to untreated. Data are presented as mean ±SEM. Student’s t-test determined statistical significance. Significance compared with control (##= p< .01, #= p< 0.05).

B) RNA was extracted 72hours following addition of DM and tBHP treatment. cDNA conversion, qPCR, and analysis were performed as in Table 2. Expression of the gene for MBP was probed. ΔΔCT ±SEM are presented from 3 independent biological replicates (n = 3). Student’s t-test determined statistical significance. A significant increase was found when precursor cells were compared with untreated-differentiated cells (#= p< 0.05), however tBHP treatment did not significantly alter MBP mRNA compared with untreated (ns).

C) Protein was harvested from oligodendrocytes at 72 hours post-treatment. Cell lysates were immunoblotted for MBP. Representative Western blot images are shown, with quantification of band intensities normalized to GAPDH loading controls. Data are presented as mean ± SEM from 4 separate cultures (n= 4). Student’s t-test determined statistical significance. A significant increase in MBP was found when OPCs were compared with untreated-differentiated cultures (###= p< 0.001). A significant reduction in MBP was observed when tBHP treated cultures were compared with untreated cultures (#= p< 0.05).
Figure 5.4: MMF reduces ROS accumulation induced by various oxidant compounds

OPCs were pre-treated with 10 μM monomethyl fumarate (MMF) for 6 hrs. Cultures were then treated with 2.5 μM tBHP (A), 100 μM hydrogen peroxide (B), or 100 μM buthionine sulfoximine (BSO) (C) for an additional 2 hours. DHE staining, imaging and analysis was performed as in Figure 1. Representative experiments shown, values are presented as mean ± SEM, n= 2. Student’s t-test determined significance for direct comparisons (#= p< 0.05, ###= p< 0.001). Significant accumulation of reactive oxygen species was observed with each compound, and in treatment condition MMF pre-treatment significantly reduced this effect.
Figure 5.5: MMF fails to rescue differentiation defect caused by oxidative insult

OPCs plated on coverslips were pre-treated with 10 µM MMF for 24 hrs. Cells were then switched into DM, supplemented with MMF and/or treated with tBHP, hydrogen peroxide, or BSO. MMF was replenished at 24 and 48 hrs. After 72 hrs, cells were fixed and stained for GalC and DAPI, and were imaged and analyzed as in Figure 4. Data are presented as mean ± SEM, from 3 separate cultures (n= 3). Student’s t-test determined significance for direct comparisons compared to untreated and MMF Vehicle (#= p< 0.05, ##= p< 0.01, ###= p< 0.001).

A) GalC+ cell number was significantly reduced with tBHP, hydrogen peroxide, and BSO. No rescue was observed with concomitant MMF treatment.

B) MBP+ cell number was significantly reduced with tBHP and hydrogen peroxide. No significant alterations in MBP cell number occurred with MMF co-treatment.

C) Total cell number was significantly reduced with hydrogen peroxide. Simultaneous MMF treatment did not rescue this cell loss.
OPCs were treated with 10 µM MMF at the time of differentiation and harvested for protein analysis along a timecourse at the following intervals: 0hr, 2hr, 6hr, 12hr and 24hr. Cell lysates were immunoblotted for HO-1 (A) and NQO1 (B), target proteins upregulated following activation of the endogenous antioxidant response. Representative Western blot images shown, with quantification of band intensities normalized to GAPDH loading controls revealing no change at any timepoint. Data are presented as mean ± SEM from 2 separate cultures (n= 2). One-way ANOVA followed by post-hoc Dunnett’s multiple comparison determined statistical significance.
5.8 REFERENCES


6.1 OVERVIEW

Approximately half of HIV-positive individuals continue to suffer from some level of neurocognitive impairment within the spectrum of HIV-Associated Neurocognitive Disorders (HAND), despite effective suppression of viral replication through antiretroviral therapy (ART) to titers below the limits of detection (Gray, 2003, Brew, 2004, Heaton, 2010, 2011). Rigorous studies have demonstrated that dendritic pruning and synaptodendritic damage remain prevalent in patients (Masliah et al., 1997, Everall et al., 1999, Zheng et al., 2001, Langford et al., 2003, Everall et al., 2005, Ellis et al., 2007, Everall et al., 2009). Additionally, perturbations in white matter have been consistently demonstrated through evidence of disruption of myelin integrity, thinning of the corpus callosum, reduction of blood flow, and loss of volume in white matter structures (Pomara et al., 2001, Ragin et al., 2004, Gongvatana et al., 2009, Wohlschlaeger et al., 2009, Hoare et al., 2010, Tate et al., 2011, Kelly et al., 2014). Reproducible evidence of these pathologies in patients with well-controlled viral replication in the periphery suggests that within the CNS compartment, effects from inflammation, antiretroviral drugs, and viral reservoirs may all play roles in contributing to the persistence of HAND.

Effects of antiretroviral compounds on cells of the CNS have not been well characterized, despite potential implications for the persistence of HAND in light of well-known peripheral toxicities (Carr, 1998, Dalakas, 2001, Cohen, 2005, Parker, 2005, Ellis et al., 2008, Vidal et al., 2010). Activation of several cellular stress signaling pathways have been identified in peripheral cells in response to antiretroviral compounds, and are likely to also similarly affect cellular lineages exclusive to the brain, which may contribute to cognitive, behavioral, and motor deficits observed in HAND (Parker, 2005, Zhou, 2005, 2006, Gupta et al., 2007). In particular, even with the implementation of ART, studies indicate that oxidative stress is pervasive and abundant in HAND patients (Reviewed in Chapter 2). In addition to oxidative stress generated by the
presence of viral proteins and inflammatory responses of infected cells, antiretroviral compounds themselves had been previously shown to be capable of generating reactive oxygen species (ROS) in peripheral cell populations, macrophages, and astrocytes (Reviewed in Chapter 2). From this accumulated evidence, we hypothesized that ART compounds could activate cellular stress response pathways in neuroglial cells leading to cellular dysfunction, and that by counteracting the activation of these pathways the negative ART-induced effects could be ameliorated. Through our investigations exploring this hypothesis, we have contributed significant evidence to the effects of antiretroviral compounds on various neuroglial cell populations. Our findings, as discussed in the remainder of this chapter, have notable implications for the persistence of HAND in the ART era.

We began by assessing the effects of antiretrovirals on neurons, through in vivo and in vitro models (Chapter 3). Similar to reports from human patients, we observed synaptodendritic damage in the brains of SIV-infected ART-treated pigtail macaques (Masliah et al., 1997, Everall et al., 1999, Zheng et al., 2001). Through an administration model in rats, we also demonstrated that this damage can occur in vivo due to ART compounds alone, in the absence of virus. In vitro studies determined that in neurons, exposure to antiretrovirals resulted in accumulation of ROS and slight EAR activation, yet culminated in cell death. Independent cultures of enriched neurons or astrocytes revealed that ROS accumulation occurred solely in neurons, with astrocytes neither accumulating ROS nor activating the antioxidant response. In our combined neuroglial cultures, monomethyl fumarate (MMF) potently activated the EAR, causing upregulation of antioxidant genes. In the context of antiretroviral exposure, this prevented ROS accumulation and subsequent neuronal death, suggesting that oxidative stress is a major contributor to ART-induced neuronal toxicity.

Our next line of inquiry was to explore potential effects of ART compounds on oligodendrocyte survival, maturation, and myelin maintenance (Chapter 4). While not directly toxic at physiological doses, we definitively established that drugs from the PI class halt oligodendrocyte differentiation in a dose-dependent, but reversible manner. Following antiretroviral exposure ROS accumulated, but unlike the rescue of neuronal toxicity observed in our previous study, co-administered MMF was unable to restore
oligodendrocyte maturation deficits. Through an in vivo ART administration model performed in mice, Ritonavir treatment led to reduction in myelin protein levels in frontal cortex following two weeks of repeated exposure. Similarly, through an evaluation of human patient prefrontal cortex samples, we concluded that HIV-positive individuals with HAND who were ART-medicated displayed significant alterations in myelin proteins compared with both ART-naïve HAND individuals and seronegative controls.

Finally, we decided to delve deeper into the activity of the endogenous antioxidant response (EAR) in oligodendrocytes, pursuing our finding that MMF failed to rescue oligodendrocyte maturation despite potently activating this pathway and providing protection from oxidant insults in neurons (Chapter 5). Oxidant application led to robust ROS accumulation in both OPCs and mature oligodendrocytes in vitro, yet expression of EAR target genes was not enhanced. This treatment also led to a reduction in cells progressing in differentiation from precursor cell to more mature lineage stages. MMF co-administration reduced the ROS accumulation induced by several oxidant compounds but did not activate the EAR or rescue the maturation deficits.

Together our results provide some of the first evidence that antiretroviral compounds could be a major contributing factor to the persistence of HAND. In summary, we have shown that: 1) ART induces neuronal toxicity in vivo and in vitro, 2) Neuronal toxicity is primarily a result of oxidative stress, which can be rectified by an antioxidant promoting EAR activity, 3) PIs prevent oligodendrocyte maturation, 4) ART causes loss of myelin integrity in vivo, and 5) The EAR is not activated in oligodendrocytes under conditions which stimulate this pathway in other cell types. These findings advance our basic understanding of the neuronal and oligodendrogial responses to antiretroviral compounds and oxidative stress, and have important clinical implications for the treatment of HIV. These topics are considered in detail in this chapter.
6.2 ANTIRETROVIRAL COMPOUNDS GENERATE OXIDATIVE STRESS AND INVOK NEURONAL TOXICITY

In many neurodegenerative diseases, including HAND, a hallmark indicator of neuronal damage and dysfunction is evidence of synaptic injury (Gupta et al., 2010). Reduction in synaptic density as indicated by dendritic pruning is a major pathological feature in HAND patients in the ART-era (Masliah et al., 1997, Everall et al., 1999, Zheng et al., 2001, Langford et al., 2003, Everall et al., 2005, Ellis et al., 2007, Everall et al., 2009, Xu and Ikezu, 2009). Elevated markers of oxidative stress such as lipid and protein oxidation, oxidative modifications to DNA, and depletion of the key antioxidant glutathione are still detectable in the brains of ART-treated patients despite effective control of viral replication (Reviewed in Chapter 2). Despite these findings, the small number of reported studies regarding antiretroviral compound pharmacokinetics and effects in the CNS did not examine pathological readouts of neuronal damage (Huisman et al., 2003, Anthonypillai et al., 2004, Anthonypillai et al., 2006).

Through our work presented in Chapter 3, we have provided evidence for ART-induced synaptic injury in two animal models. In the first, adult rats received an NRTI-boosted PI ART-regimen (AZT+Saquinavir+Ritonavir) over the course of one week. Even in this short time span, analysis of brain sections derived from hippocampus revealed that repeated ART exposure led to significant reductions in synaptophysin, an essential component of synapses, and microtubule associated protein 2 (MAP2), a neuronal protein that stabilizes microtubules and which is used commonly as an indicator of neuronal processes. Our second in vivo model extended our findings to non-human primates, through an evaluation of brains from SIV-infected juvenile pigtail macaques. We observed decreased levels of synaptophysin in SIV-infected ART-treated animals, compared with either uninfected animals or infected animals receiving a placebo regimen. We acknowledge the caveat in the design of this study, that there is a lack of uninfected animals receiving ART. This prevented a strict determination of the effects of ART in the absence of viral infection, which is a topic of future investigation that will be addressed further in Section 6.5. Despite this, we did observe a definitive contribution of ART to neuronal damage, as SIV-infected ART-treated macaques had significantly less synaptophysin staining than their SIV-infected untreated counterparts. These studies demonstrating evidence of ART-associated synaptic damage in vivo warrant future
investigations into specific effects of individual drug classes, as well as the mechanisms through which neuronal damage occurs.

Until recently, ART-induced toxicity had only been explored in cell lines, however Robertson and colleagues have now provided the first evidence for such toxicity in primary rat neurons (Cui et al., 1997, Robertson et al., 2012). Within our current study we have shown that the PIs Ritonavir and Saquinavir induce oxidative stress followed by subsequent neuronal damage/death in primary cultures at clinically relevant doses. We have also provided evidence that through activation of the EAR by MMF, we were able to prevent the generation of oxidative stress and neuronal toxicity induced by these antiretroviral compounds. Interestingly, application of the NRTI compounds AZT or stavudine (d4T) did not cause neuronal toxicity in our experimental paradigm. In agreement with these observations, a previous study in PC-12 neuron-like cells found that following extended exposure to either AZT or d4T no inhibition of cell growth or neurite regeneration were observed (Cui et al., 1997).

We have demonstrated that ART-induced effects can be successfully blocked by MMF-mediated activation of the EAR. Only moderate activation of this pathway by ART compounds alone was seen through increased levels of both mRNA and protein of EAR target genes HO-1 and NQO1. Pre-treatment with MMF resulted in further increases in HO-1 protein and provided protection against ART-induced toxicity. We confirmed that this protection was the result of increased HO-1 activity by co-applying a heme oxygenase inhibitor at the time of MMF pre-treatment. With this inhibition, the protection provided by MMF was negated. We conclude that despite activation of the EAR in response to antiretroviral compounds, this response is either insufficient or too delayed to protect neurons from ART-mediated damage and death. In support of this interpretation, enhanced MMF-mediated activation of the EAR was strongly protective against the neurotoxic antiretroviral effects. Future studies utilizing this in vitro model could be very informative to determine if other clinically utilized ART combinations result in neurotoxicity, as well as to screen additional candidate compounds for potential use as adjunctive therapeutics prior to testing in in vivo models. The success of MMF in prevention of ART-induced neuronal toxicity in vitro is of particular interest for the potential of translation of our findings to the clinical setting.
MMF as a candidate for adjunctive therapy in patients with HAND will be discussed in detail in Section 6.6 “Clinical Implications”.

In our acute model of ART application in vitro, enriched astrocytes did not show evidence of ROS accumulation or EAR activation. Astrocytes have high levels of glutathione and antioxidant enzymes, and it is likely that our finding is reflective of efficient cellular buffering of the ROS produced by antiretroviral compounds so that no accumulation occurs and no upregulation of antioxidant proteins are required (Sagara et al., 1993, Bolanos et al., 1996). In SIV-infected macaques, we found that the astrogliosis normally present after infection was resolved in ART-treated animals. While these findings suggest that astrocytes may not be robustly impacted by ART compounds, the effects of long-term exposure cannot be predicted. Astrocytes have been well characterized in their ability to provide trophic support and stress buffering capabilities to neurons, including oxidative stress, and alterations in this capacity can precipitate damage in the already susceptible neuronal population (Sagara et al., 1993, Bolanos et al., 1996, Fernandez-Fernandez et al., 2012). In our in vitro model, enriched neuronal cultures succumb to antiretroviral-mediated toxicity in less than 6 hours. In neuroglial culture however, which contains approximately 95% neurons and 5% astrocytes, viability was sustained until mitochondrial depolarization and subsequent neuronal damage/death was observed at 48 hours post-treatment. These results indicate that while astrocytes do not themselves upregulate EAR proteins in response to antiretroviral agents, even when present in small numbers they are capable of buffering neuronal susceptibility to the oxidative stress generated by these compounds, at least for a time. Ultimately however, these neuroglial cultures do succumb to antiretroviral-mediated toxic effects. Together, our data suggest that sustained oxidative stress and ROS accumulation due to extended ART exposure may lead to ART-induced neuronal injury in the CNS, leading to the persistence of clinically observed synaptodendritic damage in HIV patients in the ART-era.

The persistence of HAND despite effective viral control outside of the CNS is accompanied by a shift in pathology from severe dementia to more subtle neurocognitive impairments, implying a continuation of neuronal damage, albeit in a milder form. Through our results presented here, we propose that the mechanism of continued synaptic damage may be a combination of viral proteins and soluble factors.
released from CNS reservoirs, as well as direct damage caused by ART compounds. Comprehensive studies have detailed neuronal and synaptic damage mediated by viral proteins and factors released from infected cells, by several direct as well as indirect mechanisms involving multiple cell types, including astrocytes (Gonzalez-Scarano, 2005). Our data suggest that additional synaptic damage may be caused by direct ART-mediated effects in neurons, through mechanisms involving oxidative stress. It is likely that longitudinal exposure to ART regimens including compounds which invoke this synaptic damage contribute to a slower, yet persistent alteration of neuronal function which contributes to the chronic course of progressive neurocognitive impairment observed in HAND patients in the ART-era.

6.3: OLIGODENDROCYTE MATURATION AND MYELINATION ARE NEGATIVELY AFFECTED IN THE CONTEXT OF HIV AND ART

Through our results presented in Chapter 4, we have been the first to evaluate oligodendrocyte maturation in the context of antiretroviral drug exposure. We have rigorously demonstrated that PIs produce significant deficits in the differentiation of cells from OPC to maturing oligodendrocytes, which is independent of the oxidative stress induced by ART compounds, despite ROS being produced. We were fortunate to be able to examine a human cohort containing HIV-positive individuals with and without antiretroviral drug experience, and measured a significant reduction in myelin basic protein and a significant increase in CNPase in the HAND patients who had been ART-medicined compared to seronegative controls and HAND patients who were ART-naïve. These data have enabled us to add cellular evidence of myelin protein perturbation to the persistence of white matter pathologies in ART-treated HAND patients, which corroborates previous reports utilizing non-invasive imaging studies (Pomara et al., 2001, Ragin et al., 2004, Gongvatana et al., 2009, Wohlschlaeger et al., 2009, Hoare et al., 2010, Tate et al., 2011, Kelly et al., 2014). Our data are also in accordance with the single transcriptome analysis study performed in patients to define genes altered in HIV infection that remained dysregulated following suppression of viral replication by antiretroviral therapy, which found that mRNA for myelin specific genes: myelin-associated oligodendrocyte basic protein and myelin basic protein (MBP) were reduced (Borjabad et al., 2011).
Given these findings, we propose the following model for the effects of antiretrovirals on the maturation of oligodendrocytes. Oligodendrocyte precursors treated with antiretroviral compounds enter into differentiation and proceed with transcription factor alterations and upregulation of myelin component mRNAs as has been well documented (Miller, 2002). During this progression, cells begin to extend actin filaments and adopt the morphology of mature, elaborated oligodendrocytes. A majority of myelin component proteins are translated in the perinuclear endoplasmic reticulum and are trafficked through the secretory pathway via the Golgi apparatus to be inserted into the forming myelin membrane (Colman et al., 1982). An exception to this is MBP, in which mRNA is instead trafficked along microfilaments to sites proximal to the myelin membrane. Once in this vicinity, protein is translated from the mRNA on free-ribosomes and is rapidly inserted into the neighboring membrane (Colman et al., 1982, Ainger et al., 1997).

In the case of treatment with PIs we propose that either the translation or trafficking of myelin proteins is affected. The rapid rescue of maturation deficits suggest that required components are already present within treated cells, and that alleviation of cellular stress by drug removal allows for renewed translation and/or proper trafficking of myelin components. All myelin proteins examined, both those traversing the secretory pathway as well as MBP, are diminished at the cell surface. The most straightforward explanation for this result would be that PI treatment decreases protein synthesis. When this blockade is removed, rapid translation, trafficking, and membrane insertion can ensue. The caveat to this proposition is that protein levels remain normal as assessed by western blotting under most conditions where a deficit in membrane positivity already exists. This is particularly evident in the highest Ritonavir dose where no decreases are detected at the protein level, yet drastic decreases are seen by immunocytochemistry. While this finding is unusual, it is not unprecedented in oligodendrocytes and has been similarly reported by two independent laboratories (Maier et al., 2009, Wahl et al., 2014). Reduction in cholesterol levels can result in decreased surface expression of proteolipid protein (PLP) while maintaining normal protein level as assessed by western blotting (Maier et al., 2009). Similarly, investigations into the role of mammalian target of rapamycin in differentiation revealed a reduction in number of maturing oligodendrocytes yet maintenance of normal myelin protein levels (Wahl et al., 2014). Based on our findings and supporting evidence from these previous works, it is therefore more likely that a trafficking/membrane insertion problem exists for
myelin proteins which have already been synthesized.

Alternatively, normal protein levels observed with Ritonavir at all concentrations and most doses of Lopinavir may indicate a failure of protein degradation under conditions which would normally promote destruction of myelin proteins which were synthesized but could not effectively be inserted into the membrane. PIs have been shown in non-CNS cells to have the off-target effect of inhibiting proteasomal degradation and activating the unfolded protein response (UPR) (Andre, 1998, Zhou, 2005, 2006). The UPR potently suppresses global protein translation while upregulating specific stress-response proteins (Brown and Naidoo, 2012). While we have provided evidence that the UPR is not activated in oligodendrocytes following antiretroviral application at timepoints where such activation was evident in neuroglial culture, we have not yet addressed potential effects on translational efficacy or inhibition of degradation machinery. Following this line of reasoning, the mechanism behind lower protein levels with highest dose Lopinavir would need to be explained, particularly if the proteasome is inhibited.

Notable differences were observed in the effects of the two PIs Ritonavir and Lopinavir, which are likely the result of dissimilar metabolism and intracellular actions resulting from the unique properties of their chemical structures. There have been several previously reported instances where different effects are noted in the same cell population following exposure to independent PI compounds. Adipocyte differentiation was inhibited with Nelfinavir and Indinavir but not with Amprenavir (Caron et al., 2003). Additionally in astrocytes, ROS production, glutathione export, and toxicity differed dramatically following Nelfinavir versus Indinavir application (Brandmann et al., 2012). In spite of differing subcellular effects invoked by Ritonavir and Lopinavir, it is clear that exposure to either PI even at low concentrations would not result in the formation of a properly functioning myelin membrane based on reduced myelin protein localization to the cell surface and our observed deficit in myelin membrane elaboration.

We have demonstrated that several antiretrovirals induce ROS, mitochondrial depolarization and neurotoxicity in astrocyte-supported neuronal culture (Chapter 3) and that oxidative stress hinders the process of oligodendrocyte differentiation (French et al., 2009, Reid et al., 2012). While both NRTI AZT
and PI Ritonavir induce robust ROS accumulation, this is not a universal feature of antiretrovirals, as Lopinavir did not produce ROS accumulation even at greatly extended timepoints in oligodendrocytes. When we probed whether the EAR, which is triggered by cellular redox homeostasis imbalance, was activated in oligodendrocytes following antiretroviral exposure, no biologically relevant increases in pathway gene mRNAs or in target proteins HO-1 or NQO1 were detected (Li, 2009). Furthermore, scavenging of ROS through EAR upregulation via MMF did not rescue PI-induced decreases in oligodendrocyte differentiation despite previously demonstrated neuroprotection (Chapter 3). Thus, while PIs have been previously shown in other cell populations to induce oxidative stress and the UPR, we provide evidence that in oligodendrocytes PIs act through an alternate mechanism to affect maturation. An intriguing possibility is the potential for PIs to cause dyslipidemia, which has been demonstrated in patients and in non-CNS cell types (Carr, 1998, Haughey et al., 2004, Zhou, 2006). An effective myelin membrane is crucially dependent on proper lipid balance to ensure appropriate organization of myelin component proteins (Maier et al., 2009, Chrast et al., 2011, Lee et al., 2014). As was mentioned previously, reduction in cholesterol levels can result in decreased PLP at the surface of the myelin membrane with minimal reduction at the protein level, which mirrors our finding with MBP under conditions of Ritonavir treatment (Maier et al., 2009). Furthermore, PIs have been linked to altered regulation and localization of the master transcription factor of lipid metabolism sterol regulatory-element binding protein 1 (SREBP1), which may be involved in our differentiation phenotype (Zhou, 2006, Goulbourne and Vaux, 2010). In summary, while previous studies have implicated oxidative stress and activation of the UPR as cellular responses to antiretroviral compounds, in oligodendrocytes PIs do not seem to be acting through these pathways to induce a differentiation deficit and alternative mechanisms should be considered.

Further investigation is needed to define the mechanism by which myelin proteins are dysregulated and prevented from reaching the myelin membrane following PI application. Several potential avenues of inquiry have been suggested by our results, including analysis of translational efficiency and protein turnover by the proteasome. In order to ascertain translational efficiency of myelin proteins in the presence or absence of PIs, pulse-chase experiments can be performed with radiolabeled methionine over a brief time interval in the context of proteasomal inhibition using the compound MG-132 to prevent breakdown
of newly synthesized proteins. At the endpoint of labeling, the myelin proteins would be pulled down using immunoprecipitation, and following gel electrophoresis the amount of newly synthesized protein could be quantified according to the amount of radioactive probe incorporated. To determine if proteasomal function is inhibited as a side effect of PI application in oligodendrocytes, cells can be treated and proteasomes specifically isolated through centrifugation in a glycerol gradient (Orino et al., 1991). Activity can then be assayed by measuring fluorescent emission of a synthesized probe engineered to emit 440-460 nm waves following specific proteasomal cleavage, allowing for comparisons under normal and treated conditions, as well as under MG-132 proteasomal inhibition. Secretory pathway function can also be assessed in our in vitro culture model. Isolation of individual cellular organelles by subcellular fractionation followed by western blotting will determine if myelin component proteins such as CNP and PLP are properly transitioning from the ER to the Golgi apparatus, or if unusual accumulations are occurring in these compartments. Immunocytochemistry followed by confocal imaging will address this question using a different approach, by co-staining with organelle markers in addition to one of the myelin proteins. Quantification of myelin proteins in each subcellular fraction will allow comparison of myelin protein localization to each cellular compartment under normal and PI-treated conditions. Finally, the lipid composition of the forming myelin membrane should be evaluated to determine if an imbalance or dysregulation of lipid rather than protein is preventing correct presentation of myelin proteins to the cell surface. Studies within the laboratory have begun to explore these possibilities, through mass spectrometry analysis of cellular lipid compositions during differentiation in the presence or absence of PIs, along with assessment of the activities and levels of the transcription factor SREBP1 and its downstream gene targets involved in lipid metabolism.

Another area crucially in need of investigation is an exploration of the potential consequences of HIV-infection on oligodendrocytes. In a recent publication, a model using inducible Tat-transgene expression in astrocytes began to address this important question. Following 3 months of Tat-expression, animals displayed significant abnormalities in myelin integrity, with aberrant morphology evident in the corpus callosum, anterior commissure, caudate-putamen, and striatum (Zou et al., 2015). Furthermore, application of exogenous Tat protein to oligodendrocytes in vitro culminated in reduced viability of immature
oligodendrocytes and significant reductions in oligodendrocyte maturation (Zou et al., 2015). While this foundational study has provided initial evidence that viral proteins can affect oligodendrocyte survival and extent of myelination, it is of upmost importance to understand the effects of HIV-infection on oligodendrocytes in the context of the entire virus and cell populations that undergo productive infection in vivo. Evidence has shown that in addition to factors secreted by infected cells, inflammatory activation of neighboring, uninfected macrophages, microglia, and astrocytes also contributes to release of cytotoxic factors into the cellular milieu. Therefore, we have begun to formulate studies using a model of HIV-infection of isolated human monocyte-derived macrophages. Following infection with a CNS-isolated strain of HIV-1, supernatants will be collected at the peak of viral infection. These supernatants have been previously demonstrated to invoke neurotoxicity, at least in part through high levels of glutamate that promote N-methyl-D-aspartate receptor (NMDA) receptor-mediated excitotoxicity (O'Donnell et al., 2006). The oligodendrocyte study mentioned above from the Knapp laboratory also demonstrated a NMDA receptor-dependent mechanism of Tat-induced effects in their model, suggesting that in the context of full virus, high levels of extracellular glutamate may be negatively impacting oligodendrocytes as well as neurons. On the basis of these preliminary findings, it is expected that oligodendrocyte survival and maturation will be affected in the context of viral infection. Results investigating this possibility, in addition to the effects of antiretroviral compounds, will have great clinical and therapeutic implications.

Through our work in Chapter 4 we have pioneered the investigation of the effects of antiretrovirals compounds on oligodendrocytes in vivo, through use of a rodent model as well as human patient samples. In mice, myelin alterations were evident in as little as two weeks of repeated exposure, with significant reductions observed in both CNPase and MOG. We also performed the first reported evaluation of myelin protein levels in individuals with HAND. None of the proteins MBP, CNP, MAG, or MOG were significantly altered when individuals were stratified solely into HIV-negative and HIV-positive groups. However, when only individuals with confirmed diagnosis of HAND were assessed, and were placed into categories based on whether they had received ART, a significant reduction in MBP was observed in patients with HAND who had been ART-medicated, when compared with either the untreated HAND individuals or the HIV-negative controls. Our findings reinforce the transcriptome analysis performed by
the Volsky group, which identified genes required for oligodendrocyte maturation including MBP as
dysregulated in patients with HAND, even under effective suppression of viral replication (Borjabad et al.,
2011). While this could potentially be interpreted that synaptodendritic damage in HAND occurs with a
subsequent loss of myelin, our results indicate that this is an insufficient explanation for all of our observed
effects at the protein level. Our examination of patient samples also yielded an unexpected significant
increase in CNPase in the HIV-positive ART-medicated group, over both the HIV-negative and HIV-
positive ART-naïve groups regardless of neurocognitive status. This observation could be indicative of an
attempted cellular remyelination response that has been unsuccessful in restoring MBP levels, as CNPase is
one of the earliest myelin proteins to be expressed during maturation from precursor cell to mature
oligodendrocyte (Scherer et al., 1994). Together with our in vitro evidence, these results indicate that
disruption of myelin in the ART era may occur as a direct effect of ART compounds on oligodendrocytes,
rather than as a secondary consequence of neuronal damage. Our data suggests that in a reversal of roles,
damage to myelin, coupled with oligodendrocyte precursors that cannot successfully remyelinate affected
areas, may in turn lead to secondary synaptodendritic neuronal damage.

Based on the evidence from our investigations and those previously performed, we have formulated the
following model for the effects of HIV and antiretroviral compounds on oligodendrocytes in pediatric and
adult settings (Figure 6.1). In children (A), throughout adolescence productive myelination occurs which
allows for the ensheathment of neurons, axon potential conductivity, and plasticity of signaling networks
throughout development. HIV-infection with concomitant suppressive ART and inflammation culminates
in widespread neurological deficits including encephalopathy, leukoencephalopathy, cerebrovascular
complications, neurocognitive deficits and developmental delays. These pathologies are likely a combined
effect of reduction in effective myelin formation and disruption of myelin integrity of forming white matter
tracts. In the adult (B), myelin is essential for the facilitation of action potentials, axonal maintenance, and
neurotrophic support. Additionally, new myelin formation and plasticity/maintenance of formed myelin is
crucial for maintained biological homeostatic function and for motor learning in adulthood. HIV-infection
with persistent inflammation and treatment with lifelong ART together can result in HAND in patients.
Pathologies including dendritic pruning, astroglialiosis, loss of white matter volume and integrity, and
reduction in mRNA and protein for myelin components are evident in these individuals. Through the findings resulting from our study, we propose that the myelin pathologies observed in patients are likely a combination of perturbed maintenance of formed myelin as well as an impairment of remyelination capability.

6.4 THE ENDOGENOUS ANTIOXIDANT RESPONSE IN OLIGODENDROCYTES

It was quite intriguing that the EAR was not activated in oligodendrocytes in our investigations of effects of antiretroviral compounds despite robust oxidative stress, or following application of MMF which was known to upregulate this pathway in other cell populations. We decided to pursue this line of investigation, to examine the activity of the EAR in oligodendrocytes in Chapter 5.

In contrast to the well-characterized pathway function of the EAR in many other cell types, very little is known about this pathway in oligodendrocytes. The sole reported study, using primary rat oligodendrocytes demonstrated upregulation of HO-1 by immunoblotting following exposure to hydrogen peroxide, however this insult culminated in loss of mitochondrial function and apoptosis, suggesting that the protein upregulation resulting from EAR pathway activity was not sufficient to protect the cells (Goldbaum and Richter-Landsberg, 2001). In our work, we provide compelling evidence that exposure to sub-toxic levels of oxidants produced ROS accumulation in both OPCs as well as mature oligodendrocytes in vitro. However, unlike other CNS cell types, in both the OPCs and mature oligodendrocytes this failed to elicit activation of the EAR. As a possible result of this failure, oxidant exposure at the time of differentiation prevented OPCs from progressing in differentiation to the more mature oligodendrocyte stages, and prevented appropriate levels of myelin protein expression for insertion into the cell membrane.

In an attempt to better understand the unrectified level of oxidative stress in oligodendrocytes, we turned our focus to a demyelinating disease in which oxidative stress is a hallmark of disease and antioxidant treatment has benefited patients: Multiple Sclerosis (MS). Symptoms of MS arise from disruption of the integrity of myelin in white matter tracts in the brain, with cellular pathology including destruction of myelin sheaths and death of oligodendrocytes, as well as axonal damage and neuronal death (Criste et al.,
While initial steps leading to the autoimmune targeting of myelin are not well understood, it is evident in MS that once initiated; chronic inflammation and oxidative stress drive ongoing pathology (Friese et al., 2014, Mahad et al., 2015). Currently, therapies aimed at reducing relapse rates and restoring quality of life in patients have focused on attenuating these two persistently elevated forms of stress (Friese et al., 2014, Mahad et al., 2015). The drug Tecfidera effectively reduces relapse rates in MS patients (Fox et al., 2014, Dubey et al., 2015). This compound is suspected to act as an antioxidant, yet the mechanism by which it is effective has not been elucidated. Tecfidera is composed of the fumaric acid ester dimethyl fumarate (DMF), which is rapidly metabolized to MMF following ingestion (Litjens et al., 2004, Schmidt et al., 2007, Limmroth, 2013, Fox et al., 2014). Within minutes of administration, DMF is no longer detectable and MMF is the primary physiological metabolite (Schmidt et al., 2007). We and others have demonstrated antioxidant properties of MMF in neurons and macrophages through Nrf2-mediated EAR activation and upregulation of target genes (Chapter 3) (Cross et al., 2011, Albrecht et al., 2012, Scannevin et al., 2012). Remarkably, MMF effects on signaling pathways in oligodendrocytes have not been explored, despite the pivotal role these cells play in MS pathology.

Within our in vitro model, we attempted to exogenously activate the EAR by treatment with MMF in oligodendrocyte lineage cells. Pre-treatment with MMF successfully attenuated ROS accumulation caused by various oxidant compounds in both OPCs and mature oligodendrocytes as anticipated. However, MMF did not upregulate Nrf2-mediated EAR target genes HO1 or NQO-1 at any point during an extended 24-hour timecourse in oligodendrocytes, and was unsuccessful at rescuing the oxidant-induced maturation deficit in developing OPCs. Our finding is in line with that of the Stangel laboratory, in which animals were fed the copper chelator cuprizone to induce a specific, reversible demyelinating lesion in the corpus callosum. During the period of remyelination in this experimental model of MS, animals were given either DMF or MMF, yet only minor effects on remyelination were noted and the fumarate compounds did not protect against cell death (Moharregh-Khiabani et al., 2010). Additionally, while this group did not examine in vitro differentiation, they demonstrated that pre-application of either DMF or MMF failed to rescue an oligodendrocyte-like cell line from oxidative stress-induced toxicity following hydrogen peroxide treatment (Moharregh-Khiabani et al., 2010).
One possible explanation for the absence of EAR pathway activity in oligodendrocytes involves the regulation of glutathione synthesis and recycling. All of the oxidants utilized within our study impact cellular glutathione: tert-butyl hydroperoxide depletes levels of cellular glutathione, buthionine sulfoximine selectively inhibits glutathione synthesis, and hydrogen peroxide generates highly reactive hydroxyl radicals which interact with cellular proteins, lipids, and reducing agents such as glutathione (Meister, 1992, Sies, 1993, Back et al., 1998, Zhao et al., 2005, French et al., 2009). MMF was unable to rescue maturation deficits induced following exposure to any of these oxidants. Glutathione s-transferase (GST) is the enzyme responsible for glutathione conjugation to electrophilic compounds. The isoform of GST expressed in oligodendrocytes is GST-pi, which within the brain is unique to this cell lineage and has been used as a marker for mature oligodendrocytes in vivo (Cammer et al., 1989, Cammer and Zhang, 1992, Girolamo et al., 2011, Li et al., 2013). Immunofluorescent studies have demonstrated that GST-pi is nuclear in OPCs and attains a cytoplasmic distribution during differentiation, which may account for some of the oxidative stress sensitivity of oligodendrocytes during early lineage stages (Tamura et al., 2007). It may be that in oligodendrocytes, the glutathione system for monitoring redox homeostasis operates differently than other CNS cell populations, such that if glutathione levels are depleted the EAR is not effectively activated, particularly in OPCs and maturing cells when components of this system are confined to the nucleus.

Interestingly, both DMF and MMF have been reported to lower cellular glutathione levels in astrocytes and could potentially do the same in oligodendrocytes (Schmidt and Dringen, 2010). However in contrast to this idea, DMF has been recently reported to increase glutathione levels in a human oligodendrocyte cell line (Huang et al., 2015). Future investigation is certainly needed to resolve these conflicting theories, and will hopefully determine mechanistically how fumarate compounds affect glutathione regulation in primary oligodendrocytes. In addition to the fumarates, it will be important to determine if EAR upregulation occurs in oligodendrocytes using alternative oxidants and antioxidants which impact cellular redox sensors other than glutathione, or if this canonical pathway is truly not utilized in this cell lineage.

Another possibility is that rather than the Nrf2 pathway playing the primary role in regulating redox homeostasis in oligodendrocytes, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-
κB) pathway may take the forefront in this cell population. These two pathways are extensively interconnected and normally antagonize each other (Li et al., 2008). It would not be surprising if a cell lineage which lacks activity in one pathway to have overly robust activity in the other in response to pathway initiation. This could be potentially harmful to oligodendrocytes, as NF-κB pathway activation leads to a pro-inflammatory state (Tobon-Velasco et al., 2014). Recent evidence from the Massa laboratory may help to support this theory. This group previously found that null mutation of Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) in mice resulted in reduced myelin gene expression and hypomyelination in the CNS (Massa et al., 2004). SHP-1 modulates ROS production by negatively regulating the NADPH oxidase complex (Krotz et al., 2005), which is inducible and toxic to oligodendrocytes (Johnstone et al., 2013). In their present study, SHP-1 deficient mice display a permanent shift away from redox homeostasis, with Nrf2 nuclear localization and upregulated HO-1 expression, but this is accompanied by irreversible protein damage both in vitro and in vivo, suggesting that the observed EAR pathway responses are insufficient to resolve the enhanced intracellular levels of ROS (Gruber et al., 2015). Importantly, SHP-1 normally negatively regulates the activity of NF-κB (Mandal et al., 2010, Mariappan et al., 2010). This report also demonstrated that silencing of SHP-1 led to enhancement of downstream NF-κB pathway targets (Gruber et al., 2015). Investigating activity of this crucial protein which has the potential to impact both oxidative and inflammatory cellular states in oligodendrocytes could be very informative in regards to MS, where both of these stresses are key drivers of pathology. With these results in mind, future studies aimed at downregulating NF-κB pathway activity may be a more effective therapeutic approach than attempting to upregulate EAR activity.

Overall, our results suggest that the source of selective vulnerability of oligodendrocytes to oxidative stress is two-fold. This cell lineage begins with endogenously low levels of antioxidant and detoxifying enzymes (Halliwell, 1992, Back et al., 1998, Fern and Moller, 2000, Baud et al., 2004), and this deficit in oxidant buffering capacity may be exacerbated by an inability to appropriately activate the supposedly ubiquitous EAR pathway under conditions of redox imbalance. Our results indicate that in vivo, oligodendrocytes likely rely on neighboring cells for protection from oxidative stresses, in much the same way that neuronal protection occurs via astrocyte involvement (Fernandez-Fernandez et al., 2012). In light of our findings, it
is not likely that Tecfidera reduces MS relapse rates through a direct effect on oligodendrocytes. Rather, it is more probable that secondary effects from EAR upregulation in neurons, macrophages, and possibly astrocytes play a role in conferring protection to the oligodendrocytes (Chapter 3 and (Cross et al., 2011, Scannevin et al., 2012)). While not examined in our present study, in addition to reducing overall levels of oxidative stress, Tecfidera may also be influencing levels of CNS inflammation through modulation of secreted cytokines and inhibition of the NF-κB pathway (Lehmann et al., 2007, Albrecht et al., 2012, Gillard et al., 2015). Functional recovery of patients with demyelination, as seen in MS and HAND, will be reliant on resilient reduction of oxidative stress. Our study has provided the foundational evidence that in order for this to succeed, the concerted efforts of multiple CNS cell populations will be required to provide an oxidative stress free environment, so that the susceptible oligodendrocyte population can effectively survive, mature, and re-myelinate affected areas.

6.5 ANTIRETROVIRAL STUDIES GOING FORWARD

The in vitro and in vivo models used throughout our studies have enabled great advances in the understanding of antiretroviral effects on CNS cell populations. Continuing forward in this line of investigation, it will be advantageous to consider: additional exploration of NRTI-based effects and initial investigation of the nNRTI and integrase inhibitor drug classes, responses to ART drugs on the whole-cell level, and advanced tools for evaluating CNS effects of ART compounds.

6.5a Further Investigation of Antiretroviral Drug Classes

In our detailed studies of the impacts of antiretroviral drugs in invoking neuronal toxicity and deficits in oligodendrocyte maturation, the candidate compound tested from the NRTI class, AZT, produced ROS accumulation but did not negatively impact the cellular functions that we assessed. NRTIs have been unequivocally implicated in peripheral neuropathy through mechanisms of mitochondrial dysfunction and oxidative stress, which made our negative findings rather unexpected (Dalakas, 2001, Ellis, 2008). NRTIs are formulated as pro-drugs, and must be tri-phosphorylated by thymidine kinases in vivo in order to effectively halt HIV replication. Expression of these enzymes is cell cycle-dependent, and while oligodendrocytes and OPCs possess the particular kinase required for NRTI phosphorylation, post-mitotic
neurons most likely do not (Nygard et al., 2003, Bazzoli et al., 2010). Therefore, it is highly likely that in our model used to study neurons, AZT is not converted to its active form. We did not test whether OPCs effectively phosphorylated AZT. Given our results, synthesized versions of tri-phosphorylated AZT and other NRTIs should be obtained and tested to see if our current results truly are representative of the drug class, or if antiretroviral-induced repercussions in neurons and oligodendrocytes would be different in the context of NRTIs which had been effectively activated by other CNS cell populations.

Additionally, while we have evaluated cellular effects following application of representative candidate compounds from the NRTI and PI classes, we have not yet explored potential consequences of drugs from the nNRTI or Integrase Inhibitor families. Similar to the NRTI compounds, the efficacy of nNRTIs is based on prevention of viral reverse transcription from RNA to DNA (Pau and George, 2014). However, unlike the nucleoside/nucleotide analogs which compete as substrates for the reverse transcriptase enzyme, the nNRTI compounds directly bind to the catalytic domain of the enzyme to prevent its activity (de Bethune, 2010, Pau and George, 2014). As the nNRTIs do not compete for endogenous cellular DNA or mitochondrial DNA synthesis, mitochondrial dysfunction may not be observed with this class. However, despite this differing mechanism of action, Efavirenz has been linked to the production of superoxide, glutathione depletion, and disruption of mitochondrial membrane potential and function in a human cell line (Apostolova et al., 2010). Furthermore, due to negative neurocognitive performance associated with use of Efavirenz, it has been removed from treatment recommendations (Decloedt and Maartens, 2013). Testing of Nevirapine and Rilpivirine in neuroglial cell populations is therefore imperative, as it remains to be seen if the other commonly prescribed nNRTIs have similar effects to Efavirenz.

Another novel category of antiretroviral compounds, the integrase strand transfer inhibitors (INSTIs), has come to the forefront in treatment regimens following the introduction of Raltegravir in 2007 and subsequent approval of Dolutegravir and Elvitegravir (Pau and George, 2014, AIDSInfo, 2015). This drug class halts the HIV integrase enzyme from forming covalent bonds between host-cell and viral DNA, which prevents effective incorporation of the viral DNA into the host chromosome. Thus far, these drugs have been associated with less peripheral toxicities than other classes, which could translate to less neuroglial
toxicity. However preliminary evidence from our laboratory suggests that a dose-dependent reduction in oligodendrocyte maturation may occur with Raltegravir, a finding that should be verified and expanded upon.

Antiretroviral regimens are constantly evolving, with new compounds and drug classes being introduced while others are eliminated. It is vital to screen these compounds in efficient ways to determine whether CNS toxicities occur, particularly with a continued focus on higher brain penetrance to eliminate viral reservoirs. Our current studies have demonstrated pronounced neuroglial effects following exposure to various antiretroviral compounds, with PIs having marked deleterious effects on neurons and oligodendrocytes. Future studies using the active form of NRTIs as well as initial examination of the nNRTIs and INSTIs, are necessary to more thoroughly understand the full range of effects of ART in patients who are exposed to regimens with a mix of these various classes.

6.5b Cellular responses to Antiretroviral Compounds

Through the combined efforts of many laboratories, oxidative stress, unfolded protein response activation, proteasome inhibition, lipid dysregulation, and release of cytokines have been well documented following exposure to antiretrovirals. These pathways have been implicated in various cell populations through clinical observations in patients, animal and cell culture models, and have been validated through a candidate pathway testing approach. The direct assessment of protein and mRNA changes in brain-resident cell populations in response to antiretroviral compounds of different classes is still crucially needed. Through DNA-microarray and RNA-Seq methods, specific pathways altered following antiretroviral exposure can be identified. This will greatly assist in informing which regimens would have the least negative impact on CNS-cells, and would allow for better targeting of deleterious side-effects through specific adjunctive therapies.

6.5c Advanced Tools for Evaluating CNS Effects of Antiretroviral Compounds

Primary isolated cortical cell populations have been our principal system for analyzing the effects of antiretroviral compounds. While we have gained much knowledge through evaluating effects on specific
cell lineages in isolation, the full extent of functional implications to cellular changes cannot be extrapolated by this method. An *ex vivo* method has been developed, where organotypic slice cultures can be isolated from brain regions of young rodents and maintained for several weeks (Gahwiler et al., 1997, Opitz-Araya and Barria, 2011). Performing ART studies on these cultures will allow for extension of current studies to include evaluation of synaptic transmission, as well as functional assessment of the formation and maintenance of myelin segments. Using this physiological model will enable normal cellular interplay between CNS-resident cell populations, and allow for high throughput screening of ART compounds for potential neuroglial effects, as well as potential adjunctive compounds prior to testing in a full-scale animal model.

Our striking findings regarding the effects of PIs on oligodendrocyte maturation and myelin maintenance have prompted consideration for additional rodent models in which to study the effects of ART compounds in the absence of viral infection. The first of these would investigate the effects of ART on the process of remyelination in the adult animal. Our laboratory has experience performing the cuprizone model, which induces a specific demyelinated lesion in the corpus callosum that remyelinates in the weeks following cessation of cuprizone ingestion (Torkildsen et al., 2008). In a procedural advancement since our jugular vein administration model, animals could be outfitted with osmotic pumps to continually deliver specific ART drugs at a constant dosing during the remyelination period. Evaluation of remyelination will determine if PIs also prevent oligodendrocyte differentiation *in vivo*. This is an important question to resolve, as it would validate our conclusion that PIs produce loss of myelin integrity as well as prevent effective remyelination of the damaged areas in the adult. Furthermore, this procedure could also be merged with candidate adjunctive therapy studies to determine if ART-effects can be attenuated. At present, even less is known about viral and ART effects in children than in adults, with neurocognitive outcomes being unpredictable as they are the first generation to survive infection long-term. With our striking evidence for PI-based oligodendrocyte differentiation deficits, we would like to establish a model of pediatric ART administration in young mice. While current technology may limit when osmotic pumps may be installed, it is of critical importance to understand the effects of ART compounds on the developing brain. Treatment of pediatric animals with ART drugs of various classes followed by cognitive and
behavioral testing as well as pathological analysis will further aid in guiding treatment regimens and understanding ART-mediated effects in HIV-infected children.

One of the most challenging aspects of HIV research is that animal models have been extremely limited due to the lack of membrane surface receptors required for HIV cellular entry in rodents. However, the well-developed model of SIV-infection in macaques as was described and utilized in Chapter 3 is a remarkable tool for researchers, as the disease course and outcomes mirror that of human patients (Dinoso, 2009, Zink, 2010, Graham, 2011). While such studies are a large financial burden, well-designed experimental plans can allow for maximum data collection and translational relevance. Future studies in these animals with SIV-positive, SIV-positive/ART-treated, and SIV-negative/ART-treated groups will be instrumental in determining the independent contributions of virus versus therapy to synaptic damage, inflammation, and loss of myelin integrity. Additionally, studies in these animals can be performed over a longer duration than the available rodent ART-administration models, and will more faithfully report the longitudinal effects of viral and ART exposure in a primate. A final possibility to consider with this model is the potential for development of pre-clinical screening of adjunctive therapies, as neurocognitive function and post-mortem pathological assessment can determine efficacy in a more expedient manner than human clinical trials.

### 6.6 CLINICAL IMPLICATIONS

The studies contained within this body of work have unequivocally demonstrated that antiretroviral compounds, specifically those of the PI class, invoke neuronal toxicity through oxidative stress, halt oligodendrocyte differentiation, produce synaptodendritic damage, and lead to loss of myelin integrity. These findings necessitate crucial reconsiderations of recommended treatment regimens, as well as provide impetus to develop effective adjunctive therapies to attenuate these effects.

In order to prolong anticipated lifespan and prevent viral effects, the World Health Organization urges that all children under the age of 5 receive ART, regardless of their CD4 counts (WHO, 2013). Of paramount concern is that the currently recommended first-line regimen for all children under 3 years of age is
Lopinavir/ritonavir (WHO, 2013). Increasing documentation has revealed that treated pediatric patients are developing neurocognitive deficits and developmental delays, as well as motor, cognitive, and behavioral deficits reminiscent of the hallmarks of HAND seen in adults (Crowell et al., 2014, Whitehead et al., 2014, Wilmshurst et al., 2014). With the currently suggested treatment guidelines, these young children are being exposed to the specific ART-compounds that we have shown propagate neuronal toxicity and prevent oligodendrocyte differentiation during the peak period of neuronal development and cortical myelination in humans (Miller et al., 2012). As these children will already have the lifelong burden of effects stemming from infection and therapy, we hope our findings will be noted by clinicians responsible for amending ART guidelines and alter the drug class of first-line pediatric therapy away from the PI-based regimens.

Additionally, our studies have illuminated synaptodendritic damage and disruption of myelin integrity with PI compounds in rodent models, as well as with combination regimens given to SIV-infected macaques and HIV-infected human patients. These findings highlight that while effective at reducing viral replication, ART compounds can directly negatively affect the health and function of CNS cell populations. The persistence of viral reservoirs necessitates continued ART adherence, despite deleterious side-effects (Chun, 1997, Finzi, 1997, Wong, 1997, Persidsky, 2006). It is therefore of upmost importance to develop adjunctive therapies to ameliorate the damage caused by ART, viral enclaves, and lingering inflammation.

We have demonstrated that MMF provides effective neuronal protection against ART-induced toxicity through Nrf2-mediated induction of the EAR. Importantly, in a study by one of our collaborators, the Kolson laboratory has shown that both MMF and DMF suppress HIV replication in macrophages, in addition to activating the EAR, potently upregulating HO-1 protein, and preventing neurotoxin release (Cross et al., 2011). The specific upregulation of HO-1 responsible for the protective effects of MMF in both our neuronal and their macrophage models may be a key element in attenuating the damage induced by chronic oxidative stress found in HAND patients. Preliminary evidence from our laboratory showed elevated cytoplasmic levels of Nrf2 in HIV-infected ART-treated patients with HAND compared with those patients who remained neurocognitively normal. This increase in cytoplasmic Nrf2 could indicate a failure of pathway activation, as nuclear localization indicates a proper stress-mediated response. To bolster
this finding, HO-1 deficiency has been recently reported in HAND patients (Gill et al., 2014). Additionally, it will be crucial to determine if MMF is capable of reducing oxidative stress in the brain as a whole, in the context of viral infection and ART medication. Unfortunately, based on our findings in oligodendrocytes, unless chronic oxidative stress is eliminated from the CNS compartment, neuronal pathology caused by myelin disruption would remain. In this setting, neurons may initially be protected by direct antioxidant effects of MMF, but unless oxidative stress levels are lowered to beyond the point of preventing oligodendrocyte damage and promoting remyelination, axonal and synaptodendritic damage would continue to persist. Together, these data also suggest that the drug Tecfidera which is currently approved for MS treatment may also be a promising adjunctive therapy for use in HAND and should be tested in animal models to determine if it would be efficacious \textit{in vivo}. Going forward, the effects of antiretroviral compounds on neurons and oligodendrocytes have highlighted the need for future adjunctive therapies designed not only to alleviate neuronal damage, but also to preserve myelin integrity and remyelination capacity of oligodendrocytes in order to attenuate and potentially reverse the neuropathologic and neurocognitive deficits still observed in HIV-positive individuals in the ART-era.

6.7 SUMMARY AND CLOSING REMARKS

Taken together, the cumulative results from our studies indicate that specific antiretroviral compounds invoke drastic negative cellular consequences in multiple CNS cell populations, independent of virus infection. We demonstrated in rodent models of drug administration that synaptodendritic damage and myelin proteins are rapidly altered following repeated exposure in the absence of virus. Extending this finding to a non-human primate model, synaptodendritic damage was also observed in SIV-infected ART-treated macaques. Furthermore, myelin proteins were significantly altered in human patients diagnosed with HAND who had received ART compared to those with HAND but who were ART-naïve, and control subjects without infection. No direct effect on myelin levels were noted when infected versus uninfected subjects were compared, suggesting a potential correlation to alterations of myelin proteins as a consequence of solely the antiretroviral drug exposure.
Through neuronal cultures *in vitro*, we established that antiretrovirals led to ROS accumulation, mitochondrial depolarization, and ultimately apoptotic cell death. Moderate activation of the EAR was noted by antiretrovirals alone, and when this response was boosted by supplementation with the fumaric acid ester MMF, neuronal toxicity was prevented. In addition to investigating cellular stress response signaling pathways and survival in response to antiretroviral exposure, studies in oligodendrocytes also extended our findings to the consequences of these compounds on cellular maturation. Antiretrovirals of the PI but not the NRTI class lead to dose-dependent, reversible reductions in oligodendrocyte differentiation that are independent of oxidative stress-mediated effects. Neither the UPR or EAR are activated in the maturing cells following antiretroviral application, in contrast to previous findings in neurons and peripheral cell types. According to cytoskeletal markers and rapid reversal of effects, we have concluded that oligodendrocytes are normally primed for maturation and enter into differentiation, but myelin proteins cannot effectively be trafficked and/or inserted into the forming myelin membrane in the presence of the antiretrovirals.

Quite surprisingly, oligodendrocytes do not activate the EAR under conditions of oxidative stress induced by antiretroviral drugs, oxidants, or following stimulation with MMF. This may underlie the particular susceptibility of oligodendrocytes to oxidative stress-mediated toxicity and could help to explain the mechanism of action for therapeutics designed to alleviate oxidative stress in Multiple Sclerosis. The cellular milieu of the brain is an intricately interconnected system, where cell populations are critically dependent on one-another. Our results indicate that like neurons, which rely on neighboring cells such as astrocytes and macrophages for protection from oxidative stresses, oligodendrocytes likely behave in much the same way. It is also important to consider that while MMF is successful at rescuing ART-induced neuronal toxicity, it did not rescue the oligodendrocyte differentiation deficit. Even though the EAR was not upregulated, MMF did attenuate ROS in oligodendrocytes, suggesting that PI-based inhibition of maturation is through an independent mechanism. Future investigations will hopefully isolate the mechanism leading to this dysfunction, in order to effectively prevent the halt in differentiation.
We have discovered that PIs in particular have profound effects in perturbing oligodendrocyte maturation and invoking neuronal toxicity. We sincerely hope that our findings are used to inform decisions on the suggested pediatric ART-regimens, substituting Lopinavir/ritonavir with less toxic agents as frontline treatment during the critical period of development and myelination in children.

Much work is still needed to facilitate a better understanding of the effects of antiretroviral compounds in CNS cell populations. Once cellular consequences to antiretroviral drug exposure are more thoroughly defined, appropriately targeted adjunctive therapies will be more likely to succeed in attenuating neurological complications and reducing the severity and prevalence of HAND in HIV-infected individuals.
Figure 6.1: Model of Antiretroviral Effects on Myelin in Humans

A) Schematic of pediatric myelination and the negative consequences of HIV-infection & treatment

B) Schematic of adult myelin maintenance and the negative consequences of HIV-infection & treatment

Highlighted in red are hypothesized concepts supported through data within the Chapter 4 manuscript.
6.8 REFERENCES


APPENDIX 1

Treating Astrocytes with Antiretroviral Compounds Does Not Elicit Indirect Neuronal Toxicity or Impact Oligodendrocyte Differentiation

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Unpublished Data
**A1.1 EXPERIMENTAL RATIONALE**

In light of our findings regarding direct neuronal toxicity and altered oligodendrocyte function following antiretroviral drug exposure, we also wished to extend our findings to the possible indirect effects that secreted factors from astrocytes may have in each of these scenarios. To do this, enriched astrocytes and neurons were grown *in vitro* as described in Chapters 3, with oligodendrocytes generated from Sprague Dawley rat pups instead of mice following the same protocol as in Chapter 4 to maintain the same species throughout all experiments. Primary astrocyte cultures were then treated with a panel of antiretroviral compounds in a timecourse of 6, 12, and 24 hours (n=3 independent biological replicates). At each endpoint, supernatants were collected to determine potential effects on neurons and oligodendrocytes. The following antiretrovirals were tested: NRTIs AZT (25 µM), d4T (25 µM), ddC (25 µM); and Protease Inhibitors Ritonavir (10 µM), Saquinavir (1.0 µM), and Indinavir (25 µM). Thapsigargin at 1.0 µM served as a positive control, as release of ER-calcium stores should invoke the release of factors from astrocytes which may harm neurons.

To begin, these supernatants were applied to 14DIV enriched neuronal cultures for 48 hours at a 1:20 dilution. Following this period a MAP2 ELISA assay was performed to assess neuronal damage/death as described in Chapter 3. As can be seen in Figure A1.1, none of the supernatants from antiretroviral treated astrocytes at any point during the timecourse produced an effect on neuronal viability (n=3 biological replicates of neurons, each treated with the triplicate astrocytes derived samples). The only treatment which caused a significant decrease in MAP2 fluorescence was the sample group from 6 hour Thapsigargin treated-astrocytes (p <0.001), however this effect is resolved if samples are not taken until 12 or 24 hours suggesting that at later timepoints the astrocytes have successfully dealt with the ER-stress.

To assess potential contributions to oligodendrocyte maturation and survival, astrocyte derived supernatants were also applied at the start of differentiation at a 1:20 dilution. After 72 hours in differentiation medium, maturation was assessed by immunofluorescence as in Chapter 4. Similar to the findings on neurons, no effects on oligodendrocyte differentiation or cell number were observed following exposure to supernatants from antiretroviral treated astrocytes at any point during the timecourse (n=3
biological replicates of oligodendrocytes, each treated with the triplicate astrocytes derived samples). Thapsigargin again produced a significant effect, with a reduction in cells progressing to express either immature (GalC) or mature (MBP) markers. This effect is found in oligodendrocytes treated with supernatants collected from all antiretroviral exposure times for the astrocytes. There is also a trend towards a significant decrease in total cell number, suggesting a potential toxic effect in addition to a halt in differentiation (Figure A1.2).

A1.2 MATERIALS AND METHODS


Additional antibodies: anti-galactocerebroside mouse hybridoma supernatant (GalC H8H9), anti-myelin basic protein rat hybridoma supernatant (MBP, gift of Virginia Lee, University of Pennsylvania).

Primary Cortical Neuroglial Cultures. All experiments were performed in accordance with guidelines set forth by The University of Pennsylvania Institutional Animal Care and Use Committees (IACUC). Primary rat cortical neuroglial cultures were isolated from embryonic day 17 Sprague Dawley rat pups, as previously described (Chapter 3). Cortical cell suspensions isolated from pups were plated on poly-L-lysine.
coated T25 tissue culture flasks (2 x 10^6 cells/flask) or 96-well tissue culture plates (0.5 x 10^5 cells/well).

To attain pure astrocytic cultures, the cortical cell suspensions were initially plated in 10% fetal bovine serum (FBS) in DMEM and maintained at 37°C with 5% CO_2 for 7-12 days. At this stage the cultures are confluent and astrocytes constitutes approximately 90% of cells. Cells were harvested from these cultures and re-plated onto poly-L-lysine coated 60mm dishes, with experiments conducted once cells had achieved 75% confluency. Neuronal cultures were maintained in Neurobasal media with B27 supplement at 37°C with 5% CO_2, with 10µM Ara-C added 24 hours after plating to establish pure neuronal cultures. Half of the media was replaced with fresh media every 7 days, with experiments conducted at 14 DIV.

**Primary Cortical Oligodendrocyte Cultures**

All experiments were performed in accordance with the guidelines set forth by The Children's Hospital of Philadelphia and The University of Pennsylvania Institutional Animal Care and Use Committees (IACUC). Primary rat oligodendrocyte precursor cell (OPC) cultures were isolated from postnatal day 4 Sprague Dawley rat pups obtained from Charles River Laboratories, as previously described in Chapter 4 for mouse oligodendrocyte isolation. Cortical cell suspensions isolated from rat pups using standard protocols were plated on poly-D-lysine coated 10-cm dishes in Neurobasal medium with B27 supplement at 37°C with 5% CO_2. Cultures were switched to growth medium consisting of Neurobasal medium with B27, which contained 10 ng/ml bFGF, 2 ng/ml PDGF-AA, and 1 ng/ml NT-3 24 hours after initial plating. Following a week of growth, confluent cells were purified to 90-95% OPCs and 5-15% astrocytes using a gentle washdown procedure (Feigenson et al., 2009), and sub-cultured onto poly-lysine-coated coverslips. For differentiation, growth medium was replaced with differentiation medium, consisting of 50% DMEM, 50% Ham’s F12, Pen/Strep and 2 mM glutamine with 50 µg/ml transferrin, 5 µg/ml putrescine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 µg/ml insulin, 0.4 µg/ml T4, 0.3% glucose, and 10 ng/ml biotin.

**MAP2 Cell-Based ELISA**

To quantify neuronal damage/death, a MAP2 cell-based ELISA was performed as previously described (Chapter 3). At the end of treatments, neuronal cultures in 96-well plates were fixed with 4%
paraformaldehyde and 4% sucrose for 30 minutes. Following blocking for 1 hour in PBS+5% normal goat serum, plates were incubated with MAP2 antibody overnight at 4°C. Plates were washed with PBS+0.1% Tween-20 (PBS-T), then incubated with goat anti-mouse secondary antibody conjugated to beta-lactamase TEM-1 for 30 minutes. Following subsequent PBS-T washes, the plate was incubated with fluorocillin green substrate for 1 hour in the dark. Fluorescence intensity was measured using a Fluoroskan Ascent plate reader (Thermo Electron, Waltham, MA) with excitation at 485 nm and emission at 527 nm.

**Immunofluorescence**

Oligodendrocytes were processed for detection of specific antigens as described in Chapter 4. Live cells were labeled for cell surface marker detection anti-GalC (mouse hybridoma supernatant, undiluted). For detection of internal antigens following acid alcohol fixation and permeabilization, anti-MBP (rat hybridoma supernatant, 1:2 dilution) was used. Secondary antibodies (1:100 dilution) were Rhodamine or Fluorescein conjugated. Vectashield with DAPI was used to mount coverslips and to stain all nuclei. Antigen-positive and DAPI-positive cells were counted in 10 fields in each of 3 coverslips using a Leica DM6000B fluorescence microscope at 40x magnification. A total of approximately 2000 cells were counted per condition.
Figure A1.1 Indirect Neuronal Toxicity Does Not Occur Following Incubation with Conditioned Medium from Antiretroviral-Treated Astrocytes

Primary rat enriched neuronal cultures aged 14 days in vitro (DIV) on coverslips were exposed for 48 hours to a 1:20 dilution of supernatants derived from astrocytes which had been treated for 6 hours (A), 12 hours (B), or 24 hours (C) with Vehicle, AZT, Ritonavir, Saquinavir, Indinavir, ddC, d4T, or Thapsigargin. A MAP2 cell-based ELISA was performed to assess neuronal damage/death (n=3, vehicle: 0.02% DMSO, ###p<0.001, One-Way ANOVA with post-hoc Newman-Keuls determined statistical significance).
Figure A1.2 Conditioned Medium from Antiretroviral-Treated Astrocytes Does Not Alter Oligodendrocyte Differentiation

Primary rat oligodendrocyte cultures were exposed to a 1:20 dilution of supernatants derived from astrocytes which had been treated for 6 hours (A), 12 hours (B), or 24 hours (C) with Vehicle, AZT, Ritonavir, Saquinavir, Indinavir, ddC, d4T, or Thapsigargin during the 72 hour course of differentiation. Cells were fixed and stained with antibody to GalC, and MBP, with DAPI for total cell nuclei. Epifluorescent images were captured, with 10 fields on three coverslips per condition. Immature oligodendrocytes (GalC+ cells, left-hand column), mature oligodendrocytes (MBP+ cells, middle column), and total cell number (right-hand column) were counted using ImageJ software, represented as percentage normalized to untreated. Data are presented as mean ±SEM. (n=3, vehicle: 0.02% DMSO, ###p<0.001, ##p<0.01, One-Way ANOVA with post-hoc Newman-Keuls determined statistical significance).