HIV AND ANTIRETROVIRALS IN THE CENTRAL NERVOUS SYSTEM: MOLECULAR MECHANISMS OF COGNITIVE IMPAIRMENT

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ABSTRACT

HIV AND ANTIRETROVIRALS IN THE CENTRAL NERVOUS SYSTEM: MOLECULAR MECHANISMS OF COGNITIVE IMPAIRMENT

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Kelly Jordan-Sciutto

HIV-associated neurocognitive disorder (HAND) describes a wide range of cognitive impairments experienced by up to 55% of HIV+ individuals despite viral suppression by combined antiretroviral therapy. Reasons for the persistence of this disease are unknown, but may be related to both the presence of HIV-infected macrophages in the central nervous system as well as neurotoxicity of antiretroviral drugs. In this thesis, we identified two independent mechanisms of HIV-associated and antiretroviral-associated toxicity that may each contribute distinctly to HAND neuropathogenesis. First, we showed that β-site amyloid precursor protein cleaving enzyme 1 (BACE1), which may play a role in the onset and progression of Alzheimer's Disease, was both increased in HIV+ patient brains and required for HIV-associated neurotoxicity *in vitro*. The BACE1 cleavage target amyloid precursor protein (APP) also

mediated toxicity and was required for neuroprotective effects of BACE1 inhibition. Second, we showed that two frontline treatment antiretroviral drugs have neurotoxic potential *in vitro* and that neurotoxicity of antiretrovirals is highly variable both across and within drug classes. Neurotoxicity of one drug, lopinavir, was mediated by oxidative stress. Taken together, these data indicate that HIV and antiretrovirals may contribute to HAND persistence and that both BACE1 inhibitors and drugs targeting oxidative stress may be effective as adjunctive therapeutics in HIV+ patients.

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CHAPTER 1: INTRODUCTION

Human immunodeficiency virus (HIV) affects 36.9 million people worldwide (UNAIDS 2015) and is currently incurable. Although the disease can be well managed with combined antiretroviral therapy (cART), cART fails to eradicate the virus from the body even after decades of use (Mzingwane and Tiemessen 2017), has severe side effects (Bhatti *et al.* 2016), and does not decrease the incidence of HIV-associated cognitive symptoms (Saylor *et al.* 2016). Moreover, due to the prevalence of HIV in resource poor areas such as sub-Saharan Africa, 22 million HIV+ individuals do not have access to cART (UNAIDS 2015). Hence, the quests for an HIV cure (Melkova *et al.* 2017) and vaccine (Pegu *et al.* 2017) remain urgent priorities across the globe.

Based on the insurmountable nature of these challenges thus far, scientists and doctors are in constant efforts to advance the landscape of antiretroviral drugs (ARVs). Improvements since the advent of cART in 1996 have been numerous, including introduction of ARVs with better viral suppression, more convenient dosing regimens, and lower incidence of side effects (Günthard *et al.* 2016). Somewhat bafflingly, however, none of these advances nor the advent of cART itself has been

able to reduce the incidence of neurological complications associated with the HIV diagnosis (Saylor *et al.* 2016). There are likely multiple modes by which these complications occur (Etherton *et al.* 2015; Kaul 2008), and this multifactorial causality in addition to the unique challenges posed by CNS-focused HIV research (Ellis and Letendre 2016) have hindered the ability to confidently identify therapeutic targets. This dissertation will encompass a detailed discussion on the potential factors driving cognitive symptoms in HIV as well as provide original data and interpretation regarding new therapeutic avenues to pursue.

The dynamic diagnosis of HIV-associated neurocognitive disorder

Neurological complications diagnosed in HIV+ individuals are currently termed HIV-associated neurocognitive disorder, or HAND (Antinori *et al.* 2007). Although the prevalence of HAND did not decrease with the advent of cART in 1996, the nature and severity of the disease has dramatically shifted (Saylor *et al.* 2016; Gelman 2015). Moreover, as doctors and scientists navigate the novel landscape of a majority HIV+ population over 50 (Valcour 2013), combined effects of aging and

HAND promise to further complicate efforts toward precise diagnosis and treatment (Hellmuth *et al.* 2014).

The first published description of HAND, which was then called AIDS dementia complex (ADC), was written by Navia *et al.* in 1986. At that time, progression to AIDS due to an HIV+ diagnosis was almost certain, and authors reported that nearly two thirds of autopsied cases in their study had signs of dementia that were unexplained by CNS opportunistic infection. Cognitive impairment, behavioral changes, and motor disturbances were all common features of ADC. For most patients, these symptoms began at mild stages and progressed steadily over weeks to months until very little cognitive capability remained. At end stage disease, it was not uncommon for ADC patients to be bedridden, incontinent, and generally unaware of their surroundings.

In a companion article concurrently released by Navia *et al.*, the most frequently observed neuropathological features of ADC were reported. Gross cerebral atrophy was the most prominent observation across mild, moderate, and severe dementia cases. Other abnormalities were white matter pallor and the presence of multinucleated giant cells (MGCs), which are produced by fusion of infected macrophages (Sutton and Weiss 1966). The majority of pathological observations were in the basal ganglia, pons, and other subcortical structures, with the cortex

being relatively spared. These symptomatic and neuropathological features were repeatedly observed throughout the next decade, along with activated microglia, myeloid cell infiltration, synaptodendritic damage, and astrocytosis (Petito *et al.* 1986). It became evident that HIV, even in the absence of opportunistic infection or other comorbidities, directly caused CNS disease (Price 1996).

Throughout this same period, however, manifestations of ADC were already beginning to shift with the introduction of the first antiretroviral drug azidothymidine (AZT) in 1986. It became immediately clear that in addition to dramatically lowering viral load in HIV+ individuals (Yarchoan *et al.* 1986; Fischl *et al.* 1987), AZT also decreased the rate and severity of neurological complications (Yarchoan *et al.* 1987; Schmitt *et al.* 1988). However, AZT was not an ideal therapeutic intervention given its side effect profile and the ability of the virus to mutate and eventually escape suppression by the drug (Larder and Kemp 1989).

To address these issues, scientists developed novel antiretroviral agents for use first in combination with and eventually in lieu of AZT. By combining several different ARVs with unique mechanisms of action targeting viral replication, HIV+ individuals were able to keep viral load low, improve immune function, and live longer (Schmit and Weber

1997; Brodt *et al.* 1997). This new standard of care, now known as cART, also markedly improved neurological functioning (Ferrando *et al.* 1998; Price *et al.* 1999) and decreased the incidence of ADC (Sacktor *et al.* 2001). Perhaps most importantly, the most severe stages of ADC were rarely observed in patients with access to cART (McArthur *et al.* 2003).

However, it was reported soon after the introduction of cART that the rate of ADC did not decline as quickly as rates of other AIDS-defining illnesses (Dore *et al.* 1999), and one report found that among two cohorts of patients with advanced infection, rates of neurocognitive dysfunction were unchanged by cART (Sacktor *et al.* 2002). The authors concluded that despite marked overall improvements, selected neurocognitive deficits remained prevalent.

These shifted symptomatic features characterizing the disease in the post-cART era predominantly consisted of memory and executive function deficits, with less impact on motor skills and verbal processing than had been observed in ADC (Heaton *et al.* 2011). Despite the reduced prevalence of severe ADC in particular, these distinct impairments have persisted in the decades following cART introduction. They form the current classification of HAND and comprise defined subsets according to severity (Antinori *et al.* 2007). Patients diagnosed

with asymptomatic neurocognitive impairment (ANI) demonstrate impaired performance on neuropsychological testing within at least two cognitive domains, but note no interference with everyday functioning, whereas a diagnosis of mild neurocognitive disorder (MND) indicates similarly impaired testing performance but with added interference in daily life. Diagnosis of the most severe form of HAND, HIV-associated dementia (HAD), requires that the patient present with moderate-to-severe impairment within at least two cognitive domains in addition to markedly impaired functioning (Rosca *et al.* 2012; Antinori *et al.* 2007).

Currently, estimates regarding the overall persistence of HAND among HIV+ individuals range from 15 – 55% (Sacktor *et al.* 2016). This is controversially high given that up to 70% of these patients fall within the ANI category, and it has been suggested that ANI may be overestimated (Gisslén *et al.* 2011). An alternative view, however, is that MND is often misdiagnosed as ANI due to patients not recognizing the impact their impairments have on daily functioning (Valcour 2013). Regardless, ANI patients remain a critical demographic to target because these individuals are 2 – 6 times more likely to develop moderate or severe forms of HAND versus patients with no impairment (Grant *et al.* 2014a).

Neuropathological features of the disease have also dramatically shifted since the advent of cART. In either HIV- vs. HIV+ individuals or in individuals with HAND, differences in several cerebrospinal fluid (CSF) factors have been identified, including increased immune and cytokine activation markers (Kamat *et al.* 2012; Lyons *et al.* 2011) and changes in factors associated with neuronal damage such as neurofilament light chain protein (NFL) and tau (Abdulle *et al.* 2007; Angel *et al.* 2012; Peluso *et al.* 2013). However, many of these markers are only relevant either for patients with HAD or patients in late stages of immune complication (Saylor *et al.* 2016).

Some similarities in *post mortem* observations exist between recent studies and the early reports of Navia *et al.* 1986, including subtle changes in white matter and the presence of HIV-infected perivascular macrophages (Brown 2015; Fischer-Smith *et al.* 2008; Saylor *et al.* 2016). Overall, however, HAND patients with viral suppression by cART and an absence of other comorbidities look very similar to both neurocognitively normal and HIV- controls upon autopsy (Gelman 2015). This lack of an obvious pathological feature to indicate potential therapeutic targets has presented a dilemma for the field, and as yet no adjunctive therapies have been approved for use in HAND patients (Rosca *et al.* 2012; Saylor *et al.* 2016).

As an added complexity in the navigation of this changing disease, increased lifespan afforded by cART (Lai *et al.* 2006) means the average age of HIV+ individuals is increasing. Currently over half of HIV+ individuals in the United States are over 50 years of age, and many of these patients have been living with HIV for decades (Valcour 2013). Thus, physicians and scientists have the novel task of understanding effects of both aging and prolonged duration of infection on cognitive functioning. Some studies have indicated that aging may not exacerbate HAND symptoms (Milanini *et al.* 2017; Vance *et al.* 2016), but potential confounds and inconsistent results have left the individual contributions of aging and HIV difficult to resolve (Hellmuth *et al.* 2014).

Abnormal aging is also a concern, with particular attention paid to whether HIV+ individuals are more likely to develop Alzheimer's Disease (AD). Of concern, the genetic risk factor for AD ApoE ϵ4 is also predictive of poorer cognition and increased brain atrophy in HIV+ patients over 60, indicating the possibility of mechanistic overlap between AD and HAND (Panos *et al.* 2013; Wendelken *et al.* 2016). However, the data regarding the similarities and differences between the two diseases have been mixed. The following section includes a detailed discussion of AD-like symptoms, biomarkers, and pathology among HIV+ individuals.

Common features of Alzheimer's Disease and HAND: a focus on amyloid precursor protein

With an aging HIV+ population, HAND and AD are now emerging in an overlapping demographic of men and women over 60. HAND in its current form shares symptoms in common with AD including personality changes, forgetfulness, and attention difficulties (Meehan and Brush 2001). In some cases, these similarities impede the ability of physicians to distinguish AD from HAND (Xu and Ikezu 2008; Szirony 1999).

Soluble markers in cerebrospinal fluid and plasma

One diagnostic strategy is to measure soluble factors in the CSF that can serve as unique disease biomarkers. The defining CSF biomarker of AD is decreased concentrations of the soluble peptide amyloid-β 42 (Aβ42) (Dickerson *et al.* 2013; Blennow *et al.* 2015), and it has been suggested that these changes are specific to AD and can be used to distinguish from HAND (Ances *et al.* 2012; Steinbrink *et al.* 2013; Mäkitalo *et al.* 2015). In fact, one study reported *increased* levels of CSF Aβ42 in HIV+ patients as compared to HIV- controls (Peluso *et al.* 2013). However, three other studies (Brew *et al.* 2005; Clifford *et al.* 2009b; Krut *et al.* 2013) identified a pattern of CSF Aβ42 decrease in HIV+ individuals similar to that seen in AD patients, implying both less

potential utility as a unique biomarker as well as possible overlapping mechanistic features of the two diseases. Importantly, decreases were observed even in patients on cART, and they were specific to Aβ42 while other AD biomarkers such as phosphorylated tau and total tau were unchanged. These contradictory results have made it difficult to clearly identify biomarkers of HAND as well as to determine whether mechanisms involving Aβ42 are relevant for HAND neuropathogenesis.

Amyloid plaque formation and the role of β-amyloid cleaving enzyme in Alzheimer's Disease

In AD, CSF Aβ42 concentration is anticorrelated with Aβ42-related pathology in the brain, characterized by amyloid deposition. Amyloid deposition is a hallmark feature of AD and describes the accumulation of dense amyloid plaques in the extracellular space of hippocampus, cortex, and other regions depending on progression of disease (Fagan *et al.* 2006; Roe *et al.* 2013).

Amyloid plaques are formed through proteolytic processing of amyloid precursor protein (APP). Under normal physiological conditions, APP is trafficked through the secretory pathway to the cell surface, where in its full-length form it serves a variety of functions involving cell-cell adhesion and synaptic regulation (Müller *et al.* 2017). It is also

sequentially cleaved by the α-secretase a disintegrin and metalloproteinase domain-containing protein (ADAM10) (Lammich *et al.* 1999) and the γ-secretase presenilin 1 (PS-1) (Selkoe and Wolfe 2000), resulting in the production of cleavage products with additional unique roles in cellular function. One of these cleavage products in particular, soluble APPα (sAPPα), has been identified as a neuroprotective factor through multiple downstream mechanisms (Obregon *et al.* 2012; Milosch *et al.* 2014; Furukawa and Mattson 1998). APP is also constitutively endocytosed, and in endosomes it is cleaved by the β-secretase β-site amyloid precursor protein cleaving enzyme 1 (BACE1) to form sAPPβ and C-terminal fragment-β (CTFβ) (Kandalepas and Vassar 2014). CTFβ is then cleaved by PS-1 to generate an APP intracellular domain (AICD) fragment and the Aβ42 or Aβ40 monomer. Aβ42 monomers readily oligomerize and can eventually form dense fibrils and amyloid plaques. Although the generation of Aβ42 monomers is not abnormal, the observation of plaque formation is strictly seen in pathological cases and forms one of the major defining features of AD (Blennow *et al.* 2015). Critically, however, plaque formation is not necessarily correlated with cognitive impairment in AD (Perrin *et al.* 2009), and stronger correlations are observed when comparing cognition with the accumulation of Aβ42 oligomers at the synaptic cleft

(Haass and Selkoe 2007). These and other observations in animal models suggest that toxicity may be driven by the oligomeric Aβ species rather than plaques themselves (Kayed and Lasagna-Reeves 2013; Sengupta *et al.* 2016).

Expression of BACE1 protein is increased by approximately two-fold in AD patient brains (Fukumoto *et al.* 2002; Holsinger *et al.* 2002; Yang *et al.* 2003; Johnston *et al.* 2005), while expression of ADAM10 is decreased in both brain and CSF (Colciaghi *et al.* 2002; Bernstein *et al.* 2003; Olsson *et al.* 2003; Fellgiebel *et al.* 2009). This imbalance drives APP processing toward the amyloidogenic rather than the non-amyloidogenic pathway in AD, resulting in decreased levels of neuroprotective sAPPα (Almkvist *et al.* 1997; Lannfelt *et al.* 1995) as well as increased β-amyloid oligomers and the formation of plaques. Shifts in secretase expression and activity may play a causal role in cognitive impairment. Indeed, one genetic mutation predicting early onset AD is a change in the amino acid sequence of APP that confers higher affinity for BACE1 (Citron *et al.* 1992). Genetic loss of BACE1 in animal models essentially abolishes Aβ production (McConlogue *et al.* 2007), and in several rodent models of AD either genetic or pharmacologic BACE1 inhibition improves synapse function and memory (Ohno *et al.* 2004; Singer *et al.* 2005; Fukumoto *et al.* 2010; Chang *et*

al. 2011; Devi *et al.* 2015). Based on these studies and others, BACE1 inhibitors have been developed by several pharmaceutical companies and are currently in phase II and III clinical trials to assess efficacy in treating AD patients (Yan and Vassar 2014; Ghosh and Tang 2015).

Whether similar BACE1 upregulation or BACE1-dependent impairments are present in HAND or animal models of HIV is not known. *In vitro* studies suggest that the viral protein trans-activator of transcription (tat) can stimulate BACE1 colocalization with APP in the endolysosome and increase its proteolytic activity (Chen *et al.* 2013; Kim *et al.* 2013), and viral protein glycoprotein 120 (gp120) can increase BACE1 transcription (Bae *et al.* 2014). However, relevance of these particular mechanisms in mixed neuroglial environments or *in vivo* remains unclear.

Amyloid Precursor Protein Processing in HAND

Despite the lack of research focused on a specific role for BACE1 in HAND, many studies have attempted to address whether pathological APP processing is present in patients. Reports have conclusively identified diffuse amyloid plaques or other amyloid pathology in HIV+ patients prior to the availability of cART (Raja *et al.* 1997; Esiri *et al.* 1998; Izycka-Swieszewska *et al.* 2000; Rempel and Pulliam 2005;

Achim *et al.* 2009). In cohorts that included patients taking cART, amyloid pathology has also been observed (Green *et al.* 2005; Soontornniyomkij *et al.* 2012; Levine *et al.* 2016), and in one study patients with access to cART actually had increased amyloid deposition as compared to patients in the pre-cART era (Green *et al.* 2005). However, this could be due to several factors, including the parsimonious explanation that patients taking cART live longer following seroconversion.

Although these *post mortem* studies converge on agreement that altered APP processing is present in HIV+ individuals, it remains unclear A) whether APP pathology is a feature of HAND or of HIV in general, B) whether the observed accumulation represents amyloid, full-length APP, or an intermediate species such as Aβ42 oligomers, and C) whether the observed accumulation is extracellular (as observed in AD) or intracellular. Although very few data exist to address the first two points, current evidence from analysis of brain tissue samples suggests that intracellular vs extracellular pathology is more commonly observed (Green *et al.* 2005; Achim *et al.* 2009). These findings are corroborated by a series of positron emission tomography (PET) scan experiments using Pittsburgh compound B ([^{11}C] PiB) to specifically recognize fibrillar amyloid deposits in the extracellular space. Three of such studies (Ances

et al. 2010; Ances *et al.* 2012; Ortega and Ances 2014) found no evidence of extracellular deposition in HIV or HAND specifically, even in older patient populations.

Hence, the difficulty in determining the potential role of APP processing in HAND lies in its unique presentation. Ample evidence suggests that APP processing differs in HIV+ individuals versus HIV- controls; however, neither CSF nor parenchymal observations mimic those characteristic of AD. A more detailed characterization of the specific APP pathology associated with HIV and HAND is needed in order to assess the precise mechanisms implicated in AD that may also be relevant in HAND. A better understanding of this overlap has potential to facilitate A) distinction between the two diseases when diagnosing older patients with HIV, B) rational drug design for adjunctive therapies in HAND, and C) determination of disease-causing pathological features as distinct from epiphenomena. This last potential benefit is often underappreciated but should not be overlooked; if scientists are able to more precisely identify common pathology across neurodegenerative diseases with similar symptoms, there will be a higher likelihood of identifying features directly related to those symptoms. Critically, this approach may accelerate advances in AD as well as in HAND.

Although a more precise clarification of APP pathology in HAND will be useful, it is also important to note that additional APP species rather than amyloid plaques *per se* may mediate toxicity in AD (Kayed and Lasagna-Reeves 2013). Thus, HAND neuropathogenesis may still be related to amyloidogenic processing of APP even if extracellular plaques are not formed in HIV+ patient brains.

HIV or antiretrovirals: which is responsible for HAND persistence?

To explain the persistence of HAND despite viral suppression with cART (Saylor *et al.* 2016), two distinct theories have emerged: either A) CNS penetrance and/or efficacy of antiretroviral drugs (ARVs) is not high enough to sufficiently reduce viral load in the brain, or B) ARVs themselves contribute to the development of HAND (Etherton *et al.* 2015). Given that these two possibilities have exactly opposite implications for how to set new therapeutic goals, identifying the true cause of HAND persistence has been a high priority over the past decade.

Evidence for the role of HIV

The most convincing point in support of a role for HIV rather than ARVs in HAND persistence is that for most ARVs, with the exception of efavirenz (Avery *et al.* 2013) and dolutegravir (Letendre *et al.* 2014), concentrations in CSF are substantially lower than those measured in plasma (Best *et al.* 2009; Best *et al.* 2012; Eisfeld *et al.* 2013). Moreover, the primary cells in the brain responsible for viral replication are macrophages, and effects of ARVs on HIV in macrophages are not as pronounced as effects on peripheral T cells (Aquaro *et al.* 2002). Finally, one study of 53 patients found that in some cases HIV in the CSF developed resistance to ARVs independently from virus in the plasma (Cunningham *et al.* 2000). Perhaps due to one or more of these factors, viral RNA has been identified in CSF even when plasma viral loads are undetectable (Edén *et al.* 2010; Canestri *et al.* 2010; Peluso *et al.* 2012), a phenomenon termed viral escape. In cases of high viral load in the brain, HIV may cause neurological impairments through direct effects of viral proteins on neurons, alterations in glial function with indirect effects on neurons, or a combination of both routes (Kaul 2008; Kaul and Lipton 2006; Kovalevich and Langford 2012).

HIV does not directly infect neurons, but factors shed from the virus may still affect neurons through interactions with surface receptor proteins (Kaul *et al.* 2001). HIV canonically enters cells by interaction

with CD4 as well as either the CXCR4 or CCR5 chemokine receptor; these chemokine receptors are expressed in neurons (Asensio and Campbell 1999), and *in vitro* evidence suggests they may mediate neurotoxicity induced by HIV (Chen *et al.* 2002; Kaul and Lipton 1999). Several viral proteins cause neuronal damage *in vitro* including tat (New *et al.* 1997), gp120 (Brenneman *et al.* 1988; Kaul *et al.* 2001), and nef (Koedel *et al.* 1999). Neurotoxic potential of these proteins persists in primary neuronal cultures without glia (Meucci *et al.* 1998) as well as in neuroblastoma cell lines (Hesselgesser *et al.* 1998), indicating that direct effects on neurons are a possible mediator of neuronal damage induced by HIV.

However, ample evidence suggests that when the neuronal environment better reflects the brain by including glial populations, HIV-induced neurotoxicity is primarily mediated by other cell types (Kaul *et al.* 2001). Macrophages in particular play a critical role, as they sustain productive infection by HIV beginning soon after seroconversion (Ho *et al.* 1985; Koenig *et al.* 1986). In 1990, scientists observed that *in vitro* these infected myeloid cells secrete factors that induce potent neurotoxicity (Giulian *et al.* 1990). Moreover, direct stimulation of uninfected myeloid cells with gp120 was also sufficient to induce release of neurotoxins (Giulian *et al.* 1993), indicating that effects of viral

proteins in many *in vitro* experiments may be explained by glial involvement. Indeed, several later studies confirmed that macrophages were a key driver of HIV-induced neurotoxicity under various experimental paradigms and through multiple downstream mechanisms (Kaul and Lipton 1999; Viviani *et al.* 2001; Medders *et al.* 2010; Festa *et al.* 2015). Astrocytes also make important contributions to the neurotoxicity of HIV both *in vivo* and *in vitro* (Genis *et al.* 1992; Vázquez-Santiago *et al.* 2014).

Evidence for the role of antiretroviral drugs

Depending on their mechanism of action, ARVs can be divided into six classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors (FIs), and entry inhibitors (EIs). The Department of Health and Human Services (DHHS) current recommends that frontline treatment for HIV include two NRTIs in combination with a PI or an INSTI (DHHS 2016a). This particular regimen is not optimally effective for all patients nor available across the globe to all HIV+ individuals, and therefore all classes are still routinely prescribed depending on the circumstances.

Ample evidence also suggests that ARVs may contribute to HAND neuropathogenesis. In *in vitro* and animal studies, ARVs from various drug classes can induce mitochondrial dysfunction, ER stress, and oxidative stress, ultimately leading to synaptodendritic damage and neuronal death (Akay *et al.* 2014; Brown *et al.* 2014; Robertson *et al.* 2012b; Vivithanaporn *et al.* 2016; Gannon *et al.* 2017).

The early NRTIs, and in particular the first drug introduced to treat HIV, zidovudine (AZT), induced potent mitochondrial toxicity after chronic exposure (Lewis and Dalakas 1995). This potential may be due to an inhibitory effect of nucleoside analogues on the γ-subunit of DNA polymerase (Côté *et al.* 2002; Apostolova *et al.* 2011), as several other NRTIs have since been found to damage mitochondrial DNA as well (Dalakas 2001). Indeed, in one study the NRTI efavirenz caused a loss of ATP and depolarization and fragmentation of mitochondria in both primary neurons and a neuroblastoma cell line (Purnell and Fox 2014). Mitochondrial toxicity has also been observed across cell types including neurons with both NNRTIs and PIs, although the mechanisms are unclear (Apostolova *et al.* 2011). Interestingly, however, in one experiment probing both mitochondrial damage and neurotoxic potential of ARVs across classes, the effects of ARVs on mitochondrial respiration were not related to neurotoxic potential (Robertson *et al.*

2012b). This indicates that at least for some ARVs, damage occurs through additional mitochondria-independent pathways.

An additional mechanism of neurotoxicity induced by ARVs may be endoplasmic reticulum (ER) stress, resulting from an accumulation of misfolded proteins and causing activation of the unfolded protein response (UPR). In one recent report, the NRTI efavirenz caused activation of UPR signaling proteins protein kinase R-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1 (IRE1) in brain endothelial cells (Bertrand and Toborek 2015), indicating a potential mechanism by which ARVs may disrupt the blood-brain barrier (BBB). The effects of PIs on ER stress have also been the subject of investigation given that PIs ritonavir (André *et al.* 1998), saquinavir (Pajonk *et al.* 2002), and nelfinavir (Hamel *et al.* 2006) can directly inhibit the proteasome causing ER protein accumulation. Our previous work has demonstrated that ritonavir leads to PERK activation both *in vivo* and *in vitro*, resulting in increased BACE1 expression and neuronal damage (Gannon *et al.* 2017). Potential relevance of this pathway in patients is highlighted by our earlier work demonstrating UPR activation in *post mortem* HIV+ brains (Lindl *et al.* 2007; Akay *et al.* 2012), although these results cannot distinguish between effects of ARVs and possible effects of HIV itself on ER stress.

ARVs across classes can increase production of reactive oxygen species (ROS) leading to oxidative stress in many cell types. This phenomenon has received most attention in the periphery due to its role in many of the common side effects associated with ARVs (Elias *et al.* 2013; Ivanov *et al.* 2016). In HIV+ patients, indicators of oxidative stress in plasma are increased by HIV itself and further increased by ARVs, including PIs in particular (Ngondi *et al.* 2006; da Cunha *et al.* 2013). Studies addressing oxidative stress in the brain specifically are less abundant, but one report demonstrated that the NRTI efavirenz increased ROS in a neuroblastoma cell line, which was associated with increases in BACE1 expression and activity (Brown *et al.* 2014). In addition, we have shown that PIs ritonavir and saquinavir potently increase ROS in primary neuronal cultures and that alleviation of oxidative stress with monomethyl fumarate (MMF) is protective against PI-induced neuronal damage. In the same study, relevant combinations of ARVs including a PI induced neuronal damage and neuronal loss in both non-human primates and rats (Akay *et al.* 2014), indicating ability of ARVs to injure neurons *in vivo* regardless of HIV infection.

Although *in vitro* and animal studies have provided valuable insights into the possible mechanisms of ARV-induced neurotoxicity, the most powerful evidence in support of neurotoxic potential for cART is

observed in the clinic. Indeed, neuropsychiatric side effects are associated with ARVs across several classes, including NRTIs lamivudine (Song *et al.*) and abacavir (Colebunders *et al.* 2002; Palacin *et al.* 2006; Foster *et al.* 2004), NNRTIs efavirenz (Mollan *et al.* 2014; Clifford *et al.* 2009a; Ma *et al.* 2016; Arendt *et al.* 2007) and nevirapine (Wise *et al.* 2002; Morlese *et al.* 2002); and INSTIs raltegravir (Harris *et al.* 2008; Eiden *et al.* 2011; Gray and Young 2009) and dolutegravir (Kheloufi *et al.* 2015; Hoffmann *et al.* 2017). In one surprising study, patients taking either NRTIs, NNRTIs, PIs, or a combination of all three drug classes improved their psychological testing scores following temporary ART discontinuation (Robertson *et al.* 2010). One important caveat in the interpretation of these studies is that neuropsychiatric side effects of ARVs may be due to indirect effects on the brain caused by peripheral toxicities (Troya and Bascuñana 2016), and therefore human data alone cannot directly implicate ARV effects on neurons. Also of critical note is that these studies do not negate the overwhelming benefits of cART nor indicate that patients should discontinue medication under any circumstances (Saylor *et al.* 2016). They should, however, provide potential cause for reevaluation of treatment strategies with the goal of maintaining optimal cognitive function.

Barriers to identifying drug regimens that minimize cognitive impairment

Scientists are eager to determine whether HIV or ARVs is the primary contributor to HAND in order to better understand disease persistence, and clinicians have the added urgency of needing to provide the optimal regimens to their patients. In an attempt to elegantly address both of these needs, clinicians developed a measure of how effectively different ARVs can access the brain, which is called the CNS penetration effectiveness score (CPE) (Letendre *et al.* 2008). Unfortunately, however, studies comparing CPE scores of drug regimens with neurocognitive functioning have not provided clarity regarding whether or not high drug concentrations in the CNS are beneficial. At least three studies have reported improved cognition with increased CPE score (Tozzi *et al.* 2009; Smurzynski *et al.* 2011; Carvalhal *et al.* 2016), indicating that in these patients cognitive impairment may have been caused by HIV rather than ARVs. In contrast, however, two studies reported improved cognition associated with lower CPE regimens (Marra *et al.* 2009; Caniglia *et al.* 2014), and Caniglia *et al.* 2014 specifically identified an increased risk of HAD as CPE score increased. Finally, two additional experiments yielded no evidence of a significant relationship between CPE score and cognitive functioning (Robertson *et al.* 2012a;

Ellis *et al.* 2014). Given the diversity of these results, in clinical practice it is not advised to consider CPE score when designing an ART regimen (Saylor *et al.* 2016).

One possible reason for the mixed evidence gathered from CPE studies is likely to be heterogeneity of ARVs both within and between classes. Because no such drugs exist that are identical with the exception of their CPE score, effects of CNS penetrance cannot truly be compared directly in human subjects. Moreover, presentation of HAND is heterogeneous as well. The ideal tests for screening and diagnosis of HAND both in research and in clinical practice are not yet standardized and continue to produce highly variable data regarding nature and severity of cognitive symptoms, particularly during longitudinal follow-up (Kamminga *et al.* 2013; Kamminga *et al.* 2017b; Kamminga *et al.* 2017a). In addition to these obstacles, however, perhaps a clear consensus cannot be reached because both HIV and ARVs play important additive or synergistic roles in the persistence of HAND (Sanchez and Kaul 2017).

A third possibility: combined contributions

The data presented in this dissertation suggest that both HIV and ARVs may contribute to HAND through distinct mechanisms. This may

partially explain why data in human patients have been mixed, and increasing CNS penetrance of ART regimens does not consistently improve or impair cognitive function. Because of this complexity, the best clinical goals may not be related to increasing or decreasing CNS ARV concentrations, but rather designing therapeutic regimens with low neuropsychiatric side effect profiles despite high viral suppression in the CSF.

Modeling HIV with a focus on NMDA receptors

Both macrophages and astrocytes are likely to play a key role in HAND neuropathogenesis given that they are susceptible to HIV infection and are required for regulation of extracellular glutamate concentrations (Vázquez-Santiago *et al.* 2014). Indeed, patients with HAD have increased CSF glutamate concentrations that are correlated with cognitive impairment (Ferrarese *et al.* 1997; Ferrarese *et al.* 2001). In an animal model of HIV, researchers identified disruptions in astrocytic glutamate reuptake due to effects of HIV on microglia as a mediator of neuronal damage (Moidunny *et al.* 2016). Direct glutamate release and/or impaired reuptake from HIV-infected macrophages also causes neuronal damage *in vitro* (Jiang *et al.* 2001) and impaired

hippocampal synaptic plasticity and memory deficits in an animal model of HIV encephalitis (Zink *et al.* 2002).

The multiple paths to NMDA receptor activation in HAND

Several glutamate receptors are expressed in neurons including metabotropic receptors (mGluRs 1-8) and ionotropic N-methyl-d-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), and kainate receptors. Several of these receptor subtypes have been implicated in disease pathogenesis (Nicoletti *et al.* 2011; Traynelis *et al.* 2010), but evidence suggests a more prominent role for NMDARs in neurodegenerative mechanisms due to their mediation of excitotoxicity (Lipton and Rosenberg 1994; Mehta *et al.* 2013; Carvajal *et al.* 2016; Kocahan and Doğan 2017). Indeed, excitotoxic damage downstream of NMDAR activation is another common feature of AD (Kocahan and Doğan 2017) that may also be implicated in HAND.

In HIV+ patient *post mortem* brain samples, NMDAR subunit-encoding genes are downregulated in frontal cortex along with other genes involved in synaptic signaling and transmission (Masliah *et al.* 2004); this may indicate a negative feedback mechanism to regulate

excitatory post-synaptic potentials during repeated NMDAR stimulation. Moreover, in an animal model of HIV, neuronal damage induced by increased extracellular glutamate concentration was dependent upon NMDARs (Moidunny *et al.* 2016).

In an early *in vitro* model of HIV in which human myeloid cells were infected and the conditioned media was used to treat primary chick ciliary neurons, NMDAR activation was required for neurotoxicity (Giulian *et al.* 1990). Although in this circumstance the NMDAR activation was likely due to glutamate release by infected cells (Jiang *et al.* 2001), it is important to note that NMDARs can be activated by additional secreted factors, such as chemokines, or by viral proteins directly. One of the first studies to suggest this possibility showed that gp120-induced neuronal damage was accompanied by increased intracellular calcium concentrations and was prevented by a calcium channel antagonist (Dreyer *et al.* 1990). Later experiments implicated NMDARs more directly in gp120-induced calcium influx and toxicity (Lannuzel *et al.* 1995) and demonstrated that gp120 acts as an NMDAR agonist by occupying the glycine binding site (Fontana *et al.* 1997). Ability of gp120 to bind NMDARs and increase intracellular calcium concentration in hippocampal cells was selectively enhanced by extrasynaptic NMDAR2B receptor subunits (Zhou *et al.* 2016), which

play a particularly critical role in excitotoxic disease mechanisms (Bading 2017). Synaptic damage caused by exposure to the viral protein tat can also be reversed by inhibition of NMDA receptors (Shin *et al.* 2012), and NMDA antagonists are protective in rodent models of HIV involving either gp120 or tat (Mucke *et al.* 1995; Anderson *et al.* 2004; Nakanishi *et al.* 2016). Considered together, these studies indicate that regardless of whether neurotoxicity is caused by direct interactions with viral particles or glutamate imbalance due to glial infection/activation, NMDARs are likely to play a role. Unfortunately, although NMDARs received attention in the past as a potential therapeutic target in HAND, failed phase III trials of the NMDAR antagonist memantine has decreased momentum for this approach in recent years (Schifitto *et al.* 2007; Zhao *et al.* 2010).

An in vitro *model of HIV-induced neurotoxicity: critical roles for glutamate and NMDAR signaling*

The two main challenges in designing a cell culture model relevant to HAND are that A) HIV does not infect neurons, and B) HIV does not infect non-human cells. The first point can be seen as an opportunity rather than an obstacle, however, because macrophages, which do sustain productive viral infection *in vivo* (Rappaport and Volsky 2015),

are more readily available from human donors. As discussed above, HIV-associated effects on neurons are likely to at least in part be mediated indirectly through macrophage infection (Kaul *et al.* 2005); hence, scientists are able to use macrophages in order to model HIV-induced neurotoxicity.

This type of model was created in 1990 by Giulian et al., who infected human macrophages with isolated strains of T-cell-propagated HIV and periodically collected conditioned media from the infected cell cultures. This media was then used to treat primary neurons isolated from chickens. Treatment caused severe neuron loss that was entirely prevented by inhibition of NMDARs but unaffected by inhibition of AMPARs or kainate receptors. The same group subsequently observed that gp120-induced neuronal damage could be similarly blocked by selective NMDAR inhibition (Giulian *et al.* 1993).

The model was later adapted into the HIV-infected monocyte-derived macrophage (HIV/MDM) model used for experiments presented in this dissertation (Chen *et al.* 2002). In this model, monocytes are isolated from blood samples collected from healthy human donors and are differentiated into macrophages in culture. Isolated HIV strains are used to infect these macrophages, and conditioned media is collected over a period of several days. The conditioned media can be used to

treat neurons from either cell lines or primary isolations of multiple species including rats, mice, and humans (Chen *et al.* 2002; O'Donnell *et al.* 2006). Careful characterization of this model has revealed that neurotoxicity is mediated specifically by NMDAR2A and NMDAR2B subtypes, and that glutamate in the conditioned media is the primary but not the sole factor leading to cell loss (O'Donnell *et al.* 2006).

The daunting diversity of mechanisms in HAND

On the surface, it would seem that determining mechanistic responsibility for HAND should be a relatively simple task. Unlike in idiopathic diseases such as AD, for instance, the cause is essentially known: it must be either HIV or ARVs. This determination is complicated, however, by the opinion proposed herein and elsewhere that causes are multifactorial and may differ among patients.

In the following chapter, the role of HIV will be addressed, with a specific focus on mechanistic overlap between HAND and AD. Although the data thus far indicate that neuropathogenesis in HAND is distinct from AD in terms of APP processing overall, it remains critical to identify the precise features that may be shared. This would not only accelerate progress in HAND by utilizing the breadth of information gathered from decades of AD research, but also would provide valuable insights into

common pathways of neurodegeneration that could be targeted across diseases.

In a subsequent chapter, the potential contributions of ARVs to HAND are addressed *in vitro*. Here, the attempt to identify a cause of HAND persistence is further complicated by the diversity of ARV classes as well as the diversity of drugs within a single class. It is likely that mechanisms contributing to neurotoxicity of one drug may be not be relevant to other ARVs.

Because of drug diversity as well as variability among patients with HIV, it may not be feasible to define a single rationale for HAND persistence. Instead, scientists can hope to provide doctors with increasingly powerful tools that will aid in the personalization of cART regimen design. However, those mechanisms that do have overlapping relevance either across ARV classes, across patients with inconsistent serum or CSF profiles, or across diseases are critical to identify because they will provide the most promising therapeutic targets.

CHAPTER 2: BACE1 MEDIATES HIV-ASSOCIATED AND EXCITOTOXIC NEURONAL DAMAGE THROUGH AN APP-DEPENDENT MECHANISM

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Abstract

HIV-associated neurocognitive disorder (HAND) has been reported to share symptoms and neuropathological features with Alzheimer's Disease (AD), which is characterized by amyloid-β (Aβ) plaques in patient brains. Plaques are formed by aggregation of Aβ oligomers, which may be the toxic species in AD pathogenesis, and oligomers are generated by cleavage of amyloid precursor protein (APP) by β-site amyloid precursor protein cleaving enzyme 1 (BACE1). BACE1 inhibitors reverse neuronal loss and cognitive decline in animal models of AD. Although some studies have also found evidence of altered APP processing in HIV+ patients, it is unknown whether increased BACE1 or Aβ oligomers is a feature of HAND. Moreover, it is unknown whether BACE1 or APP is implicated in the excitotoxic, NMDA receptor-dependent

component of HIV-associated neurotoxicity *in vitro*. Thus, we hypothesize that HIV-associated neurotoxicity is mediated by NMDAR-dependent elevation of BACE1 and subsequent altered processing of APP. Supporting this, we observed elevated levels of BACE1 and Aβ oligomers in CNS of HIV+ patients. In a model of HIV-associated neurotoxicity in which primary rat neurons are treated with supernatants from HIV-infected monocyte-derived macrophages (HIV/MDMs), we observed NMDAR-dependent elevation of BACE1 protein levels. NMDA treatment also increased BACE1, and both pharmacological BACE1 inhibition and genetic loss of APP were partially neuroprotective. Moreover, in $APP^{-/-}$ neurons, toxicity was BACE1-independent, indicating that the role of BACE1 is directly related to cleavage of APP. These findings suggest that increased BACE1 and resultant Aβ oligomer production may contribute to HIV-associated neuropathology, and inhibition of BACE1 may have therapeutic potential in HAND.

Significance

HIV-associated neurocognitive disorder (HAND) is a range of cognitive impairments affecting approximately 50% of HIV+ individuals.

The cause of HAND is unknown, but evidence suggests that HIV-infected macrophage infiltration into the brain may cause neuronal damage. Herein, we show that neurons treated with conditioned media from HIV-infected macrophages have increased expression of β-site amyloid precursor protein cleaving enzyme 1 (BACE1), a protein implicated in Alzheimer's Disease (AD) pathogenesis. Moreover, inhibition of BACE1 prevented neuron loss due to conditioned media exposure, but it had no effect on HIV-associated neurotoxicity in neurons lacking its cleavage target amyloid precursor protein (APP). We also observed increased BACE1 expression in HIV+ patient brain tissue, confirming the potential relevance of BACE1 as a therapeutic target in HAND.

Introduction

HIV-associated neurocognitive disorder (HAND), which persists in 15% - 55% of HIV+ individuals despite viral suppression by ART, is a constellation of cognitive, behavioral, and motor impairments (Sacktor *et al.* 2016). HAND has some clinical (Sacktor and Robertson 2014) and pathological (Clifford *et al.* 2009b; Borjabad and Volsky 2012; Levine *et al.* 2013; Ortega and Ances 2014) features in common with Alzheimer's Disease (AD), and in an aging HIV+ population (Valcour 2013) it is

increasingly difficult to distinguish AD from the combined effects of age and HIV (Xu and Ikezu 2008). Understanding the common pathways involved in neuropathology will maximize the efficacy of treatment.

In AD, increased amyloid precursor protein (APP) cleavage by the β-secretase β-site amyloid precursor protein cleaving enzyme-1 (BACE1) leads to overproduction of amyloid-β (Aβ) peptides (MacLeod *et al.* 2015). Aβ peptides can oligomerize and ultimately form extracellular aggregates called plaques (Trojanowski *et al.* 1995). Importantly, although extracellular plaques are a defining feature of AD, Aβ oligomers are more likely to be the neuropathogenic species (Sengupta *et al.* 2016; Kayed and Lasagna-Reeves 2013). In HAND, evidence of a role for amyloid is more mixed, but the majority of studies (Esiri *et al.* 1998; Rempel and Pulliam 2005; Achim *et al.* 2009; Clifford *et al.* 2009b; Brew *et al.* 2005) indicate that there is altered APP processing and metabolism of some form, even in patients on effective ART regimens (Green *et al.* 2005; Soontornniyomkij *et al.* 2012). Unlike in AD, however, ART-treated HAND patients do not form extracellular plaques. Rather, evidence suggests diffuse intracellular accumulation of either full-length APP, Aβ, or oligomers (Ortega and Ances 2014; Xu and Ikezu 2008). Despite its proposed role in AD neuropathogenesis (Sengupta *et al.* 2016; Kayed and Lasagna-Reeves 2013), a lack of

antibody specificity and wide variation across study designs have made it difficult thus far to determine whether oligomeric Aβ specifically is altered in HAND.

BACE1 is elevated in AD brains (Yang *et al.* 2003; Johnston *et al.* 2005), and BACE1 inhibition effectively decreases plaque burden and improves cognition in animal models of AD (Singer *et al.* 2005; Ohno *et al.* 2004; Chang *et al.* 2011). Evidence from *in vitro* experiments in primary rodent neurons shows that BACE1 activity and localization are also affected by treatment with HIV proteins trans-activator of transcription (tat) and glycoprotein 120 (gp120) (Chen *et al.* 2013; Kim *et al.* 2013; Bae *et al.* 2014). However, it remains unknown what role BACE1 plays in HIV-associated neurotoxicity and neuropathogenesis.

Macrophages sustain productive viral infection in HIV patient brains (Petito *et al.* 1986; Koenig *et al.* 1986), and infected macrophages may mediate HIV-associated neurotoxicity by secreting factors that include viral proteins, chemokines, and glutamate (Kaul 2008). Glutamate release in particular has been linked to neuronal damage and cognitive dysfunction in HIV both *in vivo* and *in vitro* (Jiang *et al.* 2001; Zink *et al.* 2002). Similarly to AD pathology (Mehta *et al.* 2013), *in vitro* evidence suggests that glutamate may cause neuronal damage in HIV through NMDA receptor (NMDAR)-dependent

mechanisms of excitotoxicity (Giulian *et al.* 1990; Chen *et al.* 2002; O'Donnell *et al.* 2006). Consequently, we employed a previously developed and well-characterized *in vitro* model of HIV-associated neurotoxicity (Chen *et al.* 2002; O'Donnell *et al.* 2006) in which cultured rat neurons are exposed to supernatants collected from HIV-infected human monocyte-derived macrophages (HIV/MDMs). In this model, neurotoxic injury induced by HIV/MDM supernatants is entirely dependent on NMDAR activation (Giulian *et al.* 1990; Jiang *et al.* 2001; Chen *et al.* 2002; O'Donnell *et al.* 2006).

Based on the similarities observed thus far between AD and HAND in relation to amyloid metabolism (Ortega and Ances 2014), we hypothesized that Aβ oligomers and BACE1 protein levels are increased in HAND patient brains. Moreover, we hypothesized that *in vitro* neurotoxicity induced by HIV/MDM supernatants is dependent upon NMDAR-mediated upregulation of BACE1 and a resultant increase in amyloidogenic APP processing.

Materials and Methods

Chemicals and Reagents. The following antibodies used in this study were purchased from the indicated vendors: Cell Signaling Technology

(Danvers, MA): β-site amyloid precursor protein cleaving enzyme 1 (BACE1; 5606S), presenilin 1 (PS-1; 5643), β-actin (3700); BD Transduction Laboratories (San Jose, CA): binding immunoglobulin protein (BiP; 610978); Abcam (Cambridge, MA): amyloid precursor protein (APP; ab32136), a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10; ab1997), microtubule-associated protein 2 (MAP2; ab5392); Sigma Aldrich (St. Louis, MO): Actin (A2066); BioLegend (San Diego, CA): MAP2 (801801). The mouse monoclonal antibody against BACE1 (3d5) was a generous gift from Dr. Robert Vassar (Feinberg School of Medicine, Northwestern University, Chicago, IL). The antibody against Aβ-oligomers (Nab61) was kindly provided by Dr. Virginia Lee (The Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA). The following chemical reagents used in the study were purchased from the indicated vendors: Citifluor (London, UK): 4’,6-diamidino-2-phenylindole (DAPI); Invitrogen (Carlsbad, CA): Dulbecco’s Modified Eagle’s Medium (DMEM), neurobasal medium, B27 supplement; BioRad (Hercules, CA): Bradford protein assay dye, polyvinylidene fluoride (PVDF) membrane, prestained broad range molecular weight ladder; Sigma Aldrich (St. Louis, MO): Tween 20, Triton X-100, Fast Green FCF, protease inhibitor cocktail, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), cytosine β-D-

arabinofuranoside hydrochloride (AraC); Peptides International (Louisville, KY): Poly-L-Lysine; Scytek Labs (Logan, UT): normal antibody diluent (NAD); Thermo Fisher Scientific (Waltham, MA): Hank's Balanced Salt Solution (HBSS), Trypsin, GlutaMAX; Millipore (Temecula, CA): Luminata Classico ECL, β-secretase inhibitor (BSI) II & IV; Tocris Bioscience (Bristol, UK): amino-5-phosphonovaleric acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), dizocilpine (MK-801). All horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Thermo Fisher Scientific, and all fluorescent dye-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA).

Immunofluorescence of human tissue. Paraffin-embedded tissue sections from the hippocampus of control and HIV(+) human autopsy cases obtained from the National NeuroAIDS Tissue Consortium (NNTC) were prepared for immunofluorescent staining with minor modifications of previously described protocols (Lindl *et al.* 2007). The age, neurocognitive status, sex, and postmortem interval of each human specimen was provided by the NNTC (Table 1). Glass slides containing paraffin-embedded tissue sections (10 μM) were heated overnight to 55°C, deparaffinized in histoclear and rehydrated in 100%, 95%, 90%,

and 70% ethanol washes. Tissue was then incubated in 3% H_20_2 in methanol to inactivate endogenous peroxidase activity. Antigen unmasking was performed with target retrieval solution at 95°C for 1 h. Sections were then blocked with 10% normal goat serum and incubated with primary antibody overnight at 4°C. Tyramide amplification was used to detect BACE1 and DNA was visualized with DAPI staining. Slides were washed with phosphate buffered saline plus 0.1% Tween-20 (PBS-T) and mounted in Citifluor AF1 and analyzed by laser confocal microscopy at 600x on a Radiance 2100 equipped with Argon, Green He/Ne, Red Diode, and Blue Diode lasers (BioRad). Post-acquisition analysis was performed using Metamorph 6.0 (Universal Imaging, Downingtown, PA). Total intensity for MAP2 was determined by the measurement of integrated pixel intensity per z-stack image, where integrated pixel intensity is defined as total pixel intensity per image times the area of pixels with positive MAP2 signal. Data were analyzed using GraphPad Prism statistical software (version 5.0; GraphPad, San Diego, CA), and data are expressed as mean ± standard error of the mean (SEM).

Immunoblotting of human tissue. Flash-frozen whole-brain tissue samples from HIV (-) control (n=4), HIV (+) neurocognitively normal

(n=8), and HIV (+) HAND (n=6) human autopsy cases were obtained from the tissue banks of the NNTC. Frontal cortex was dissected from Brodmann areas 9 or 10. Tissue was prepared for western blotting as described previously (Lindl *et al.* 2007). Briefly, frozen brain tissue (100 mg) was homogenized and solubilized in ice-cold tissue extraction buffer (50 mM Tris pH 7.5, 0.5M NaCl, 1% NP-40, 1% SDS, 2 mM EDTA, 2 mM EGTA, 5 mM NaF, 0.4 mM Na3VO4, 1 mM dithiothreitol (DTT) and 1:100 protease inhibitor cocktail). Extracts were then centrifuged at 12,000 *g* at 4oC for 20 min. 30 µg protein for each sample was loaded into each lane of 10% Bis-Tris gels and transferred to PVDF membranes followed by blocking with tris buffered saline plus 0.1% Tween-20 (TBS-T) with 5% BSA for 30 min at room temperature. Membranes were probed with various primary antibodies overnight at 4°C.

Preparation of primary rat cortical neuron cultures. Primary rat cortical cultures were prepared from embryonic day 18 Sprague-Dawley rat embryos (Charles River Laboratories, Seattle, WA). Brains were isolated, and dissected cortices were incubated for 40 minutes in DMEM + 0.027% trypsin as described previously (Wilcox *et al.* 1994). Cells were then washed in saline, triturated, resuspended in neurobasal media supplemented with B27, and plated on poly-L-lysine-coated 6-well (9.4-

cm^2 growth area) or 24-well (1.9-cm^2 growth area) plates (USA Scientific, Ocala, FL) at a concentration of 750,000 cells/ml. After 48 h, cells were treated with 10 μM AraC to remove dividing glial cells. Cultures were maintained in neurobasal media supplemented with B27 at 37°C with 5% CO_2 as described previously (Gannon *et al.* 2017; Akay *et al.* 2011). On 10 days *in vitro* (DIV), 20% fresh media was added. Cells were treated on DIV 14–21.

Preparation of primary mouse cortical neuroglial cultures (MCCs). Primary mouse cortical cultures were prepared from ED 16-18 c57/BL6 wild type or $APP^{-/-}$ mouse embryos (The Jackson Laboratory, Bar Harbor, ME). Brains were isolated, and dissected cortices were incubated for 15 min in HBSS + .025% trypsin. Cells were then washed with saline, triturated, and resuspended in neurobasal media + B27 supplement + GlutaMAX before plating on poly-L-lysine coated 6-well (9.4 cm^2 growth area) or 24-well (1.9cm^2 growth area) plates (USA Scientific) at a concentration of 250,000 cells/ml. Cells were maintained at 37°C in 5% CO_2 as described previously (Gannon *et al.* 2017; Akay *et al.* 2011) and were treated on DIV 14.

Drug treatments. Cells were treated for the time and dose specified for each experiment with either NMDA dissolved in H_2O, MK-801 dissolved in DMSO, AP-5 dissolved in H_2O, or CNQX dissolved in DMSO; or β-secretase inhibitor (BSI) II or IV dissolved in DMSO. Pretreatments with MK-801, AP-5, and CNQX were 1 h prior to treatment with HIV/MDM supernatant for 16 or 24 h. Pretreatments with BSI were 1 h prior to 24 h treatment with HIV/MDM supernatant or NMDA.

HIV/MDM supernatants. Monocytes were isolated from healthy human donors and differentiated into macrophages before infection with HIV-1 as previously described (Cross *et al.* 2011). Briefly, macrophages were exposed to HIV-1 T-cell propagated virus (89.6) for 24 h before virus was removed and cells were rinsed thoroughly with DMEM. Supernatants were then collected every 3 days, and macrophage infection was confirmed by HIV reverse transcriptase (RT) assay. DIV 14-21 rat neuronal cultures were treated with a 1:20 – 1:80 dilution of HIV/MDM supernatant; results presented are those in which dilutions led to ~50% MAP2 loss after 24 h.

Immunofluorescence of primary neuron cultures. Following treatment, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 20

min. Cells were then rinsed twice in PBS and three times in PBS-T, followed by a 30-min incubation with a blocking/permeabilization solution containing 0.2% BSA + 0.1% Triton-X in PBS. Cells were rinsed three times in PBS-T and incubated with MAP2 primary antibody diluted at 1:4000 in NAD for 2 h at room temperature. Following three washes in PBS-T, cells were then incubated with a FITC-conjugated goat anti-mouse secondary antibody diluted at 1:500 in NAD for 30 min at room temperature. Cells were then imaged using a Keyence BZ-X-700 digital fluorescent microscope (Keyence Corporation, Itasca, IL) affixed with UV, FITC, Cy3, and Cy5 filters. Images captured at ×20 magnification were analyzed with the BZ-X Keyence software to quantify the number of neurons. Specifically, the number of neurons, identified as cells expressing MAP2, was averaged across a total of 25 fields/well, with 2–4 wells/treatment condition for each biological replicate. Data were analyzed using GraphPad Prism statistical software version 7.0, and data are expressed as mean fold change from untreated (UT) ± SEM.

Immunoblotting of cultured samples. Following treatment, cells were rinsed twice with PBS and lysed with whole cell lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 0.4 mM NaF, 0.4 mM Na3VO4, and 1:100 protease inhibitor cocktail). Protein supernatants were collected

with centrifugation at 20,000 *g* for 10 min at 4°C. Protein concentrations were determined using the Bradford method, and 3-5 µg total protein per condition was loaded into each lane of precast 10% Bis-Tris NuPAGE Novex gels (Thermo Fisher Scientific). Proteins were then transferred to PVDF membranes, which were blocked with 5% BSA in TBS-T for 1 h at room temperature and incubated overnight with primary antibodies at 4°C. Following three washes in TBS-T, membranes were incubated with HRP-conjugated secondary antibody (1:5000 in 5% BSA + TBS-T) for 30 min at room temperature. Bands were visualized by chemiluminescence with Luminata Classico ECL, and images were captured by film development or ChemiDoc Touch imaging system (BioRad). Equal loading and even transfer of samples were confirmed using fast green staining of the membranes. Densitometric analysis of band intensities was conducted using ImageJ software (v1.44, NIH), and all bands were normalized to fast green stain. Data were analyzed using GraphPad Prism statistical software version 7.0, and data are expressed as mean fold change from UT ± SEM.

Rat Aβ ELISAs. Media was collected from NMDA-treated primary rat neurons at several time points. Samples were centrifuged at 4°C 20,000 rcf for 10 min, and undiluted supernatants were assayed with a rat $A\beta_{42}$-

or $A\beta_{40}$-specific sandwich ELISA (Wako Chemicals, Richmond, VA) according to manufacturer's protocols. Luminescence was quantified using a 96-well plate reader measuring at 450nm. Raw pmol/L concentrations were normalized to MAP2(+) cell counts for each treatment group. Data were analyzed using GraphPad Prism statistical software version 7.0, and data are expressed as mean fold change from UT ± SEM.

Results

Aβ oligomers are elevated HIV(+) hippocampus. Previous studies have shown accumulation of neurodegenerative proteins such as APP and/or its cleavage products in the brains of HAND patients. To investigate the mechanisms underlying age-related pathologies that are increasingly observed in HAND, we first assessed levels of Aβ oligomers (Aβ oligo), the Aβ species suggested to be responsible for CNS neurotoxicity in AD, in the hippocampus of HIV(+) patients and age-matched HIV(-) controls (see Table 1 for a summary of cases). We observed elevated levels of intraneuronal Aβ oligomers in HIV(+) patients (Fig. 1A, Table 1). Importantly, however, we found no evidence of senile or diffuse Aβ plaque deposition in the hippocampus using the

BC05 antibody, an Aβ42-specific antibody generated by Dr. Virginia Lee (not shown).

BACE1 is increased in both hippocampus and frontal cortex of HIV(+) individuals. As oligomeric Aβ is derived from cleavage of APP by BACE1, we determined whether BACE1 expression was altered in the same cohort of samples. When we stained for BACE1 in distinct regions of the hippocampus, we observed significantly elevated BACE1 immunoreactivity in HIV(+) patients compared to HIV(-) controls in CA1 and CA3, consistent with our finding of elevated Aβ oligomers, despite decreased microtubule-associated protein 2 (MAP2) expression (Fig. 2A). However, no significant changes in BACE1 were observed in the dentate gyrus. To confirm our immunostaining findings, we assessed protein levels of BACE1 in whole brain lysates from the mid-frontal cortex of HIV(-) and HIV(+) individuals by immunoblotting. As shown in Fig. 2B, BACE1 was elevated in HIV(+) individuals compared to uninfected controls. We also replicated this result in a second independent cohort of HIV(-) and HIV(+) individuals, confirming increased BACE1 in frontal cortex with HIV (Fig. 2C, D).

BACE1 is increased in primary neurons treated with supernatants from HIV-infected human macrophages (HIV/MDMs). Based on our human data demonstrating a correlation between HIV seropositive status and elevated BACE1 levels, we used an *in vitro* model to ask whether a causal relationship exists between HIV and BACE1. In this model, which has been described in detail previously (O'Donnell *et al.* 2006; Cross *et al.* 2011), macrophages derived from monocytes of healthy human donors are infected with HIV-1. Supernatants from infected macrophages (HIV/MDM) or uninfected macrophages (Mock) are then used to treat primary rat cortical neurons. As previously shown, HIV/MDM supernatant treatment caused loss of MAP2(+) neurons after 24 h (Fig. 3A). To test whether HIV/MDM supernatants induced BACE1 in neurons, we treated neuronal cultures with HIV/MDM supernatants for 16 or 24 h. By 16 h, levels of BACE1 protein were increased in HIV/MDM- but not Mock-treated neurons (Fig. 3B, C). We then tested whether effects on BACE1 were consistent across multiple supernatants. We collected supernatants generated from 3 individual healthy macrophage donors and treated neurons with Mock or HIV/MDM supernatants for 16 h. Levels of BACE1 protein were increased by treatment with all 3 supernatants tested (Fig. 3D, E).

BACE1 increase by HIV/MDM supernatants is dependent on NMDA receptor (NMDAR) signaling. Increased glutamate and activation of NMDAR glutamate receptors are critical components of neurotoxicity in HAND and AD (Kaul *et al.* 2001; Kocahan and Doğan 2017) as well as in our model (O'Donnell *et al.* 2006). Therefore, we asked whether NMDARs are required for BACE1 induction. To test this, we pretreated primary rat neurons with either NMDAR inhibitor MK801 or AP-5 or glutamate receptor AMPAR inhibitor CNQX 1 h prior to 16 or 24 h treatment with HIV/MDM or Mock supernatants. As observed previously, neurotoxicity of HIV/MDM supernatant treatment was blocked by pretreatment with MK801 or AP-5, while CNQX had no effect after 24 h (Fig. 4A). Correspondingly, induction of BACE1 by HIV/MDM supernatant treatment was blocked by pretreatment with MK801 or AP-5 but not CNQX after 16 h (Fig. 4B). These results are quantified in Figs 4C, D. Having demonstrated the necessity for NMDAR signaling in BACE1 upregulation, we next asked whether NMDAR activation was sufficient for upregulation of BACE1. Indeed, we observed a dose-dependent increase in BACE1 protein levels in rat primary neurons following 16 h treatment with NMDA alone (Fig. 4E, F).

NMDAR activation shifts APP processing toward the amyloidogenic pathway. In neurons, full length APP can be cleaved by either ADAM10 or BACE1, and in either case is subsequently cleaved by PS-1. Cleavage by ADAM10/PS-1 generates the non-amyloidogenic fragment P3, while BACE1/PS-1 cleavage generates Aβ monomers that oligomerize to form disease-associated peptides, fibrils, and plaques. Because we found that NMDAR signaling was both necessary and sufficient for BACE1 induction and neurotoxicity induced by HIV/MDM supernatants, in the next series of experiments we treated primary rat neurons directly with NMDA for 10 min, 2, 8, 16, 24, or 48 h to further assess changes to APP processing and secretase expression. Because NMDA is a synthetic compound, we also treated neurons with the endogenous NMDA ligand glutamate to ensure physiological relevance of our results. Treatment with either NMDA or glutamate for 16 h increased both BACE1 and PS-1 protein levels while dramatically decreasing ADAM10 protein levels (Fig. 5A-D). Corresponding neuronal cultures were fixed 10 min, 2, 8, 16, 24, or 48 h following NMDA treatment. Consistent with changes in protein expression playing a role in NMDA-induced neurotoxicity, significant loss of MAP2(+) cells did not occur until 24 h following treatment with NMDA (Fig. 5E). Next, we asked whether changes in secretase expression patterns were

accompanied by changes in the production of BACE1 cleavage product $A\beta_{42}$. To test this, we collected supernatants from primary rat neurons treated with NMDA for 16 or 24 h and measured the concentration of secreted monomers of $A\beta_{42}$ with a high sensitivity ELISA. After 24 h, $A\beta_{42}$ concentration normalized to MAP2+ cells was increased in NMDA-treated neuron supernatants (Fig. 5F).

Neurotoxicity induced by either NMDA or HIV/MDM supernatants is partially dependent on BACE1 activity. Because NMDA treatment caused both BACE1 increases and neurotoxicity, we asked whether BACE1 activity plays a role in the mechanism of neuronal death induced by NMDA. Rat primary neurons were pretreated with a pharmacological BACE1 inhibitor (BSI) for 1 h prior to 24 h treatment with NMDA, and BSI pretreatment significantly decreased NMDA-induced neurotoxicity (Fig. 6A, B). To further confirm the role of BACE1 in neurotoxicity of our *in vitro* HIV model, we also pretreated rat primary neurons with BSI for 1 h prior to 24 h treatment with HIV/MDM or Mock supernatants. Again, neurotoxicity was blocked by BSI treatment (Fig. 6C, D).

BACE1-mediated NMDA-induced neurotoxicity is dependent on the expression of APP. BACE1 cleaves several protein targets in addition to APP, including sodium and potassium channels, cell adhesion related proteins, and others (Kim *et al.* 2007; Munro *et al.* 2016). Thus, we asked whether the critical function of BACE1 responsible for its role in NMDA-induced neurotoxicity was dependent on the presence of APP. To test this, neuroglia were isolated from either wild type (WT) or APP knock-out ($APP^{-/-}$) mouse cortex at ED 16-18. Neuroglial cultures isolated from $APP^{-/-}$ mice were viable and had undetectable levels of APP (Fig. 7A). Consistent with our observations in rat neuronal cultures, NMDA induced BACE1 in both WT and $APP^{-/-}$ mouse neuroglia, indicating that cells were similarly responsive to NMDA at the level of changes in BACE1 expression (Fig. 7B). At the level of neurotoxicity, however, $APP^{-/-}$ cultures showed a striking resistance to NMDA-induced insult as compared to WT cultures, with both higher NMDA concentrations and longer treatments needed to cause the same degree of MAP2 loss (Fig. 7C, D). Specifically, while 5 μM NMDA led to approximately 50% MAP2+ cell loss following 24 h treatment in WT cultures, 10 μM NMDA was required to induce the same level of toxicity in $APP^{-/-}$ cells and 5 μM NMDA treatment had no effect (Fig. 7E). To determine whether APP was specifically required for the role of BACE1 in NMDA-induced toxicity, we

then asked whether the remaining portion of NMDA-induced neurotoxicity in $APP^{-/-}$ cells was BACE1-dependent. To test this, we first confirmed BACE1 dependence of NMDA-induced neurotoxicity in WT mouse cultures by pretreating cells with BSI for 1 h prior to 24 h treatment with 5 μM NMDA. Similarly to our observations in primary rat neurons, BACE1 inhibition significantly decreased neurotoxicity (Fig. 7F, H). We then repeated this experiment in $APP^{-/-}$ cells, with the exception that 10 μM NMDA was used in order to induce a similar level of toxicity (~50% MAP2+ cell loss) as 5 μM NMDA treatment in the WT (Fig. 7E). In $APP^{-/-}$ cells, BACE1 inhibition had no effect on NMDA-induced neurotoxicity after 24 h (Fig. 7G, I).

Discussion

In the present study, we show that both BACE1 and Aβ oligomers are increased in brains of ART-treated HIV+ patients, and also demonstrate a mechanistic role for both BACE1 and APP in HIV-associated *in vitro* neurotoxicity (overview presented in Fig. 8). Moreover, by clearly showing a necessity and sufficiency of NMDAR activation in engaging this mechanism, we implicate a role for BACE1 in classic excitotoxicity pathways relevant not only to HAND but to

neurodegenerative diseases more broadly (Lipton and Rosenberg 1994; Carvajal *et al.* 2016; Kocahan and Doğan 2017).

These data add to a growing body of evidence that there are overlapping neuropathological mechanisms in AD and HAND. Aβ oligomers are thought to be the toxic species promoting neuropathogenesis in AD (Kayed and Lasagna-Reeves 2013), and our data indicate that they may play a similar role in HAND. BACE1 is increased in *post mortem* brains of AD patients (Yang *et al.* 2003; Johnston *et al.* 2005), and in animal models of AD BACE1 inhibitors reverse neuronal loss and cognitive deficits (Ohno *et al.* 2004; Singer *et al.* 2005; Chang *et al.* 2011). To our knowledge, our study provides the first evidence that BACE1 is similarly altered in HIV+ patient brains, and that exposure to cultured media from HIV-infected MDMs can increase BACE1 levels in neurons. Strikingly, we and others have also shown that BACE1 is increased by antiretroviral drugs of at least two classes (Brown *et al.* 2014; Gannon *et al.* 2017), implying that ART-treated HIV+ patients may have additive increases in BACE1 due to influence of both the viral infection and the therapeutic intervention. In addition, we provided evidence here for a mechanistic role of BACE1 in HIV-associated neurotoxicity *in vitro*. Future studies should thus investigate

whether BACE1 inhibitors reverse neuronal damage and cognitive deficits in rodent and non-human primate models of HAND.

Interestingly, we found that the HIV-associated increase in BACE1 was mirrored by an increase in the APP γ-secretase PS-1 (Selkoe and Wolfe 2000) and a corresponding decrease in the APP α-secretase ADAM10 (Lammich *et al.* 1999). This is consistent with a previous study showing that NMDAR activation increased sAPPα concentration in neurons and conditioned media, although the authors did not investigate direct influence on ADAM10 protein levels (Lesné *et al.* 2005). Our observation highlights another potential similarity with neuropathogenesis in AD, given that ADAM10 is decreased in both *post mortem* brain and CSF of AD patients (Colciaghi *et al.* 2002; Bernstein *et al.* 2003; Olsson *et al.* 2003; Fellgiebel *et al.* 2009). Changes in ADAM10 are likely to play an important role in disease given that ADAM10 cleavage product sAPPα affords neuroprotection in a variety of contexts (Habib *et al.* 2017), and moreover a decrease in ADAM10 likely exacerbates the shift towards amyloidogenic APP cleavage due to increased BACE1 and PS-1.

Although BACE1 is well known for its role in APP cleavage, it has other cleavage targets including proteins involved in development, synaptic function, and cell-cell adhesion (Munro *et al.* 2016). Hence, it

is of critical note that in our study we determined that the mechanistic role of BACE1 in NMDA-induced neurotoxicity was dependent on the presence of APP, indicating that APP cleavage was indeed the relevant BACE1 function responsible for toxicity in our model. Consistent with this, genetic loss of APP alone also conferred resistance of neurons to NMDA-induced toxicity. Within the context of abnormal APP processing observed in brains of HIV patients here and in other studies (Green *et al.* 2005; Ortega and Ances 2014), these results indicate a potential pathogenic role for altered APP processing in HAND independent of plaque formation.

In apparent contradiction, however, previous studies have demonstrated both decreased cell viability and increased sensitivity to excitotoxic insult in cultured neurons lacking APP (Perez *et al.* 1997; Han *et al.* 2005). Others have reported no differences in susceptibility (Harper *et al.* 1998; White *et al.* 1998), and another study found similar results to ours, albeit with copper-induced neurotoxicity rather than direct excitotoxic insult (White *et al.* 1999). This discrepancy may be due to critical differences in the downstream effects of NMDA receptor activation depending on the dose and time course in question. Indeed, NMDA exerts opposing effects on extracellular Aβ accumulation *in vivo* depending on the dose, with lower doses increasing amyloidogenic APP

processing while higher doses cause a decrease (Verges *et al.* 2011). While Han and colleagues used a 300 μM dose of NMDA for 15 minutes, we used doses within the 5-20 μM range for longer exposures, attempting to mimic a more chronic disease process. Depending on the precise neuronal microenvironment and pathological stage, both mechanisms are likely to play a role given that the acute protective effects of sAPPα must compete against the more chronic detrimental effects of the amyloidogenic pathway (Hefter and Draguhn 2017).

Also consistent with our observation that loss of APP confers resistance to NMDA-induced neurotoxicity, one series of studies has demonstrated a role for APP in trafficking NMDARs to the cell surface and increasing postsynaptic excitatory currents (Cousins *et al.* 2009; Hoe *et al.* 2009; Innocent *et al.* 2012; Cousins *et al.* 2015). This may be a contributing factor to our results as well, particularly given that NR2B subunit localization is selectively affected by APP (Hoe *et al.* 2009) and in large part NR2B subunits mediate the neurotoxicity in our *in vitro* HIV model (O'Donnell *et al.* 2006). Important to note, however, is that although loss of APP shifted the toxicity dose curve of NMDA, the effect of NMDA on BACE1 expression was not changed. This indicates that toxicity resistance was not entirely due to changes in surface

expression, and may instead have resulted from decreased BACE1-dependent toxic mechanisms.

Classical pathways of excitotoxic injury are well characterized, but there are several ways that a role for BACE1 may fit within these pathways or within a parallel apoptotic or necrotic mechanism. For instance, Lesné *et al.* 2005 found that changes in APP cleavage product concentrations induced by NMDA were prevented by either a calcium chelator or inhibition of calmodulin/calmodulin kinase. Given our observation of increased BACE1 expression following NMDA treatment, one possibility is that BACE1 levels are directly affected by either calcium or calcium-dependent enzymes. BACE1 is also increased *in vitro* by calpain (Dong *et al.* 2005; Liang *et al.* 2010), another critical factor in excitotoxic injury. Possible parallel mechanisms of BACE1 mediated toxicity due to NMDAR activation include oxidative stress-dependent pathways (Tong *et al.* 2004) and the unfolded protein response (O'Connor *et al.* 2008), which mediates BACE1 upregulation in response to HIV protease inhibitors (Gannon *et al.* 2017). Because several mechanisms can potentially increase BACE1 activity and/or upregulate its expression, the particular pathway engaged likely depends on multiple factors and the possibilities are not mutually exclusive.

Aside from identifying a specific role for BACE1, our study adds to a body of literature implicating critical roles for glutamate and NMDA signaling in HIV neuropathogenesis. Indeed, NMDARs are downregulated in brains of HIV encephalitis patients (Masliah *et al.* 2004), and HIV dementia patients have increased CSF glutamate levels that are correlated with the degree of neurocognitive impairment (Ferrarese *et al.* 1997; Ferrarese *et al.* 2001). In the recently developed EcoHIV mouse model, HIV-stimulated release of cytokines from microglia impaired astrocytic reuptake of glutamate, increasing glutamate in the extracellular space and causing NMDA-dependent excitotoxicity (Moidunny *et al.* 2016). In addition to glutamate, viral proteins gp120 and tat can also directly activate NMDARs (Fontana *et al.* 1997; Shin *et al.* 2012; Zhou *et al.* 2016), and NMDA antagonists are neuroprotective in gp120 and tat rodent models of HIV (Mucke *et al.* 1995; Anderson *et al.* 2004). This indicates that both increased glutamate and free viral particles may act in parallel to disrupt NMDA signaling in HIV-infected individuals.

In summary, we have provided further evidence for the involvement of BACE1 and altered APP cleavage in HAND, with a potential role for Aβ oligomers in particular. Few studies in the era of combined ART have provided clear neuropathological differences

between virally suppressed HIV+ individuals and HIV- controls (Gelman 2015), further highlighting the significance of this work and the potential importance of BACE1 as a biomarker or therapeutic target. Moreover, by identifying both NMDAR signaling and altered APP processing as critical factors mediating the role of BACE1 in HIV-associated neurotoxicity, we provide support for the potential efficacy of several avenues for therapeutic intervention in HAND in addition to direct pharmacological targeting of BACE1.

Table 2-1. Summary of human cases used for immunofluorescence staining in figure 2-1

[A]NNTC, National NeuroAIDS Tissue Consortium identification. [B]HIV infection status. [C]Neurocognitive diagnosis. [D]Postmortem interval (hours). [E]Patients medicated on antiretroviral drugs for greater than 12 months. [F]Extent of hippocampal Aβ oligomer burden as assessed by Nab61 staining. [G]Not available. F = female, M = male.

NNTC ID[A]	HIV[B]	Neurocog[C]	Age	Gender	PMI[D] (h)	ART > 12 mo.[E]	Aβ Oligo[F]
3009	-	Normal	53	F	N/A[G]	-	-
3012	-	Normal	50	M	N/A	-	-
7665	-	Not Tested	47	F	19.18	-	-
8087	+	Normal	50	M	18	+	-
6771	+	Normal	46	M	2.75	+	+
6568	+	HAD	32	M	14	+	++
7680	+	HAD	34	F	5	+	++
8270	+	MCMD	49	M	67.33	+	+
8382	+	MCMD	37	M	11.5	+	+
6683	+	MCMD	31	M	8.83	+	++
6050	+	HAD	40	M	N/A	+	+
6040	+	HAD	34	M	N/A	+	++
4002	+	HAD	35	M	N/A	+	+

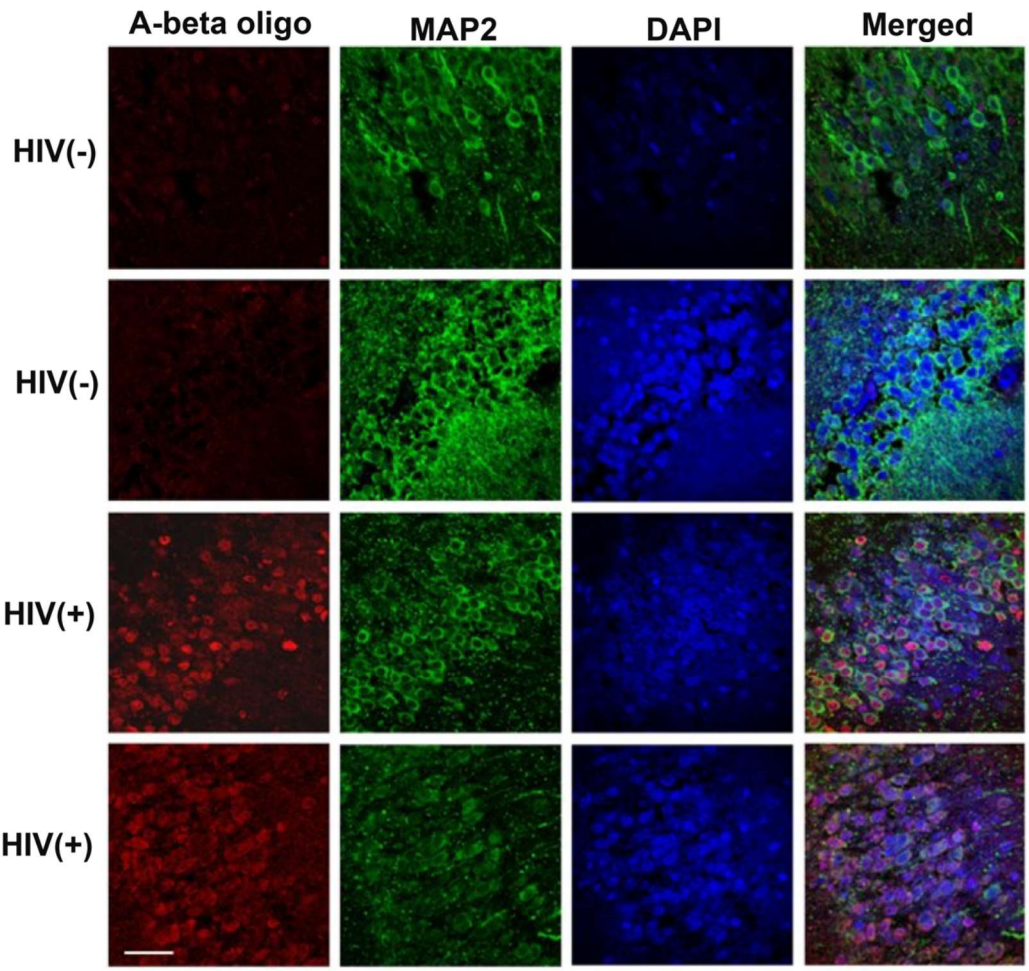

Figure 2-1. Aβ oligomers are elevated in the brains of HIV(+) cases

Paraffin-embedded tissue sections from hippocampus of HIV(-) and HIV(+) individuals were prepared for immunofluorescent analysis and visualized by laser confocal microscopy. Representative images are shown from hippocampal sections triple-labeled for Aβ oligomers (red), MAP2 (green), and nuclei (blue). Red and green colocalization appears yellow.

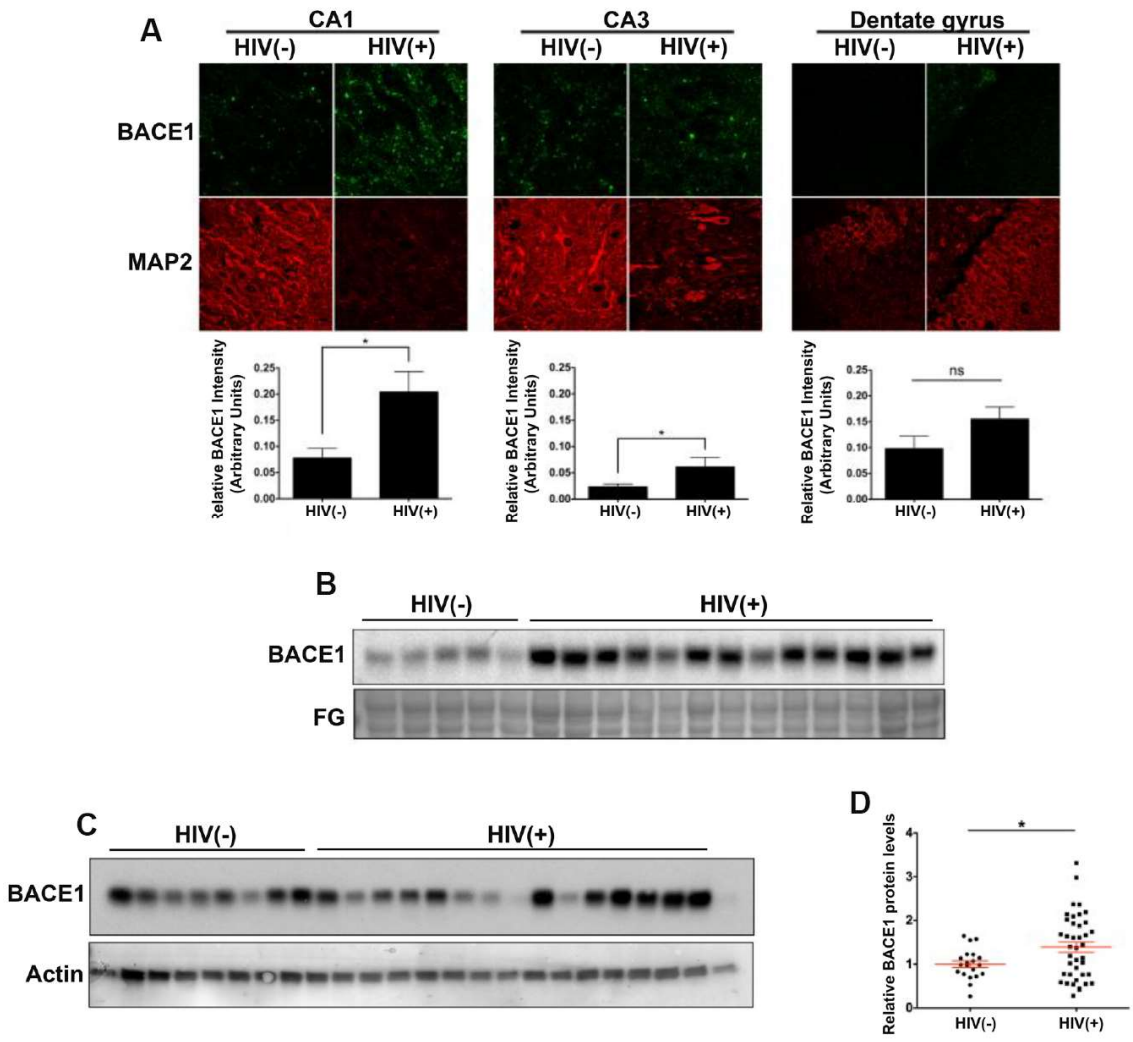

Figure 2-2. BACE1 protein is increased in hippocampus and frontal cortex of HIV+ individuals

A) CA1, CA3, and dentate gyrus hippocampal sections were double-labeled for BACE1 (green) and MAP2 (red). Representative images and quantification are shown (two sample t-test, HIV(-) n=3, HIV(+) n=10, *p<0.05, ns: not significant). **B)** Lysates from the mid-frontal cortex of HIV(-) controls (n=5) and HIV(+) cases (n=13) were prepared for immunoblot and probed for BACE1. Representative blots are shown. FG = fast green loading control. **C)** In an additional cohort, lysates from mid-frontal cortex of HIV(-) controls (n=20) and HIV(+) cases (n=40) were prepared for immunoblot and probed for BACE1 and actin as a loading control. Representative blots are shown. **D)** Results from second cohort are quantified across all blots (ANOVA followed by Newman-Keuls, *p<0.05). Red lines indicate mean ± SEM.

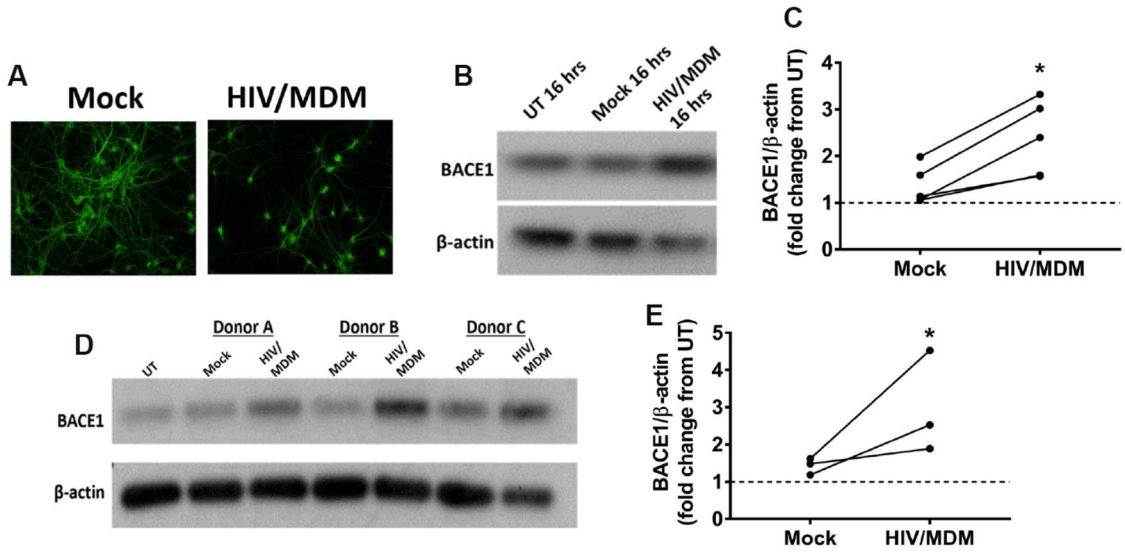

Figure 2-3. BACE1 is increased in primary neurons following treatment with HIV-infected monocyte-derived macrophage supernatants (HIV/MDMs)

A) Primary rat neurons were treated with Mock or HIV/MDM supernatants at a dilution causing 50% MAP2 loss after 24 h. Representative images of MAP2 (green) are shown. **B)** Whole cell lysates were collected following 16 or 24 h treatment with Mock or HIV/MDM supernatants, and lysates were probed for BACE1. Representative blots are shown. **C)** Densitometric analysis of western blots revealed a significant increase in BACE1/β-actin following 16 h HIV/MDM treatment (paired t-test, n=5, *p<0.05). **D)** HIV/MDM supernatants were generated from 3 separate healthy monocyte donors and were used to treat primary neurons for 16 h. Representative blots are shown. **E)** Increases in BACE1 by 16 h HIV/MDM treatment were consistent across HIV/MDM and Mock supernatants generated from multiple macrophage donors (paired t-test, n=3, *p<0.05). Dotted lines represent UT cultures.

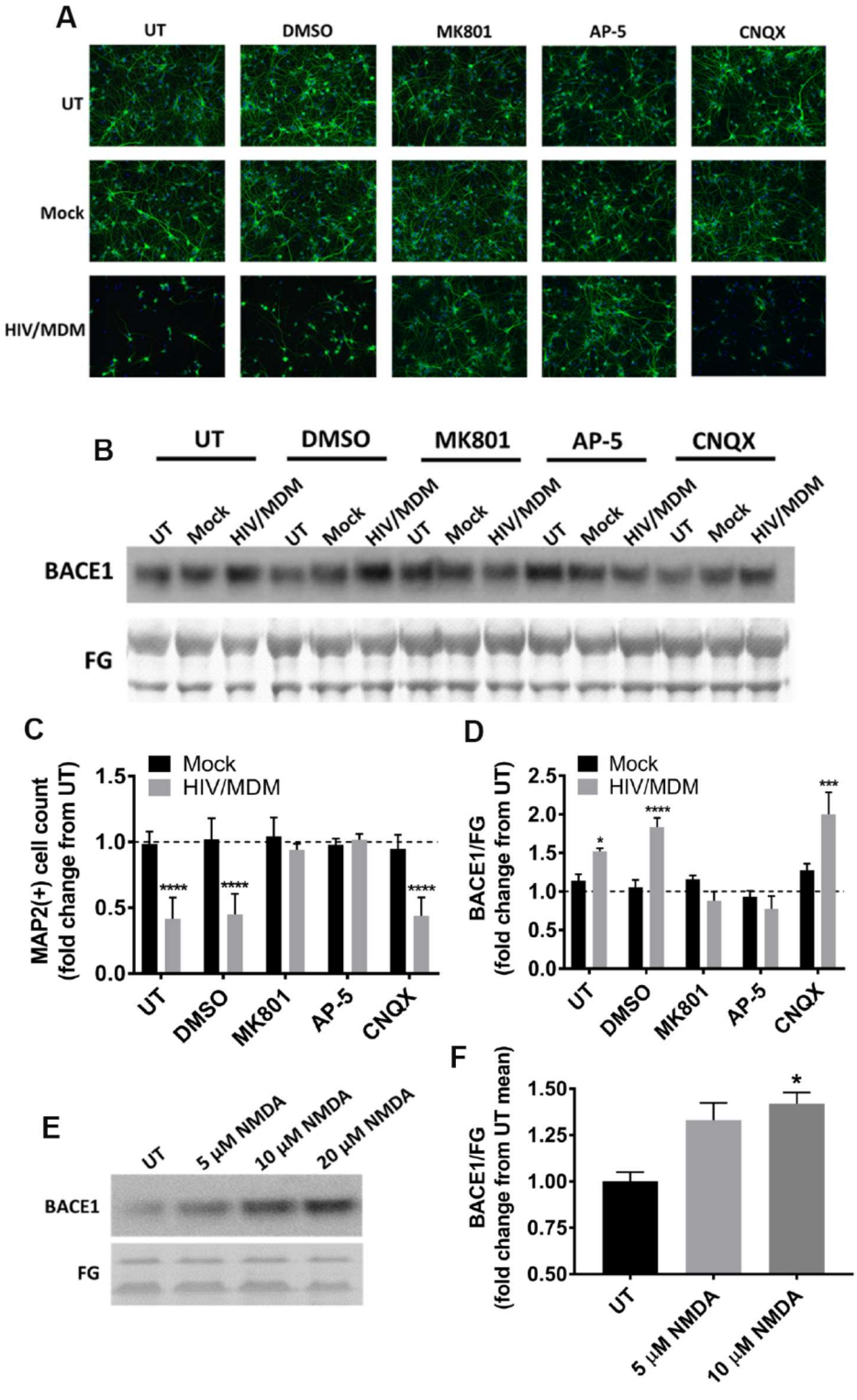
A
UT
DMSO
MK801
AP-5
CNQX
UT
Mock
HIV/MDM
B
UT
DMSO
MK801
AP-5
CNQX
UT Mock HIV/MDM UT Mock HIV/MDM UT Mock HIV/MDM UT Mock HIV/MDM UT Mock HIV/MDM
BACE1
FG
C
Mock
HIV/MDM
MAP2(+) cell count (fold change from UT)
1.5
1.0
0.5
0.0
****
****
****
UT
DMSO
MK801
AP-5
CNQX
D
Mock
HIV/MDM
BACE1/FG (fold change from UT)
2.5
2.0
1.5
1.0
0.5
0.0
*
****
***
UT
DMSO
MK801
AP-5
CNQX
E
UT
5 μM NMDA
10 μM NMDA
20 μM NMDA
BACE1
FG
F
BACE1/FG (fold change from UT mean)
1.50
1.25
1.00
0.75
0.50
*
UT
5 μM NMDA
10 μM NMDA

Figure 2-4. NMDAR signaling is necessary and sufficient for BACE1 upregulation by HIV/MDM supernatants

A) Primary rat neurons were pretreated for 1 h with 10 μM NMDAR antogonist MK801, 100 μM NMDAR antagonist AP-5, or 30 μM AMPAR antagonist CNQX prior to 24 h treatment with Mock or HIV/MDM supernatants. Representative images are double labeled for MAP2 (green) and nuclei (blue). **B)** Identical pretreatments were performed for a separate of cultures harvested following 16 h treatment with Mock or HIV/MDM supernatants. Representative blots are shown. **C)** Number of MAP2+ cells was quantified across 24 h treatment groups (repeated measures two-way ANOVA followed by Dunnett's test, n=3, ****p<0.0001). **D)** Densitometric analysis of BACE1/FG was used to quantify levels of BACE1 protein across 16 h treatment groups (repeated measures two-way ANOVA followed by Dunnett's test, n=3, *p<0.05, ***p<0.001, ****p<0.0001). **E)** Cultures were treated with 5, 10, or 20 μM NMDA for 16 h and assessed for BACE1 protein levels. Representative blots are shown. **F)** Densitometric analysis of BACE1/FG was used to quantify levels of BACE1 protein (repeated measures one-way ANOVA followed by Dunnett's test, n=4-5, *p<0.05). Dotted lines represent UT cultures. FG = fast green loading control.

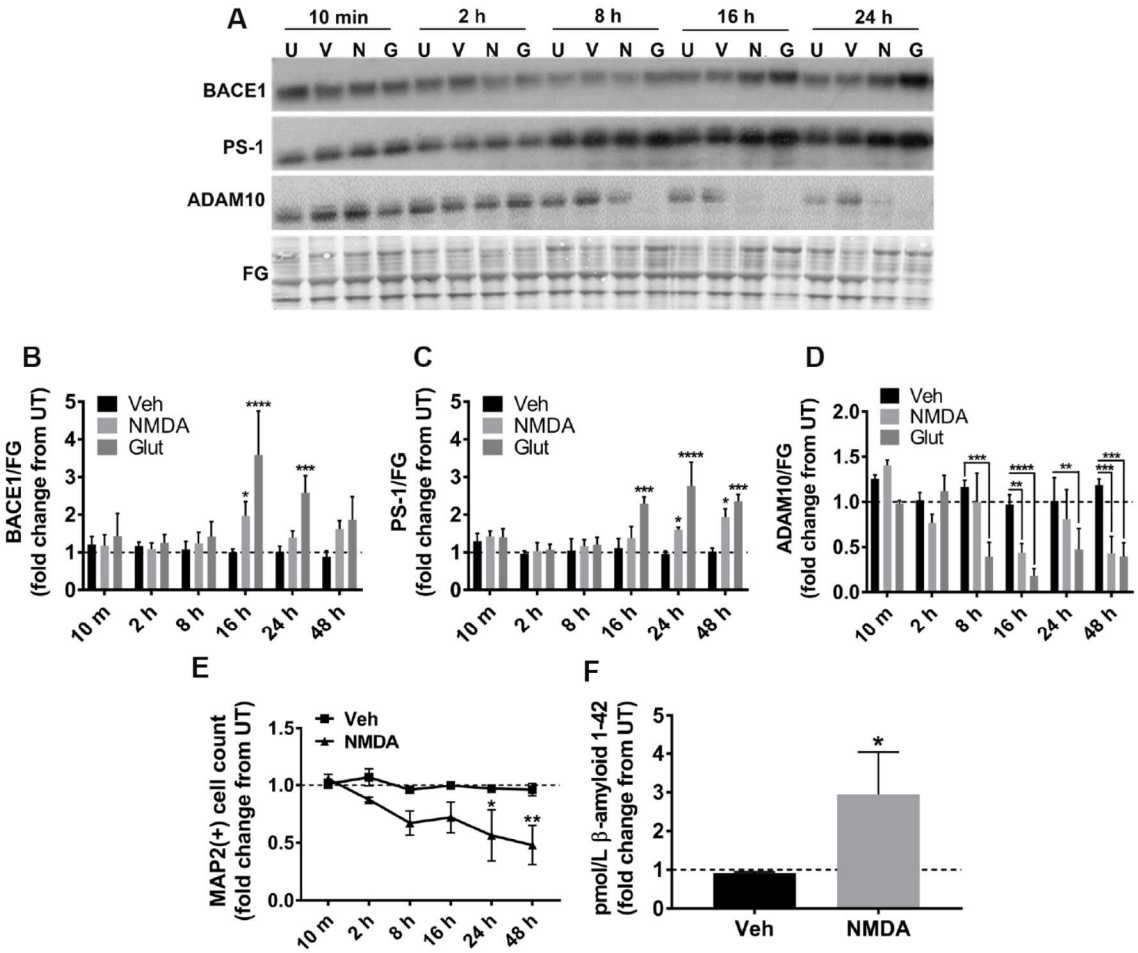

Figure 2-5. NMDAR signaling shifts APP cleavage toward the amyloidogenic pathway

A) Primary rat neurons were treated with 10μM NMDA or 10μM glutamate for 10 min, 2, 8, 16, 24, or 48 h. Whole cell lysates were collected and assessed for BACE1, PS-1, and ADAM10 expression; representative blots are shown. **B-D)** Densitometric analysis of each secretase compared to FG was used to quantify protein levels across time points (repeated measures two-way ANOVA followed by Dunnett's test, n=3-4, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). FG = fast green loading control. **E)** Number of MAP2+ cells was quantified following 10 μM NMDA treatment (repeated measures two-way ANOVA followed by Sidak's test, n=4, *p<0.05, **p<0.01.) **F)** Conditioned media from primary rat neurons was collected following 24 h treatment with 10 μM NMDA, and sample concentrations of Aβ42 were measured using a sandwich ELISA. Concentrations were normalized to the number of MAP2+ cells in identically treated cultures within each biological replicate (paired t-test, n=3, *p<.05). Dotted lines represent UT cultures.

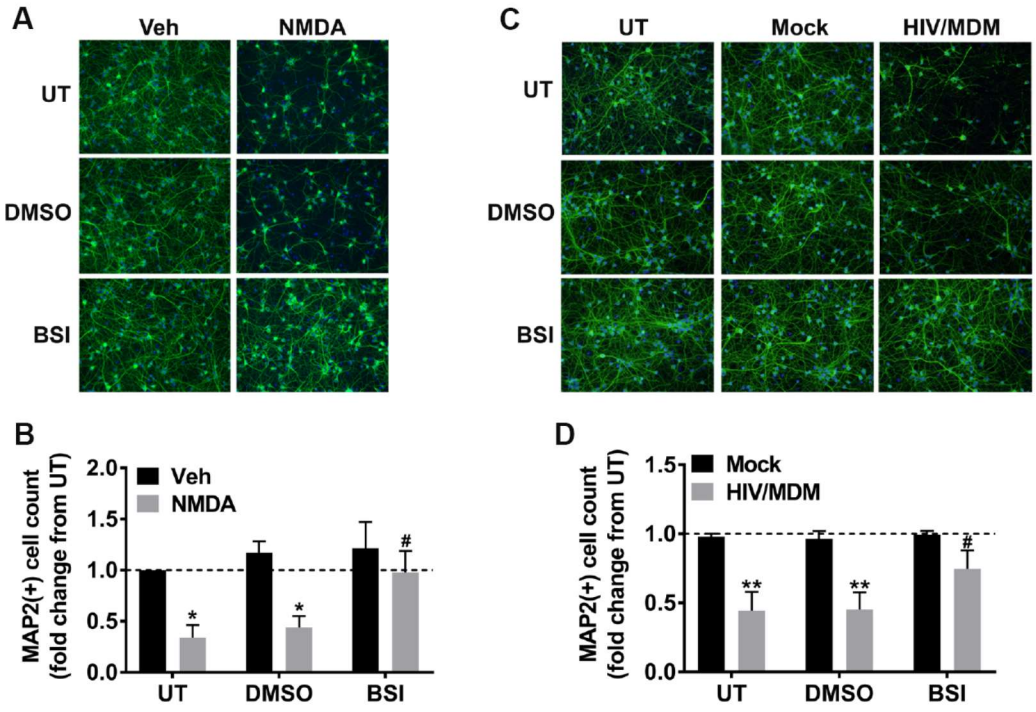

Figure 2-6. Neurotoxicity induced by either NMDA or HIV/MDM supernatants is dependent on BACE1 activity

A) Primary rat neurons were pretreated with DMSO vehicle or 100 nM BSI II for 1 h prior to 24 h treatment with 10 μM NMDA. Representative images are double labeled for MAP2 (green) and nuclei (blue). **B)** Number of MAP2+ cells was quantified across treatment groups (repeated measures two-way ANOVA followed by Tukey's test, n=4, *p<0.05 vs treatment vehicle, #p<0.05 vs pretreatment vehicle). **C)** Primary rat neurons were pretreated with DMSO vehicle or 5 μM BSI IV for 1 h prior to 24 h treatment with Mock or HIV/MDM supernatants. Representative images are double labeled for MAP2 (green) and nuclei (blue). **D)** Number of MAP2+ cells was quantified across treatment groups (repeated measures two-way ANOVA followed by Tukey's test, n=4, **p<0.01 vs Mock, #p<0.05 vs pretreatment vehicle). Dotted lines represent UT cultures.

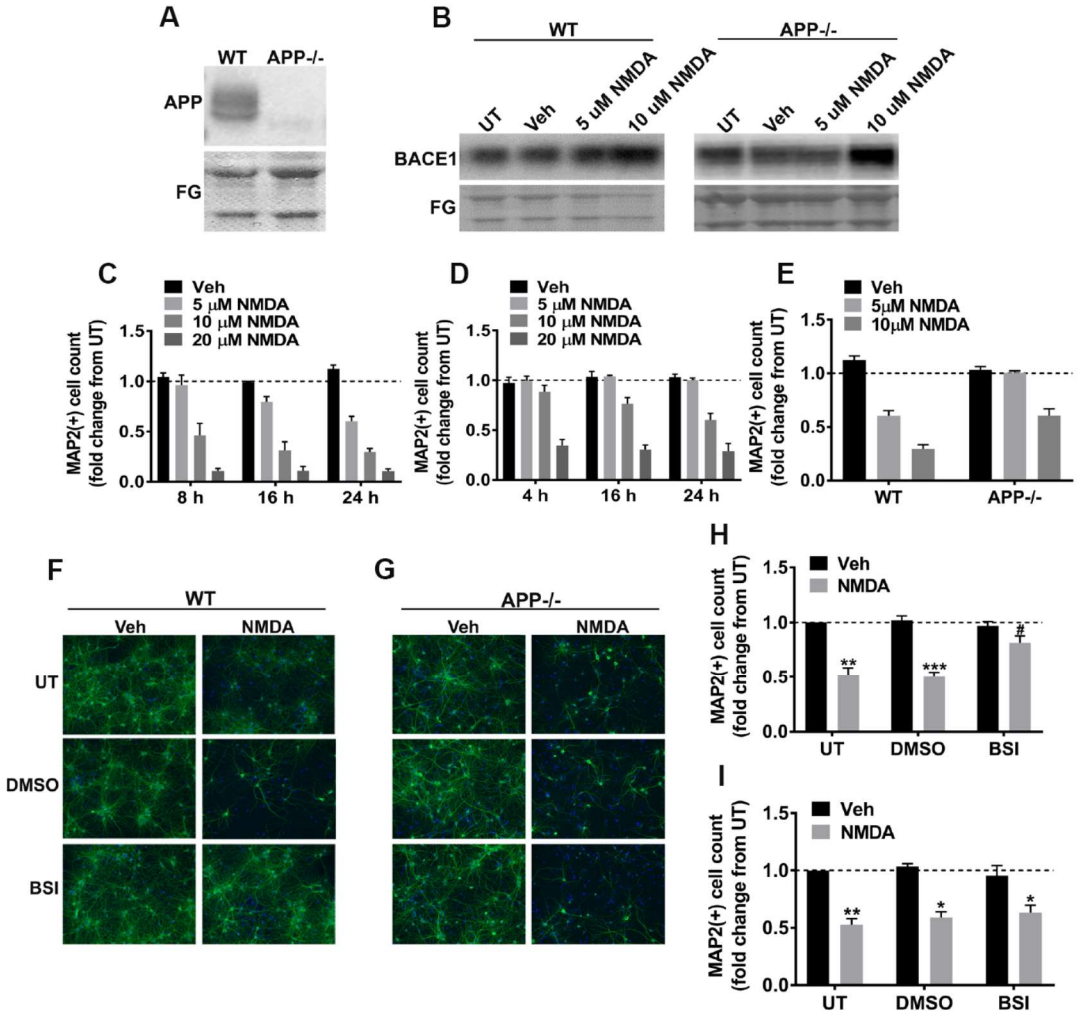

Figure 2-7. APP is required for BACE1-dependent neurotoxicity.

A) Primary neuroglia were isolated from WT or APP$^{-/-}$ mice and assessed for levels of APP by western blot. Representative blots are shown. **B)** WT or APP$^{-/-}$ neuroglial cultures were treated with 5 or 10 μM NMDA for 16 h and whole cell lysates were collected and assessed for BACE1. Representative blots are shown. FG = fast green loading control. **C)** WT and **D)** APP$^{-/-}$ cultures were treated with 5, 10, or 20 μM NMDA for 4, 16, or 24 h. Number of MAP2+ cells was quantified across treatment groups. **E)** Data from C and D 5 and 10 μM 24 h NMDA treatment groups only were replotted to show direct comparison between WT and APP$^{-/-}$ responses. **F)** WT and **G)** APP$^{-/-}$ cultures were pretreated with DMSO vehicle or BSI for 1 h prior to 24 h treatment with 5 μM (WT) or 10 μM (APP$^{-/-}$) NMDA. Representative images are double labeled for MAP2 (green) and nuclei (blue). **H-I)** Number of MAP2+ cells was quantified across treatment groups (repeated measures two-way ANOVA followed by Dunnett's test, n=3-4, *p<0.05, **p<0.01, ***p<0.001). Dotted lines represent UT cultures.

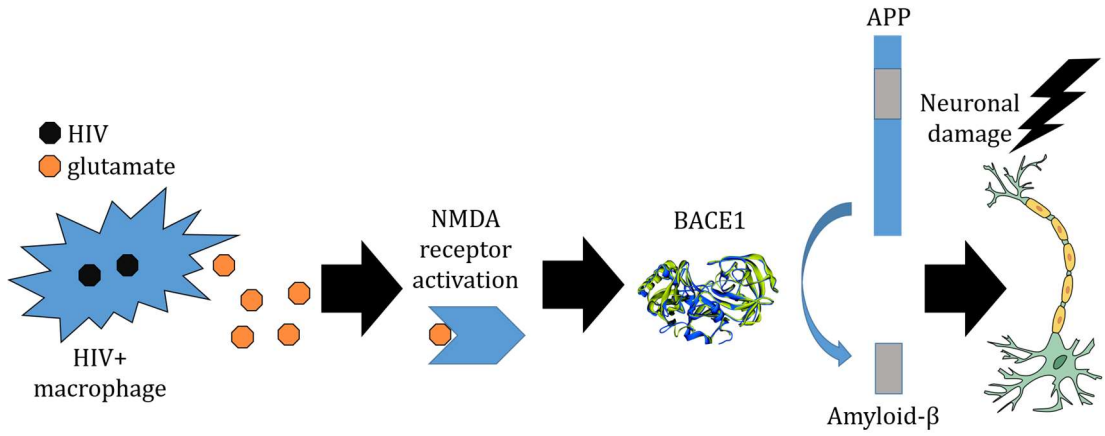

Figure 2-8. Hypothesis overview: BACE1 and APP mediate NMDAR-dependent neuronal damage associated with HIV.

Data presented herein support the hypothesis that BACE1 mediates neuronal damage induced by NMDA receptor activation. Specifically, we propose that in an HIV+ individual, HIV-infected macrophages in the brain release increased levels of glutamate and/or fail to adequately regulate glutamate levels in the extracellular space. Increased extracellular glutamate leads to chronic activation of NMDA receptors on neurons, which causes increased expression of BACE1 protein. Increased BACE1 expression then results in increased processing of APP and higher concentrations of amyloid-β, which lead to neuronal damage over time.

CHAPTER 3: DIFFERENTIAL EFFECTS OF ANTIRETROVIRALS ON NEURONS *IN VITRO*: A ROLE FOR THE ENDOGENOUS ANTIOXIDANT RESPONSE

Anna L Stern, Rebecca N Lee, Nina Panvelker, Jiean Li, Jenna Harowitz, Kelly L Jordan-Sciutto, and Cagla Akay-Espinoza

Abstract

Mounting evidence suggests that antiretroviral drugs may contribute to the persistence of HIV-associated neurocognitive disorder (HAND), which impacts 30%–50% of HIV-infected patients in the post-antiretroviral era. We previously reported that two first generation HIV protease inhibitors, ritonavir and saquinavir, induced oxidative stress and the unfolded protein response, with subsequent neuronal death *in vitro*, which was reversed by augmentation of the endogenous antioxidant response by monomethyl fumarate. We herein determined whether two newer-generation PIs, darunavir and lopinavir, were deleterious to neurons *in vitro*. Further, we expanded our assessment to include three integrase strand transfer inhibitors, raltegravir, dolutegravir, and elvitegravir. We found that only lopinavir and

elvitegravir were neurotoxic to primary rat neuroglial cultures as determined by the loss of microtubule-associated protein 2 (MAP2). Intriguingly, lopinavir but not elvitegravir led to oxidative stress and induced the endogenous antioxidant response. Furthermore, neurotoxicity of lopinavir was blocked by pharmacological augmentation of the endogenous antioxidant heme-oxygenase 1 (HO-1), expanding our previous finding that protease inhibitor-induced neurotoxicity was mediated by oxidative stress. Intriguingly, we found that neurotoxicity was induced only by a subset of protease inhibitors and integrase strand transfer inhibitors, providing evidence for class- and drug-specific neurotoxic effects of antiretroviral drugs. Future *in vivo* studies will be critical to confirm the neurotoxicity profiles of these drugs for incorporation of these findings into patient management. The endogenous antioxidant response is a potential access point for the development of adjunctive therapies to complement antiretroviral therapies and limit their contribution to HAND persistence.

Introduction

Human immunodeficiency virus (HIV) affects 36.9 million people globally (UNAIDS 2015) and 1.1 million people in the United States

alone (Centers for Disease Control and Prevention 2016). Left untreated, HIV replicates in blood and tissues, eventually leading to debilitating loss of immune function defined as acquired immune deficiency syndrome (AIDS). AIDS patients are susceptible to opportunistic infections which are often lethal. However, the introduction of combination antiretroviral therapy in 1996 transformed HIV diagnosis from a death sentence into a chronic, manageable condition with minimal to no effect on life expectancy in the absence of comorbidities (Lai *et al.* 2006; Teeraananchai *et al.* 2016).

Despite the pronounced benefits of antiretroviral therapy, the incidence of neurological complications among HIV-infected individuals has not declined. In fact, HIV-associated neurocognitive disorder (HAND) remains prevalent, with estimates ranging between 15% and 55% in HIV-infected patients (Saylor *et al.* 2016). Although the diagnosis of severe neurological dysfunction, termed HIV-associated dementia (HAD), is now rare, the incidence of both asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) has increased (Sacktor *et al.* 2016). Additionally, while approximately 70% of HAND patients are asymptomatic, ANI patients are two to six fold more likely than neurocognitively normal patients to progress to symptomatic disease (Grant *et al.* 2014b). Moreover, with

the expected increase in life expectancy of HIV-infected individuals afforded by antiretroviral therapy, age-related changes in the central nervous system (CNS) may exacerbate HAND symptoms (Gelman and Schuenke 2004; Cohen *et al.* 2015; Tan *et al.* 2013).

Persistence of HAND despite viral suppression by antiretroviral therapy is not well understood. The cause is likely multifactorial, with contributions from HIV-related factors (Kaul and Lipton 2006; Chen *et al.* 2014) as well as from antiretroviral drugs (ARVs) themselves (Treisman and Soudry 2016; Shah *et al.* 2016). Several studies found that ARVs with higher CNS penetration effectiveness were associated with more frequent neurological symptoms, indicating a role for ARV toxicity (Marra *et al*. 2009; Caniglia *et al.* 2014; but see Carvalhal *et al.* 2016; Smurzynski *et al.* 2011 for alternative results). Numerous studies also demonstrated the potential for ARVs to cause oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction, with subsequent synaptodendritic damage and neuron loss both *in vivo* and *in vitro* (Akay *et al.* 2014; Gannon *et al.* 2017; Robertson *et al.* 2012b; Brown *et al.* 2014). As neurotoxicities associated with different ARVs may vary, and given the continuing development of newer and more effective ARVs, questions remain regarding the potential for current therapies to instigate long-term adverse neurological effects.

ARVs can be broadly categorized into five classes according to their mechanism of action: entry inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), and protease inhibitors (PIs). Currently, the United States Department of Health and Human Services (DHHS) recommends that frontline treatment for adult HIV-infected individuals should include either an INSTI or a PI in combination with two NRTIs (DHHS 2016a). We previously showed that two first generation PIs, ritonavir and saquinavir, led to oxidative stress and neurotoxicity. Herein, we expanded our investigation to include three INSTIs, elvitegravir (EVG), dolutegravir (DTG), and raltegravir (RAL), as well as two commonly used PIs darunavir (DRV) and lopinavir (LPV). All are currently recommended by the DHHS as frontline treatment options for adults and adolescents, except for LPV which is recommended by the DHHS and the WHO for all children under three years of age (DHHS 2016a; WHO 2016a). We investigated the neurotoxicity profiles of these ARVs *in vitro* and examined the underlying mechanisms contributing to toxicity.

Materials and Methods

Chemicals and Reagents. The following antibodies were purchased from the indicated vendors: Enzo Life Sciences (Farmingdale, NY): heme-oxygenase 1 (HO-1, ADI-SPA-896); Abcam (Cambridge, MA): glutathione-S-reductase (GSR, ab16801); Cell Signaling Technology (Danvers, MA): phosphorylated eukaryotic initiation factor 2α (peIF2α, 9721), total eIF2α (9722); BD Transduction Laboratories (San Jose, CA): binding immunoglobulin protein (BiP, 610978); BioLegend (San Diego, CA): microtubule-associated protein 2 (MAP2, 801801). The following chemical reagents were purchased from the indicated vendors: Citifluor (London, UK): 4',6-diamidino-2-phenylindole (DAPI); Invitrogen (Carlsbad, CA): Dulbecco's Modified Eagle's Medium (DMEM), neurobasal medium, B27 supplement; BioRad (Hercules, CA): Bradford protein assay dye, polyvinylidene fluoride (PVDF) membrane, prestained broad range molecular weight ladder; Sigma Aldrich (St. Louis, MO): Tween 20, Triton X-100, Fast Green FCF, protease inhibitor cocktail, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO); Peptides International (Louisville, KY): Poly-L-Lysine; Scytek Labs (Logan, UT): normal antibody diluent (NAD); Millipore (Temecula, CA): Luminata Classico ECL; Thermo Fisher Scientific (Waltham, MA): CellRox Green, tetramethylrhodamine methyl ester (TMRM); Tocris Bioscience (Bristol,

UK): 1-(2-Cyano-3,12,28-trioxooleana-1,9(11)-dien-28-yl)-1H-imidazole (CDDO). All horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Thermo Fisher Scientific, and all fluorescent dye-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA). ARVs were kindly provided by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (Bethesda, MD).

Preparation of primary rat cortical neuroglial cultures. Primary rat cortical cultures were prepared from embryonic day 18 Sprague-Dawley rat embryos (Charles River Laboratories, Seattle, WA). Brains were isolated, and dissected cortices were incubated for 40 minutes in DMEM + 0.027% trypsin as described previously (Wilcox *et al.* 1994). Cells were then washed in saline, triturated, resuspended in neurobasal media supplemented with B27, and plated on poly-L-lysine-coated 6-well (9.4-cm^2 growth area) or 24-well (1.9-cm^2 growth area) plates (USA Scientific, Ocala, FL) at a concentration of 500,000 cells/ml. Cultures contained approximately 90% neurons and 10% astrocytes/glia and were maintained in neurobasal media supplemented with B27 at 37°C with 5% CO_2 as described previously (Gannon *et al.*

2017; Akay *et al.* 2011). On 10 days *in vitro* (DIV), 20% fresh media was added. Cells were treated on DIV 14–16.

Drug treatments. Cells were treated with individual ARVs for the times and doses as indicated. DTG, RAL, and EVG were prepared as 50 mM stock solutions in DMSO, whereas RTV, LPV, and DRV were prepared as 25 mM stock solutions in DMSO. In specific experiments, CDDO (prepared as 50 mM stock solution in DMSO) was used for 1-h pretreatment before the indicated ARV treatments.

Immunofluorescence. Following treatment, cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min. Cells were then rinsed twice in PBS and three times in PBS containing 0.1% Tween 20 (PBS-T), followed by a 30-min incubation with a blocking/permeabilization solution containing 0.2% BSA + 0.1% Triton-X in PBS. Cells were rinsed three times in PBS-T and incubated with MAP2 primary antibody diluted at 1:4000 in NAD for 2 h at room temperature. Following three washes in PBS-T, cells were then incubated with a FITC-conjugated goat anti-mouse secondary antibody diluted at 1:500 in NAD for 30 min at room temperature. Cells were then imaged using a Keyence BZ-X-700 digital fluorescent microscope

(Keyence Corporation, Itasca, IL) affixed with UV, FITC, Cy3, and Cy5 filters. Images captured at ×20 magnification were analyzed with the BZ-X Keyence software to quantify the number of neurons. Specifically, the number of neurons, identified as cells expressing MAP2, was averaged across a total of 25 fields/well, with 2–4 wells/treatment condition for each biological replicate. Data were analyzed using GraphPad Prism statistical software (version 7.0; GraphPad, San Diego, CA), and data were expressed as mean fold change from untreated (UT) ± standard error of the mean (SEM).

Immunoblotting. Following treatment, cells were rinsed twice with PBS and lysed with whole cell lysis buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40, 0.4 mM NaF, 0.4 mM Na3VO4, and 1:100 protease inhibitor cocktail). Protein supernatants were collected with centrifugation at 20,000 *g* for 10 min at 4°C. Protein concentrations were determined using the Bradford method, and 3-5 µg total protein per condition was loaded into each lane of precast 10% Bis-Tris NuPAGE Novex gels (Thermo Fisher Scientific). Proteins were then transferred to PVDF membranes, which were blocked with 5% BSA in tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated overnight with primary antibodies at 4°C. Following three

washes in TBS-T, membranes were incubated with HRP-conjugated secondary antibody (1:5000 in 5% BSA + TBS-T) for 30 min at room temperature. Bands were visualized by chemiluminescence with Luminata Classico ECL, and images were captured by film development or ChemiDoc Touch imaging system (BioRad). Equal loading and even transfer of samples were confirmed using fast green staining of the membranes. Densitometric analysis of band intensities was conducted using ImageJ software (v1.44, NIH), and all bands were normalized to fast green stain. Data were analyzed using GraphPad Prism statistical software, and data were expressed as mean fold change from UT ± SEM.

CellRox Green live cell imaging. CellRox Green oxidative stress detection reagent was purchased as a stable 2.5-mM solution dissolved in DMSO. Aliquots were stored at −20°C protected from light and with a desiccant, thawed just prior to use. Directly following drug treatments, CellRox was added to cell media at a final concentration of 5 μM and incubated at 37°C for 30 min according to manufacturer's instructions. Cells were then visualized at 20× using Keyence BZ-X-700 digital fluorescent microscope by time-lapse live cell imaging. Images of each well were captured approximately every 6 min for 1 h following the incubation period. Cell media were then removed, and cells were rinsed and fixed

with 4% paraformaldehyde for 20 min prior to immunofluorescence staining, as described above. Images of cells stained with MAP2/DAPI were then merged with CellRox Green images using Adobe Photoshop. Quantification of CellRox Green fluorescence was achieved using Keyence BZ-X analysis software. Data were analyzed using GraphPad Prism statistical software, and data were expressed as mean fold change from UT ± SEM.

Results

Elvitegravir but not dolutegravir or raltegravir is toxic to neurons *in vitro*. Given that INSTIs are an integral part of the updated frontline treatment for HIV, we first determined the effects of three commonly prescribed INSTIs, EVG, DTG, and RAL, on MAP2 expression in primary neuroglial cultures. We treated cells with individual ARVs at 0.1, 1, or 10 μM either one time for two days or every other day for four days (see Table 1 for comparison with *in vivo* concentrations of INSTIs used in this study). While 2- and 4-day treatments with EVG at lower concentrations did not lead to neuronal damage as determined by the reduction in the number of MAP2-positive cells, 10 μM EVG led to an average of 76%

MAP2 loss at 4 days (Fig. 1A, B). In contrast, neither DTG (Fig. 1A, C) nor RAL (Fig. 1A, D) was neurotoxic at any dose or time point tested.

Lopinavir but not darunavir is toxic to neurons *in vitro*. PIs are the second most commonly prescribed ARVs. Specifically, DRV is the only currently recommended frontline PI for adults in the United States, whereas LPV is the PI of choice both in the United States and globally for the treatment of children under the age of three (DHHS 2016a; WHO 2016a). Hence, we determined the effects of these drugs on MAP2 expression in primary neuroglial cultures. We treated cells with ARVs individually at either 0.1, 1, or 10 μM for 2 days (see Table 1 for comparison with *in vivo* measured concentrations of PIs used in this study). At lower concentrations, LPV was not toxic; however, 10 μM LPV led to an average of 54% MAP2 loss (Fig. 2A, B). In contrast, DRV was not toxic at any dose after 2 days. Surprisingly, treatment with 0.1 μM DRV led to an increase in the number of MAP2+ cells compared with the vehicle control (Fig. 2A, C).

In clinical practice, PIs are often administered with a low "booster" dose of RTV to inhibit the metabolism of concomitantly prescribed ARVs and increase their bioavailability. These combinations are packaged into fixed-ratio pill forms such that RTV dose is increased by the same ratio

as the primary PI if the regimen is altered. Based on our previous work demonstrating that RTV was neurotoxic at 10 μM *in vitro* (Gannon *et al.* 2017; Akay *et al.* 2014), we determined whether lower doses of RTV administered as part of an ARV regimen could alter the effects of other PIs. Cells were treated for 2 days with LPV or DRV at 0.1, 1, or 10 μM concentrations either alone or with corresponding RTV booster concentrations of 0.02, 0.2, or 2 μM, respectively. Although 2 μM RTV was neurotoxic after two days regardless of concomitant treatment with DRV, none of the combinations tested were more toxic than either ARV alone (Fig. 2D).

Lopinavir but not elvitegravir increases reactive oxygen species (ROS). We previously observed that ARVs caused oxidative stress in the CNS *in vivo* (Akay *et al.* 2014). Therefore, we next determined whether LPV and EVG led to ROS accumulation in neurons. The oxidative stress indicator CellRox Green was added to the culture media together with LPV or EVG, and live cell imaging was conducted 1 h later as described in the Methods section. The green fluorescence in the nucleus, indicating the accumulation of the oxidized compound in the presence of ROS, was significantly increased by LPV compared to DMSO vehicle control. In contrast to LPV, however, EVG did not lead to an increase in

ROS; instead, both DMSO and EVG led to a reduction in ROS levels even below those measured in untreated cultures (Fig 3A, B).

Lopinavir but not elvitegravir activates the endogenous antioxidant response. Based on our observation of oxidative stress induced by LPV, we sought to determine whether the endogenous antioxidant response was activated. To that end, we assessed the levels of heme-oxygenase 1 (HO-1), a canonical indicator of endogenous antioxidant response activation with potent antioxidant properties, in lysates prepared from cultures treated with 0.1, 1, or 10 μM LPV for 4, 8, or 20 h. As shown in Fig. 4A and B, we observed that HO-1 protein levels were increased in cultures treated with 10 μM LPV for 20 h. In contrast, EVG treatment had no significant effect on HO-1 (Fig. 4E, F). Another common mediator of cellular toxicity is ER stress, which we previously identified as a correlate of neuropathological changes in HIV-infected patients (Lindl *et al.* 2007; Akay *et al.* 2012). Thus, we determined whether two canonical ER stress markers, BiP and phospho-eIF2α (peIF2α), were altered in neurons exposed to 10 μM LPV or EVG for 4, 8, or 20 hours. Surprisingly, we observed no changes in either BiP or the ratio of peIF2α/teIF2α following LPV treatment (Fig. 4A, C, D). In

contrast, 10 μM EVG treatment led to a transient increase in peIF2α/teIF2α at 4 h followed by a decrease at 20 h (Fig. 3E, H).

Induction of heme-oxygenase 1 by CDDO is protective against lopinavir-induced neurotoxicity. HO-1 is a critical endogenous antioxidant component of the cell, and its pharmacological induction was previously demonstrated to be protective against a variety of CNS insults including RTV-mediated neurotoxicity (Chen 2014; Cross *et al.* 2011; Akay *et al.* 2014). Thus, we hypothesized that augmentation of HO-1 might be neuroprotective against LPV-induced neuronal death. CDDO is a triterpenoid known to induce the expression of antioxidant response element genes including *HO-1* via activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Yates *et al.* 2007). We determined whether CDDO could augment HO-1 expression in LPV-treated cultures. Indeed, after 20 h of treatment, HO-1 was increased in cultures exposed to CDDO or LPV alone and was further increased by the combination of both compounds (Fig. 5A, B). Surprisingly, under the same conditions, neither CDDO nor LPV led to a change in the protein levels of glutathione reductase (GSR), another component of the endogenous antioxidant response targeted by Nrf2 (Fig. 5A, C). In agreement with its ability to augment HO-1 expression,

CDDO pretreatment was able to completely block LPV-induced neurotoxicity (Fig. 5D, E), indicating both a mechanistic role for oxidative stress in LPV-mediated neurotoxicity as well as a neuroprotective role for HO-1. In contrast, consistent with our observed lack of ROS accumulation and HO-1 induction following EVG treatment, CDDO had no effect on EVG-induced neurotoxicity (Fig. 5F).

Discussion

The introduction of ART was a life-saving advancement in HIV/AIDS, and our most urgent goal is to expand access to therapy around the world (WHO 2016b). However, given the continued persistence of HAND despite effective viral suppression with ARVs, a better understanding of potential ARV neurotoxicities is necessary as patients remain on ART for decades due to increased lifespan. In particular, the most recently introduced class of ARV, INSTIs, requires further investigation as their worldwide use continues to grow.

In the present study, we investigated the *in vitro* effects of five drugs including the INSTIs EVG, DOL, and RAL and the PIs LPV and DRV, all of which are recommended as part of current frontline regimens for HIV-infected individuals in the U.S (DHHS 2016a; DHHS 2016b).

Neuronal damage was induced by EVG and LPV only, and LPV but not EVG led to oxidative stress while EVG but not LPV led to transient ER stress. Furthermore, by pharmacologically inducing the endogenous antioxidant HO-1, we were able to reverse LPV-induced neuronal damage.

Of the three INSTIs tested, only EVG caused neuronal damage in primary rat cultures. This within-class difference was dramatic, with EVG causing a 76% loss of MAP2, whereas the same dose and time course of treatment with DTG or RAL had no effect. These data corroborate previous studies showing the lack of *in vitro* toxicity of RAL (Blas-Garcia *et al.* 2014). Thus, it may be warranted for clinicians to consider this observation when deciding between the three drugs, especially in younger patients who are anticipated to take ARVs for several decades. However, it is critical to interpret these data with caution and in the full context of other studies. For instance, while we found no evidence of neurotoxicity with DTG in our model, which was consistent with many comprehensive studies of patient populations (Kanters *et al.* 2016; Singh *et al.* 2016a), other recent evidence indicates that potential CNS toxicity of DTG should not be overlooked (Hoffmann *et al.* 2017; Kheloufi *et al.* 2015; de Boer *et al.* 2016). Neuropsychiatric side effects of DTG might be due to its relatively high CNS penetration effectiveness, with

CSF concentrations reaching levels equivalent to those measured in plasma (Letendre *et al.* 2014). Moreover, many ARVs as well as their metabolites can have severe peripheral side effects including chronic inflammation (Troya and Bascuñana 2016), which may indirectly cause neuropsychiatric symptoms.

Additionally, we found a difference in the neurotoxic potential of two PIs, DRV and LPV. Specifically, LPV caused a 54% loss of MAP2, whereas DRV had no effect at the same dose and time course. The relevance of this direct comparison is highlighted by comparable recommended dosing and maximum plasma concentrations of the two drugs in patients (see Table 1). Moreover, DRV had no effect on neurons even when combined with low doses of RTV, as it is currently prescribed. Future studies should address whether combinations of DRV and cobicistat, the newest approved co-formulation, remain non-neurotoxic (Capetti *et al.* 2015). These results add to a growing body of *in vitro* evidence that DRV is a particularly safe treatment option (Robertson *et al.* 2012b; Blas-Garcia *et al.* 2014). DRV was designed to bind tightly to the HIV protease and limit drug resistance (Deeks 2014; Wensing *et al.* 2010); moreover, DRV demonstrated superior viral suppression, increased $CD4^+$ T cell counts, and a lower incidence of gastrointestinal side effects in a direct comparison study with LPV (Mills *et al.* 2009).

Despite these data, LPV is prescribed more frequently than DRV in resource-poor areas, such as Sub-Saharan Africa where over 70% of HIV-infected individuals reside (Saylor *et al.* 2016; WHO 2016a). The prevalence of LPV use in resource-poor settings is primarily because LPV is available as a generic, heat stable fixed-dose combination drug approved for once-daily dosing (WHO 2016a). Our study along with those mentioned above highlight the rationale for urgent development of a similar formulation of DRV available at lower cost.

Another relevant consideration is that LPV is recommended by the DHHS (2016b) and WHO (2016a) as the frontline treatment for newborns and children up to three years of age. In addition to the evidence of blood brain barrier disruption caused by HIV in general (Singh *et al.* 2016b; Nakagawa *et al.* 2012; Singh *et al.* 2014), data suggest that the blood brain barrier in newborns is not yet fully developed (Baburamani *et al.* 2012; Watson *et al.* 2006; but see Saunders *et al.* 2012 for an alternative view), which raises the possibility that infant brains might be exposed to higher concentrations of ARVs than adults. This consideration highlights the need for future studies on LPV and its effects on neurons *in vivo*, particularly in models of pediatric infection.

In addition to characterizing the effects of LPV and EVG on neuronal damage indicated by MAP2 expression, we investigated several mechanisms that might be involved. We expected to observe ER stress, given previous reports by us and others showing the potential for a subset of ARVs to activate the ER stress response pathway *in vivo* and *in vitro* (Gannon *et al.* 2017; De Gassart *et al.* 2016; Weiß *et al.* 2016; Borsa *et al.* 2015). Interestingly, LPV did not upregulate ER stress markers BiP or peIF2α, indicating that the mechanism driving neurotoxicity might be distinct from that of other PIs (Gannon *et al.* 2017). However, a lack of effect on BiP and peIF2α does not rule out the possibility that LPV induces ER stress. These indicators only represent two arms of the unfolded protein response (UPR), a multipartite protective cellular mechanism that is activated following ER stress, and other elements of the UPR may be preferentially induced in response to LPV. In contrast to LPV, EVG treatment transiently increased the ratio of peIF2α/teIF2α, indicating potential ER stress and activation of the UPR. This increase was no longer observed after 8 h treatment, which may reflect the ability of neurons to recover. This recovery is consistent with the observation that a single EVG treatment had no effect on MAP2 after 2 days.

In contrast to our findings related to ER stress, LPV but not EVG led to oxidative stress as evidenced by both increased ROS production and activation of the endogenous antioxidant response. These data are consistent with observations of ARV-associated mitochondrial dysfunction (Noguera-Julian *et al.* 2015) as well as our previous data linking ARVs to oxidative stress (Akay *et al.* 2014). Specifically, we found that two first-generation PIs, RTV and saquinavir, led to ROS accumulation in cultured neurons and that the neurotoxicity induced by these ARVs was blocked by augmenting the endogenous antioxidant response to reduce oxidative stress. In agreement with these observations, we herein again show a mechanistic role for oxidative stress in ARV-induced neuronal damage, as LPV-induced damage was reversed by pharmacological induction of the endogenous antioxidant HO-1. Further demonstrating the distinct mechanisms of toxicity across different ARVs, neither oxidative stress, HO-1 induction, nor protective potential of CDDO was observed with EVG treatment. A limitation of this result, however, is that because EVG induced toxicity on a different time scale than LPV, the possibility remains for oxidative stress and HO-1 induction to manifest at later time points following EVG treatment. Regardless, differences in the neurotoxic potential and underlying

neurotoxic mechanisms across ARV classes as well as within each class are evident based on our findings.

One important caution in the interpretation of our data is that these studies were done *in vitro,* and cultured neurons may not respond to ARVs in a similar manner as neurons *in vivo,* even within the same species. Moreover, there may be species differences, which indicates a need for further studies on these drugs in additional rodent strains, non-human primates, and humans. Relatedly, these studies did not identify the role of neuron-glia interaction in both the toxic and protective effects observed. Because the experiments were performed in neuroglial cultures, potential contributions of astrocytes and microglia should not be overlooked. Another legitimate concern about the relevance of our findings is whether the doses at which neurotoxic effects were observed were comparable to those expected in patients. Doses tested in the current study were designed to cover a wide range of concentrations, and the concentrations which caused neuronal damage in our *in vitro* system were higher than those observed in patient CSF samples (Table 1). Hence, one important outcome of our study was that acute administrations of the five ARVs tested were not neurotoxic *in vitro* at low doses comparable to their reported concentrations in the CSF. However, although low doses had no effect on cell number quantified by

MAP2+ cell counts, the possibility of synaptic damage remains, consistent with our previous studies (Akay *et al.* 2014). Additionally, a critical feature of our model is its attempt to assess the chronic effects of ART over decades utilizing an acute model, which necessitates higher drug concentrations than may be observed in the CSF of patients at any given time. Moreover, CSF drug concentrations may not accurately reflect ARV concentrations in the brain parenchyma (Anthonypillai *et al.* 2004). Finally, as current attempts are focused on improving the CNS penetration effectiveness of ARVs to increase viral suppression in the brain (Bertrand *et al.* 2016), an understanding of the neurotoxic potential of even relatively high drug concentrations remains important.

In summary, the significance of the present study is two-fold. First, we demonstrated that certain ARVs may have significant neurotoxic potential, which we hope will both aid clinicians in their decisions and influence momentum for ARVs with the highest safety indications to be made more accessible worldwide. Second, we showed that ARVs caused neurotoxicity through distinct cellular pathways even within a single drug class. As adjunctive therapies are developed in attempts to treat the symptoms of HAND, it may be prudent to individualize therapies and include strategies to protect patients based on their specific ARV regimens.

Table 3-1. Reported patient plasma and cerebrospinal fluid concentrations of the antiretroviral drugs evaluated

Concentration range is provided in ng/ml and the maximum measured concentration is provided in μM to allow for direct comparison with doses tested in the current study.

Drug	Plasma concentration (ng/ml)	Maximum plasma concentration (μM)	CSF concentration (ng/ml)	Maximum CSF concentration (μM)
Elvitegravir[1]	450–1700	3.8	2.4-11.7	0.03
Dolutegravir[2]	220–3340	8.0	12.6-16.2	0.04
Raltegravir[3]	1140-1502	3.4	6.0-94.2	0.21
Lopinavir[4]	154–16700	26.6	1.93–78.3	0.12
Darunavir[5]	1800–12900	23.6	15.9–212	0.39

CSF, cerebrospinal fluid
[1] (Ramanathan *et al.* 2011; Podany *et al.* 2017)
[2] (Cottrell *et al.* 2013)
[3] (Yilmaz *et al.* 2009a)
[4] (Tiraboschi *et al.* 2015)
[5] (Yilmaz *et al.* 2009b)

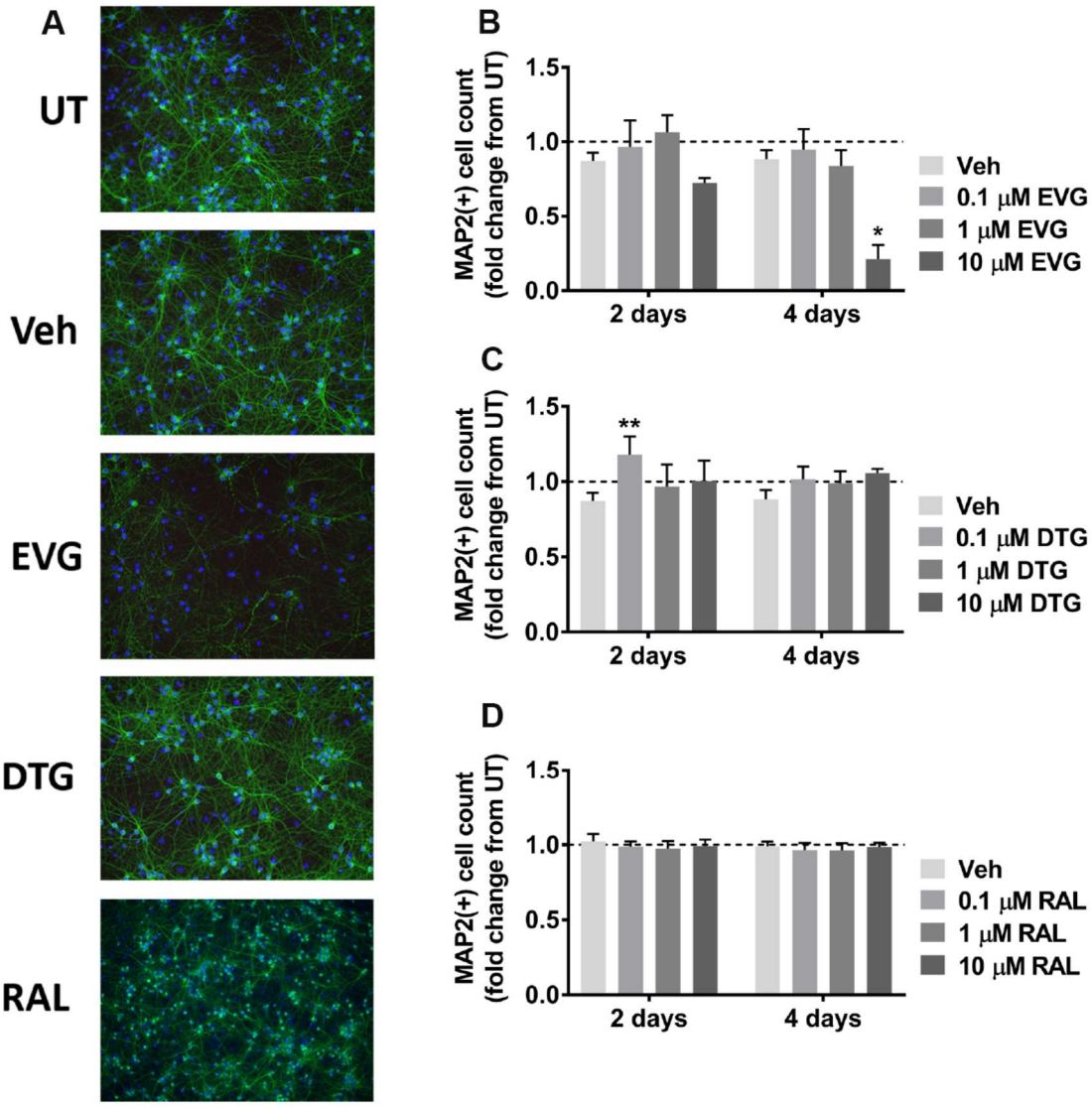

Figure 3-1. EVG but not DTG or RAL is toxic to primary rat cortical neuroglial cultures

A) Cultures were treated with DMSO vehicle or 0.1 μM, 1 μM, or 10 μM EVG, DTG, or RAL for either 2 days or every other day for 4 days. Representative images of neuroglial cultures immunostained for MAP2 (green) and DAPI (blue) after treatment with 10 μM of indicated compounds for 4 days are shown at 20× magnification. Scale bar represents 100 μM. **B-D)** Quantification of MAP2+ cells treated with indicated compounds is shown (repeated measures two-way ANOVA followed by Dunnett's test, n = 3, *$p < 0.05$, **$p < 0.01$ vs drug vehicle). Dashed lines represent untreated (UT) cultures.

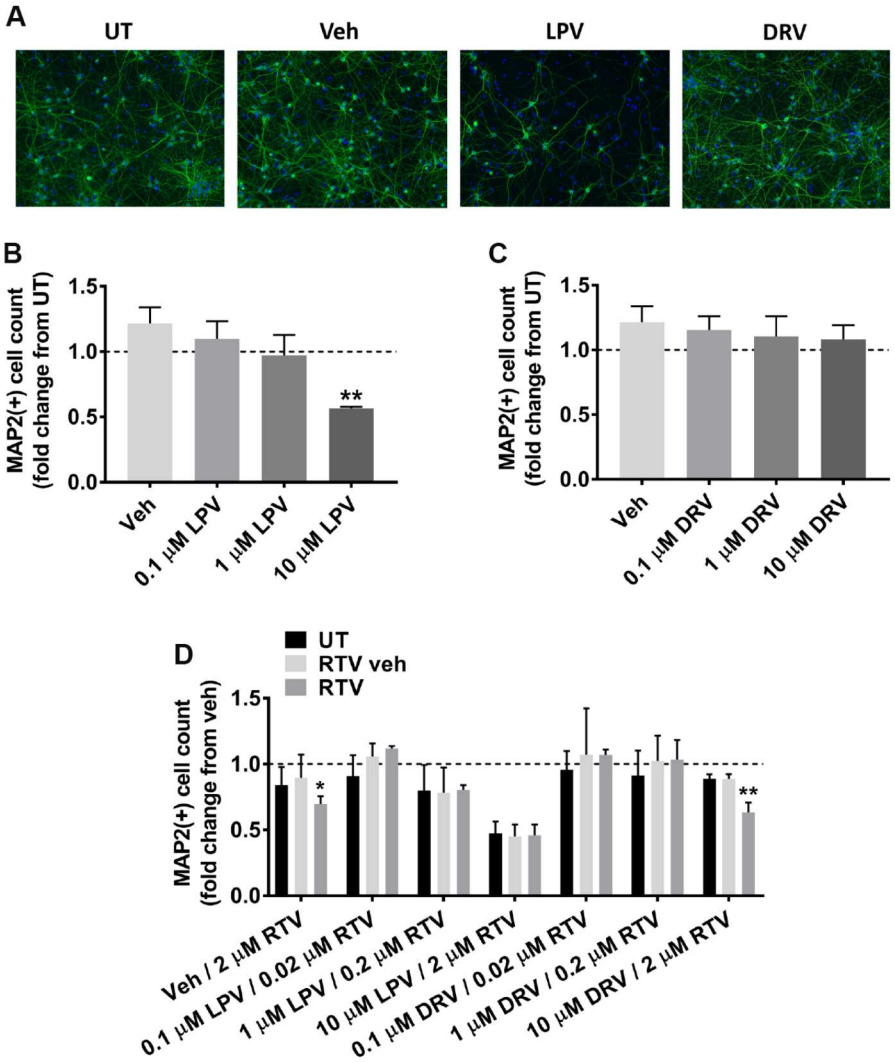

Figure 3-2. LPV but not DRV is toxic to primary rat cortical neuroglial cultures

A) Cultures were treated with DMSO vehicle or 0.1 μM, 1 μM, or 10 μM LPV or DRV for 2 days. Representative images of neuroglial cultures immunostained for MAP2 (green) and DAPI (blue) after treatment with 10 μM of the indicated compounds are shown at 20× magnification. Scale bar represents 100 μM. **B–C)** Quantification of MAP2+ cells treated with indicated compounds is shown (repeated measures one-way ANOVA followed by Dunnett's test, n = 3, **p < 0.01 vs drug vehicle). Dashed lines represent untreated (UT) cultures. **D)** Neurons were treated with LPV and DRV with or without simultaneous RTV or RTV vehicle treatment. RTV booster concentrations were given in fixed ratios with LPV and DRV concentrations. Quantification of MAP2+ cells treated with indicated compounds is shown (repeated measures two-way ANOVA followed by Dunnett's test, n = 3, *p < 0.05, **p < 0.01 vs drug vehicle). Dashed line represents untreated (UT) cultures.

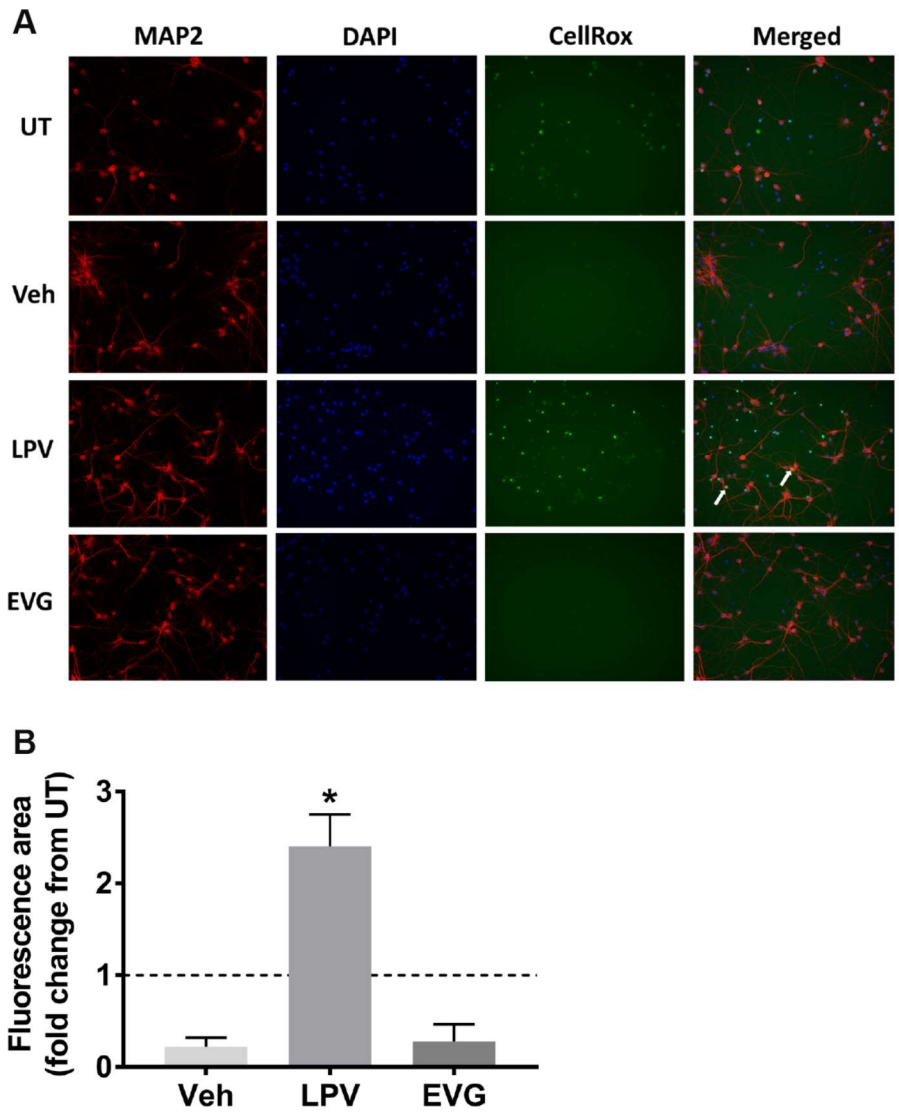

Figure 3-3. LPV but not EVG induces oxidative stress

A) Rat cortical neuroglial cultures were treated with DMSO vehicle or 10 μM LPV or EVG for 1h prior to the addition of CellRox Green reagent and live cell imaging. Images captured by time-lapse live imaging were merged with the images of the same cells that were subsequently fixed and immunostained for MAP2 and DAPI. Representative images captured 30 min following CellRox addition show cells immunostained for MAP2 (red), DAPI (blue), and CellRox green at 20× magnification. Scale bar represents 100 μM; white arrows indicate examples of neurons that accumulated CellRox green dye. **B)** Quantification of the area positive for CellRox green fluorescence normalized to untreated (UT) cultures (dashed line) is shown (repeated measures one-way ANOVA followed by Dunnett's test, n = 4, *$p < 0.05$ vs drug vehicle).

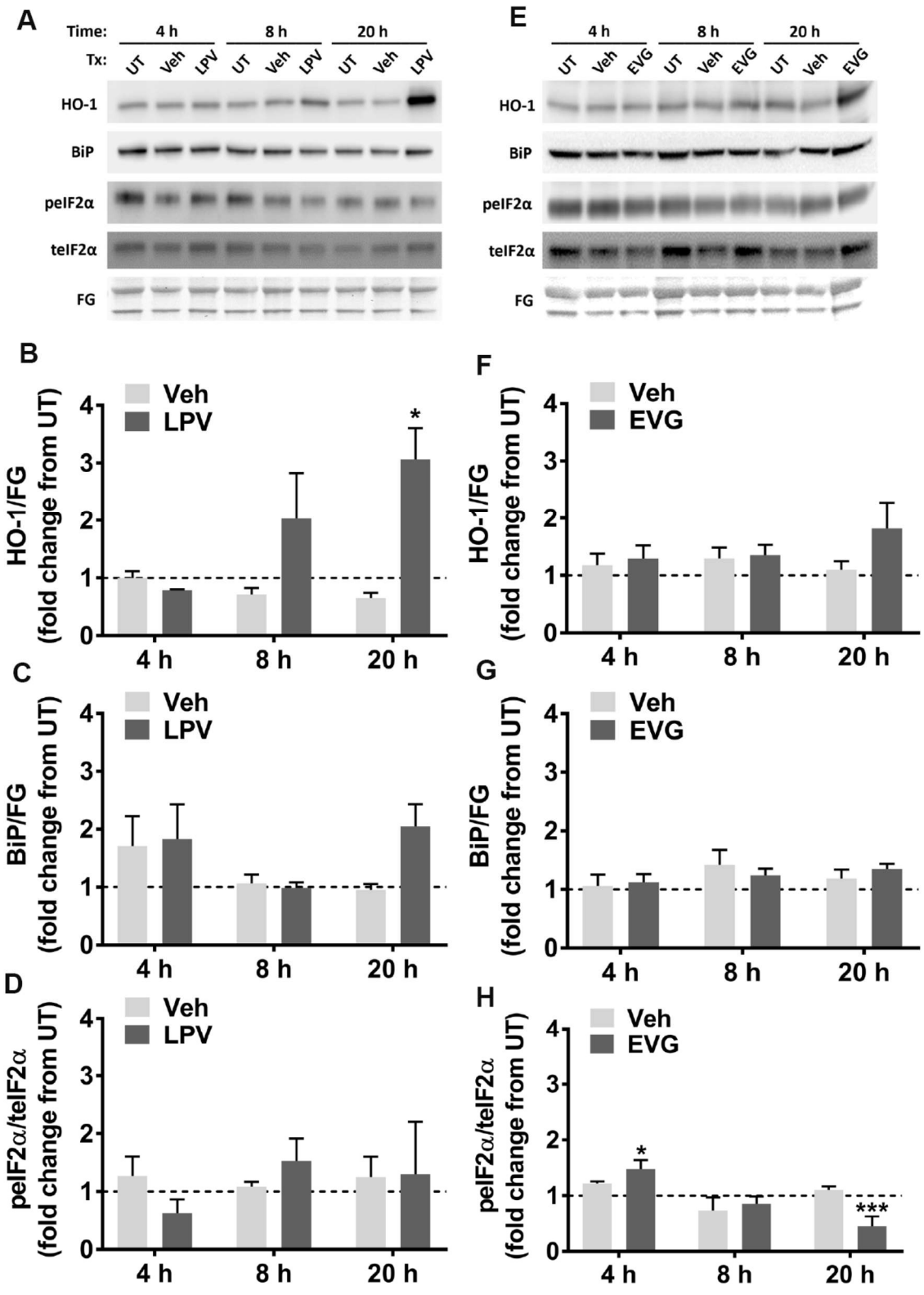
A
Time:
4 h
8 h
20 h
Tx:
UT
Veh
LPV
HO-1
BiP
peIF2α
teIF2α
FG
E
EVG
B
HO-1/FG
(fold change from UT)
Veh
LPV
*
4 h
8 h
20 h
F
C
BiP/FG
G
D
peIF2α/teIF2α
H
***

Figure 3-4. LPV but not EVG induces the endogenous antioxidant response, while EVG but not LPV transiently increases the ratio of peIF2α/teIF2α

A) Rat cortical neuroglial cultures were treated with DMSO vehicle or 10 μM LPV for 4, 8, or 20h. Representative blots are shown. FG, fast green loading control. **B–D)** Band intensities of HO-1, BiP, peIF2α, and teIF2α were quantified using ImageJ software. HO-1 and BiP are normalized to FG, and peIF2α is normalized to teIF2α (repeated measures two-way ANOVA followed by Dunnett's test, n = 3, *p < 0.05 vs drug vehicle). Dashed lines represent untreated (UT) cultures. **E)** Rat cortical neuroglial cultures were treated with DMSO vehicle or 10 μM EVG for 4, 8, or 20 h. Representative immunoblots are shown. FG, fast green loading control. **F–H)** Band intensities of HO-1, BiP, peIF2α, and teIF2α were quantified using ImageJ software. HO-1 and BiP are normalized to FG, and peIF2α is normalized to teIF2α (repeated measures two-way ANOVA followed by Dunnett's test, n = 3, *p < 0.05, ***p < 0.001 vs drug vehicle). Dashed lines represent untreated (UT) cultures.

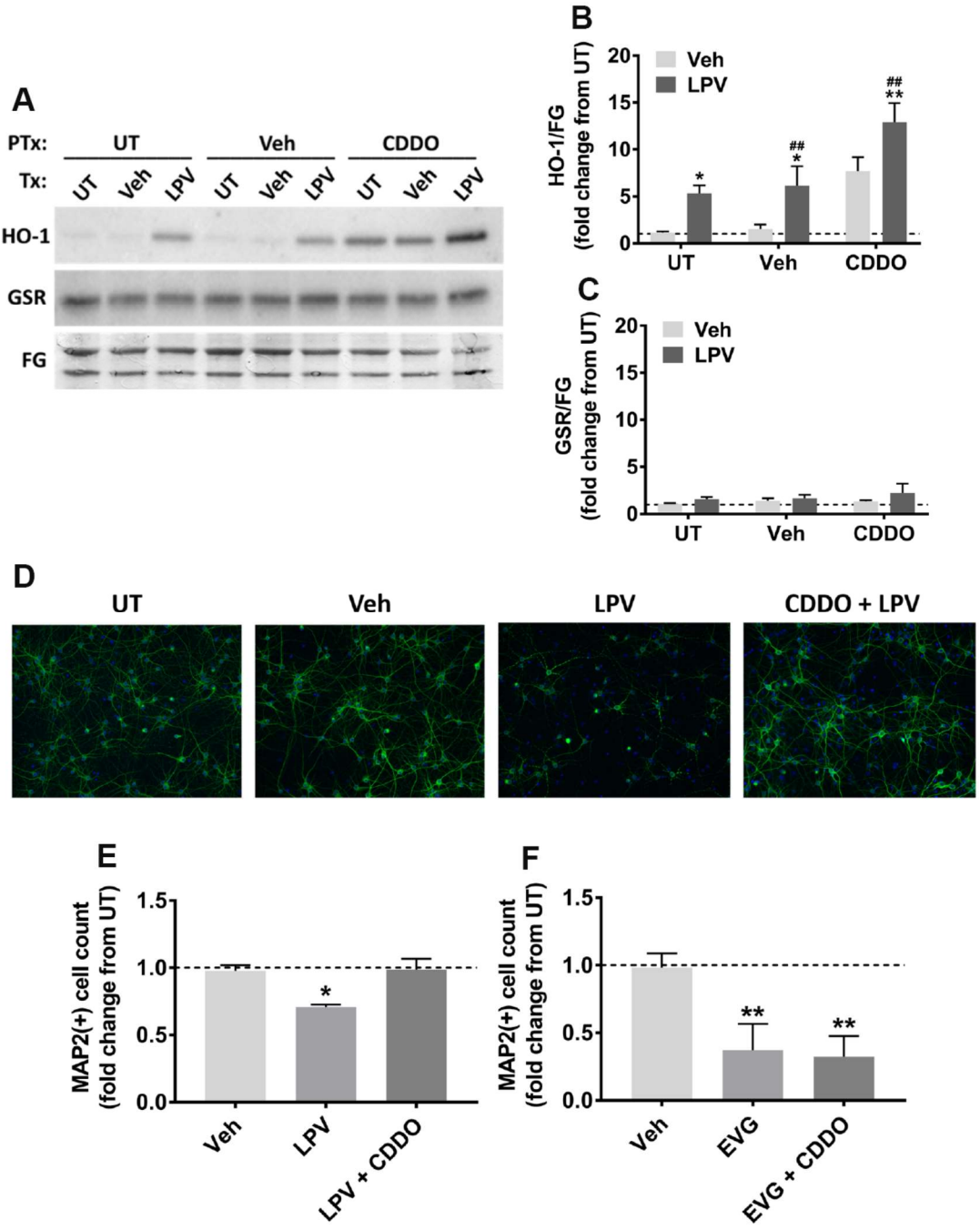
A
PTx:
UT
Veh
CDDO
Tx:
UT
Veh
LPV
HO-1
GSR
FG
B
HO-1/FG
(fold change from UT)
Veh
LPV
C
GSR/FG
(fold change from UT)
D
UT
Veh
LPV
CDDO + LPV
E
MAP2(+) cell count
(fold change from UT)
Veh
LPV
LPV + CDDO
F
MAP2(+) cell count
(fold change from UT)
Veh
EVG
EVG + CDDO

Figure 3-5. Pharmacological induction of HO-1 is protective against LPV-induced neurotoxicity

A) Rat cortical neuroglial cultures were pretreated with either DMSO vehicle or 0.1 μM CDDO for 1 h prior to 20 h treatment with DMSO vehicle or 10 μM LPV. Representative immunoblots are shown. GSR, glutathione reductase; FG, fast green loading control. **B–C)** HO-1 and GSR band intensities of were quantified using ImageJ software (repeated measures two-way ANOVA followed by Dunnett's test, n = 3, *p < 0.05, **p < 0.01 vs drug vehicle, ##p < 0.01 vs CDDO vehicle). **D)** Cultures were pretreated with either DMSO vehicle or 0.1 μM CDDO for 1 h prior to 48 h treatment with DMSO vehicle or 10 μM LPV. Representative images of LPV-treated cells immunostained for MAP2 (green) and DAPI (blue) are shown at 20× magnification. Scale bar represents 100 μM. **E)** Quantification of MAP2+ cells treated with indicated compounds is shown (repeated measures one-way ANOVA followed by Dunnett's test, n = 3, *p < 0.05 vs drug vehicle). **F)** Cultures were pretreated with DMSO vehicle or 0.1 μM CDDO for 1 h prior to 10 μM EVG treatment. After 48 h, cells were re-treated with DMSO vehicle or 10 μM EVG for another 48 h, followed by immunostaining. Quantification of MAP2+ cells treated with indicated compounds is shown (repeated measures one-way ANOVA followed by Dunnett's test, n = 3, **p < 0.01 vs drug vehicle).

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Overlapping Consequences of HIV and antiretrovirals

The studies presented in this dissertation suggest that both HIV itself and ARVs may contribute to impairments in neuronal health that underlie HAND symptoms. Among many proposed mechanisms by which HIV may indirectly affect neurons through macrophage infection, the pathway proposed herein implicates a critical role for BACE1 and APP cleavage. The mechanism of ARV-induced neurotoxicity investigated in this report involves ROS generation by protease inhibitor treatment, but this mechanism is not generalizable across ARVs.

The work presented here demonstrating a role for BACE1 in HIV-associated neurotoxicity is an extension of previous work in which the protease inhibitor ritonavir directly induced BACE1 in neurons as well (Gannon *et al.* 2017). In this study, BACE1-dependent increased amyloidogenic APP processing was also observed in response to ritonavir treatment, and pharmacological inhibition of BACE1 was neuroprotective. Hence, BACE1 is uniquely involved in mediating toxicity of both HIV and protease inhibitors, even in the absence of plaque

formation. Future studies are needed to determine if other ARVs also induce toxicity through BACE1-dependent pathways, but regardless it is promising as a potential therapeutic access point that could address multiple causes of neurotoxicity in HAND. The appeal of a BACE1 inhibition strategy is further highlighted by the success of BACE1 inhibitors thus far for the treatment of AD. Several brain-penetrant compounds with high specificity and low toxicity in animals and humans have been developed (Yan and Vassar 2014; Ghosh and Tang 2015); if any of these drugs are proven effective in AD treatment following the ongoing clinical trials, it will be particularly prudent to consider testing BACE1 inhibitors in animal models and potentially HAND patients as well.

In the second study of this dissertation, we showed that oxidative stress is mechanistically linked to neuronal damage caused by lopinavir treatment. This result is consistent with our previous report demonstrating a role for oxidative stress in both ritonavir- and saquinavir-induced neuronal damage (Akay *et al.* 2014), indicating that this may be a shared mechanism across protease inhibitors. Gannon et al. 2017 found that ritonavir also induced UPR activation that was required for BACE1 increase and neuron loss *in vitro*; thus, even a single drug may lead to neurotoxicity through multiple pathways. Indeed,

several reports have identified BACE1 upregulation in neurons due to increased ROS production (Tamagno *et al.* 2012b; Tamagno *et al.* 2008; Tamagno *et al.* 2002), and indeed one study found that both oxidative stress and the UPR were mechanistically involved in a single pathway regulating BACE1 levels (Mouton-Liger *et al.* 2012). Therefore, multiple cellular stress pathways initiated either by ARVs or HIV may be activated linearly or in parallel to converge on a similar downstream event, such as increased BACE1 and consequent neuronal loss.

Oxidative stress is likely to be another common mechanism by which HIV and ARVs additively or synergistically promote neurotoxicity. Infection of monocyte-derived macrophages by HIV increases ROS in the extracellular space (Sawada 2009; Olivetta *et al.* 2005; Olivetta *et al.* 2009), which is toxic to neurons *in vitro* (Viviani *et al.* 2001). Moreover, the virus initiates a positive feedback loop whereby ROS directly increases HIV replication (Nottet *et al.* 1994). Viral proteins gp120 and tat both increase ROS generation in brain endothelial and other cell types (Price *et al.* 2006; Toborek *et al.* 2003), and multiple reports have identified increased oxidative stress in the brains of HIV patients *post mortem* (Mollace *et al.* 2001). Because our study and others indicate that oxidative stress caused directly by ARVs can also be implicated in neuronal damage and death (Akay *et al.* 2014; Tricarico *et*

al. 2016; Shah *et al.* 2016), brain oxidative stress is likely to be induced simultaneously by both HIV and ARVs to result in increased neurotoxicity.

Under normal physiological conditions, cells can monitor and alleviate increases in oxidation by engaging the endogenous antioxidant response. This response is triggered by activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and subsequent transcription of its downstream antioxidant targets, including heme oxygenase 1 (HO-1) and glutathione reductase (GSR) (Lu *et al.* 2016). Elevation of HO-1, GSR, and other Nrf2 targets then reduces oxidant levels in the cell to maintain redox balance. Hence, in the present report, increasing HO-1 levels with CDDO was sufficient to prevent lopinavir-induced neurotoxicity associated with ROS increase. As an added complication in the oxidative stress component of toxicity, however, the ability of neuroglia to mount an endogenous antioxidant response may be compromised in the brains of HIV+ patients (Gill *et al.* 2014). Specifically, Gill et al found that viral replication in CNS macrophages correlated with decreased HO-1 expression both *in vivo* and *in vitro*, and in HAND patients, HO-1 deficiency was correlated with degree of cognitive impairment. Moreover, pharmacological manipulation of HO-1 levels in cultured macrophages was sufficient to alter glutamate content

and neurotoxicity of the conditioned media. These results in combination with the data presented in this report suggest that increased ROS in combination with decreased cellular capacity to manage oxidative stress may occur simultaneously in HIV+ patient brains to impair neuronal function.

In addition to disrupting glutamate balance through macrophage infection directly, both HIV and ARVs can alter astrocytic function. Excitatory amino acid transporter 2 (EAAT-2) expression is decreased in astrocytes following treatment with either lopinavir or amprenavir, resulting in increased extracellular glutamate (Vivithanaporn *et al.* 2016). Additionally, HIV-induced oxidative stress in astrocytes can lead to glutamate release through increased glutathione production as well as other mechanisms (Vázquez-Santiago *et al.* 2014; Markowitz *et al.* 2007). Lending further support to the proposal that HIV and ARVs act in concert to disrupt neuronal function, neurons exposed to ARVs may have heightened responses to elevated glutamate concentrations. Indeed, Robertson et al, 2012 found that certain prescribed combinations of ARVs caused neurons to have exaggerated calcium influx in response to glutamate. Altered glutamate response in addition to increased extracellular glutamate concentrations could therefore

combine synergistically to increase the likelihood of excitotoxic damage in HIV+ patient brains.

Given the likely contributions of both HIV and ARVs to HAND persistence, both high and low CNS penetrance of ARVs may be problematic depending on the particular drug and the patient viral load. Hence, scientists have the more complex goal of designing ARVs with low neurotoxic potential even in high concentrations. To this end, several new ARVs are being developed with special consideration for their function in the CNS. One avenue being pursued is nanomedicine, in which ARVs can be fused with small, lipophilic, brain-specific peptides that allow targeted CNS drug delivery. Nano-NRTIs in particular have shown recent promise in their ability to suppress brain viral load in animal models of HAND without causing the neurotoxic side effects associated with standard NRTIs (Gerson *et al.* 2014). However, clinicians should proceed with caution in the use of these drugs given that such high ARV concentrations in the CNS are unprecedented and low toxicity in animal models may not directly translate to the patient population.

Expanding upon the role of BACE1 and APP processing

Protease inhibitors induce BACE1 in neurons through ER stress and the UPR (Gannon *et al.* 2017), and herein we determined that HIV-infected macrophage supernatants and NMDAR activation induce neuronal BACE1 as well. Hence, BACE1 may provide an attractive therapeutic target for neuroprotection in HAND.

In order to further elucidate the role of BACE1 and Aβ oligomers in HAND, a detailed characterization in a larger cohort of HIV+ patients is warranted. Herein, we compared BACE1 expression in prefrontal cortex of two independent cohorts, and therefore can confidently conclude that BACE1 is increased in HIV+ patients vs. HIV- controls. We cannot, however, determine whether BACE1 is a feature of HAND or a feature of HIV serostatus regardless of cognitive impairment. BACE1 may increase susceptibility to neuronal damage and therefore play a role in HAND even if levels are not correlated with cognition, but an association between increased BACE1 and cognitive dysfunction would provide particularly strong rationale for targeting BACE1 in therapeutic interventions. Because we have also shown here that Aβ oligomers are increased in HIV+ brains and that APP processing is implicated in BACE1-mediated neurotoxicity *in vitro*, it will be critical to determine whether Aβ oligomers are increased in HAND patient brains vs.

neurocognitively normal controls. Ideally, a large and well-characterized cohort of patients across the cognitive spectrum should be used to identify differences in both BACE1 and Aβ oligomer expression between HIV-, HIV+ neurocognitively normal, ANI, MND, and HAD samples. Repetition of results will also be required as even a well-controlled cohort will include variability of cART regimen, duration of infection, and duration of treatment.

Our data strongly implicate a role for excitotoxic mechanisms in HAND, as has been suggested elsewhere (Lipton 2004; Cohen *et al.* 2015; Lindl *et al.* 2010). Although we showed that BACE1 upregulation is NMDA-dependent, further experiments are needed to address the specific NMDAR subunits involved. Typically, NMDARs are tetramers composed of at least one NR1 subunit and a combination of NR2A-D subunits or NR3A-B subunits, and subunit expression patterns differ according to both brain region and developmental stage. NR2A and NR2B receptors mediate neurotoxicity of HIV/MDMs (O'Donnell *et al.* 2006) and therefore are likely to mediate BACE1 upregulation. This prediction can be tested in future experiments by pretreating neurons with subunit specific inhibitors such as the NR2B antagonist ifendprodil, the NR2A/2B antagonist Conantokin-G, and the NR2A antagonist zinc.

Another important consideration regarding the role of NMDARs in our experiments is whether the receptors mediating toxicity are synaptic or extrasynaptic. Current evidence suggests that extrasynaptic NMDARS mediate excitotoxicity in neurodegenerative disease (Bading 2017), and consequently the most advantageous therapies should specifically target these receptors while leaving synaptic NMDARs relatively spared. Indeed, extrasynaptic NMDARs specifically promote Aβ oligomer formation, while synaptic NMDAR activity promotes non-amyloidogenic APP processing (Rush and Buisson 2014). Hence, we expect based on our observation of BACE1 increases following NMDA treatment that effects were primarily mediated extrasynaptically. This prediction may, however, prove difficult to test. The experiments described in this report were performed in cortical neurons, which typically express both NR2A and NR2B subunits across membrane substructures. Subunit selective inhibitors would not therefore provide information about synaptic localization. In hippocampal neurons, on the other hand, NR2B receptors are primarily extrasynaptic (Bading 2017). Hence, a simple way to simultaneously address subunit specificity, synaptic localization, and regional generalizability of our results would be to repeat a subset of experiments in hippocampal neurons with an additional experiment testing the effects of subtype specific NMDAR antagonists on BACE1

expression. Relevance of such an experiment is further highlighted by our observation of increased Aβ oligomers specifically in hippocampus of HIV+ patient brains.

Another remaining task is to clarify the mechanism of BACE1 upregulation in more detail. Although we showed here that increases in BACE1 protein expression were dependent on NMDAR activation, the mediator linking NMDAR activation with increased BACE1 is not known. Ritonavir-mediated BACE1 increases are blocked by inhibition of either PERK or protein kinase R (PKR) (Gannon *et al.* 2017), whereas efavirenz-mediated (Brown *et al.* 2014) and other BACE1 increases are dependent on oxidative stress (Mouton-Liger *et al.* 2012; Tamagno *et al.* 2008; Tamagno *et al.* 2012a). Cyclin-dependent kinase 5 (cdk5) (Sadleir and Vassar 2012), calpain (Liang *et al.* 2010; Dong *et al.* 2005), and Aβ oligomers themselves (Mamada *et al.* 2015) have also been implicated in mechanisms of BACE1 upregulation. One first step in identifying the mediator of BACE1 increases in our model specifically would be to determine whether HIV/MDM-induced changes in BACE1 are transcription- vs. translation-dependent. For instance, Gannon et al, 2017 showed that ritonavir-mediated BACE1 upregulation was dependent on translation, which was consistent with a mechanism involving the UPR. If translational machinery were required for BACE1

upregulation by HIV/MDMs or NMDA as well, we could assess the role of the UPR by treating neurons harvested from transgenic mice lacking PERK or other UPR signaling proteins. On the other hand, if inhibiting transcription was sufficient to prevent BACE1 upregulation by HIV/MDM or NDMA treatment, it would implicate a potential role for oxidative stress (Tamagno *et al.* 2012b). In this case, the specific mechanism could be probed by treating neurons with either free radical scavengers such as α-tocopherol (Tamagno *et al.* 2002) or compounds that activate the endogenous antioxidant response such as CDDO or monomethyl fumarate (MMF).

Another critical aspect of the toxic mechanism that has not been addressed here is the link between increased APP processing and neuron loss. This topic has plagued the AD research field for decades, and unfortunately the reasons for neurotoxicity caused by amyloidogenesis are mostly unknown (Yan and Vassar 2014). Both gain-of-function toxicity caused by Aβ oligomers (Sengupta *et al.* 2016) and loss-of-function toxicity resulting from decreased full-length APP at the synapse (Müller *et al.* 2017) remain plausible causes of neuronal dysfunction. However, due primarily to several failed clinical trials targeting Aβ species, many have proposed that neuron loss in AD is not related to APP processing (Castello *et al.* 2014) and may instead be driven by

another hallmark of AD, neurofibrillary tangles (Giacobini and Gold 2013). Importantly, although our study suggests that amyloidogenic APP processing is related to HIV-associated toxicity *in vitro*, lessons from AD research caution that mechanistic observations may not necessarily relate to HAND neuropathogenesis.

Animal models of HAND could provide a useful tool in determining whether BACE1- and APP-dependent mechanisms are relevant *in vivo*. One of the limitations of our *in vitro* system is that our primary measure of toxicity is frank neuronal loss, which is not observed in HAND in the current era (Gelman 2015). This caveat limits the potential generalizability of our findings to the more subtle damage observed in patients. Because HIV is a human-specific virus, animal models of HAND are difficult to develop and often involve multiple genetic manipulations; however, they are useful under specific circumstances (Honeycutt *et al.* 2015) and can provide powerful insights into the HIV-associated mechanisms that impact cognition. Some initial follow-up experiments, therefore, could test whether pharmacological BACE1 inhibition is able to improve memory or attention in these models. Additionally, further *in vitro* experiments could employ HIV/MDM or NMDA treatments titrated to low levels that induce synaptodendritic damage (Everall *et al.* 1999) without causing neuron loss. If blocking BACE1 activity prevents

damage under these conditions, it would further suggest a potential role for BACE1 in HAND.

Finally, a separate series of experiments could further explore the relevance of the dramatic decrease in ADAM10 we observed following NMDA treatment. ADAM10 and its cleavage product sAPPα are decreased in AD patients (Colciaghi *et al.* 2002); surprisingly though, other than identification of a role for ADAM10 in HIV-1 replication (Endsley *et al.* 2014), little attention has been paid to its potential relevance in HIV. Following our observation, we tested whether ADAM10 expression was altered in a small cohort of HIV+ vs. HIV- human samples of prefrontal cortex. ADAM10 was significantly decreased in HIV+ samples, but only in patients who had been taking cART for at least six months (data not shown). This result suggests that either A) ARVs alone are sufficient to affect ADAM10 expression through excitotoxic or other mechanisms, or B) both HIV+ serostatus and ARVs are needed in combination to decrease ADAM10. Regardless, a larger cohort would first be needed in order to verify that the effect we observed is a true feature of cART-treated HIV patients.

If human data suggest translational relevance of our ADAM10 findings, several *in vitro* experiments could be used to probe potential mechanisms linking NMDAR activation to ADAM10 regulation. One such

mechanism that has already been identified *in vitro* is a role for wnt/β-catenin signaling (Wan *et al.* 2012); however, this pathway was implicated in ADAM10 *upregulation* following a 90-minute treatment with higher NMDA doses, rather than the decrease we observed after 8-hour exposure. A more promising potential mediator of this decrease may be the UPR factor x-box binding protein 1 (XBP-1). NMDA signaling can variably affect ER stress and the UPR via changes in calcium influx (Prentice *et al.* 2015), and a canonical signaling protein in the UPR cascade is the transmembrane protein inositol-requiring enzyme 1 (IRE1), which in its activated form can splice XBP-1 mRNA to generate an active transcription factor. Spliced XBP-1 mRNA increases ADAM10 transcription *in vitro* and is correlated with ADAM10 in *post mortem* human brain tissue. Moreover, AD patient brains have decreased levels of both XBP-1 and ADAM10 in comparison with healthy age-matched controls (Reinhardt *et al.* 2014). This is in direct contrast with markers of additional UPR pathways such as PERK and activating transcription factor 6 (ATF6), which are increased in AD brain (Hoozemans *et al.* 2012).

Interestingly, a previous report from our lab suggests that XBP-1 may be implicated in HAND as well. Because it is difficult to assess mRNA levels in *post mortem* tissue, we instead reported changes in levels of

the upstream factor IRE1, which was nonsignificantly decreased in HIV+ individuals in comparison with HIV- controls (Akay *et al.* 2012). Lack of significance may have been due to the small cohort available, which only included four HIV- samples. These data suggest that HIV may cause downregulation of the IRE1 pathway, resulting in decreased XBP-1 and resultant depletion of ADAM10. Importantly, regulation of ADAM10 at the transcriptional level would be consistent with the time course of our observation that NMDA treatment does not affect protein levels until at least 8-hour exposure.

To test this prediction, experiments could first address whether a correlation exists between IRE1 or XBP-1 and ADAM10 expression in a larger cohort of HIV- and HIV+ patient brain samples. Causation could then be tested *in vitro* using both NMDA treatments and HIV/MDM treatments in primary neuronal cultures. If either siRNA knockdown or pharmacologic inhibition of IRE1 were able to prevent ADAM10 depletion by NMDAR activation, it would suggest that the IRE1 arm of the UPR at least has the potential to play a mechanistic role *in vivo*. However, ADAM10 expression is regulated by multiple pathways at the transcriptional, translational, and post-translational levels (Endres and Deller 2017), and several competing mechanisms may be at play downstream of NMDAR signaling.

A related critical pursuit would be to investigate whether loss of ADAM10 promotes neurotoxicity in HIV. Evidence suggests this may be the case, given that ADAM10 overexpression is protective against both plaque formation and memory deficits in AD mouse models (Schmitt *et al.* 2006; Postina *et al.* 2004). Functional consequences of ADAM10 loss could first be investigated by measuring sAPPα concentrations in the conditioned media of HIV/MDM- or NMDA-treated neurons. If treatments decrease sAPPα concentrations, it could suggest that loss of sAPPα contributes to toxicity in our model (Habib *et al.* 2017; Hefter and Draguhn 2017). This could be verified first by testing whether pharmacological inhibition of ADAM10 activity confers greater susceptibility to NMDA or HIV/MDM treatments in either wild type neurons or neurons lacking APP. If results demonstrate a toxicity-promoting effect of ADAM10 inhibition specifically in wild type but not APP-/- neurons, key follow-up experiments should test whether overexpression of sAPPα is neuroprotective. Manipulations of sAPPα concentration can ultimately then be tested for efficacy in animal models of HAND. Importantly, although a role for sAPPα would be scientifically interesting, BACE1 is a more attractive therapeutic target due to the myriad of relatively selective pharmacologic inhibitors that have already been developed and tested for safety (Ghosh and Tang 2015).

This dissertation has addressed two important debates in the field of HAND research and treatment: A) whether HAND persistence is primarily due to HIV or ARVs, and B) whether pathological features of HAND are similar enough to AD that it would be rational to pursue similar avenues for treatment of the two diseases. Regarding the first point, our data here add to a growing body of evidence that HAND is a multifactorial disease driven by both viral infiltration into the CNS as well as neurotoxic side effects of therapy. Regarding the second point, our data do suggest that BACE1 inhibitors similar to those being tested in AD may improve cognition in HAND. Although pathological findings are still unclear and HAND brains differ substantially from AD (Ortega and Ances 2014), BACE1 is an attractive therapeutic target regardless of the disease-specific amyloid pathology. We are hopeful that current clinical trials of BACE1 inhibitors in AD will yield positive results, and furthermore that these results along with the data presented herein will inspire further investigation into the potential benefits of targeting BACE1 in HAND.

REFERENCES

Abdulle S., Mellgren A., Brew B. J., Cinque P., Hagberg L., Price R. W., Rosengren L., Gisslén M. (2007) CSF neurofilament protein (NFL) -- a marker of active HIV-related neurodegeneration. *J. Neurol.* **254**, 1026–32.

Achim C. L., Adame A., Dumaop W., Everall I. P., Masliah E. (2009) Increased accumulation of intraneuronal amyloid beta in HIV-infected patients. *J. Neuroimmune Pharmacol.* **4**, 190–9.

Akay C., Cooper M., Odeleye A., Jensen B. K., White M. G., Vassoler F., Gannon P. J., et al. (2014) Antiretroviral drugs induce oxidative stress and neuronal damage in the central nervous system. *J. Neurovirol.* **20**, 39–53.

Akay C., Lindl K. A., Shyam N., Nabet B., Goenaga-Vazquez Y., Ruzbarsky J., Wang Y., Kolson D. L., Jordan-Sciutto K. L. (2012) Activation status of integrated stress response pathways in neurones and astrocytes of HIV-associated neurocognitive disorders (HAND) cortex. *Neuropathol. Appl. Neurobiol.* **38**, 175–200.

Akay C., Lindl K. A., Wang Y., White M. G., Isaacman-Beck J., Kolson D. L., Jordan-Sciutto K. L. (2011) Site-specific hyperphosphorylation of pRb in HIV-induced neurotoxicity. *Mol. Cell. Neurosci.* **47**, 154–165.

Almkvist O., Basun H., Wagner S. L., Rowe B. A., Wahlund L.-O., Lannfelt L. (1997) Cerebrospinal Fluid Levels of alpha-Secretase-Cleaved Soluble Amyloid Precursor Protein Mirror Cognition in a Swedish Family With Alzheimer Disease and a Gene Mutation. *Arch. Neurol.* **54**, 641–644.

Ances B. M., Benzinger T. L., Christensen J. J., Thomas J., Venkat R., Teshome M., Aldea P., et al. (2012) 11C-PiB imaging of human immunodeficiency virus-associated neurocognitive disorder. *Arch. Neurol.* **69**, 72–7.

Ances B. M., Christensen J. J., Teshome M., Taylor J., Xiong C., Aldea P., Fagan A. M., et al. (2010) Cognitively unimpaired HIV-positive subjects do not have increased 11C-PiB: a case-control study. *Neurology* **75**, 111–5.

Anderson E. R., Gendelman H. E., Xiong H. (2004) Memantine Protects Hippocampal Neuronal Function in Murine Human Immunodeficiency Virus Type 1 Encephalitis. *J. Neurosci.* **24**, 7194–7198.

André P., Groettrup M., Klenerman P., Giuli R. de, Booth B. L., Cerundolo V., Bonneville M., et al. (1998) An inhibitor of HIV-1 protease modulates proteasome activity, antigen presentation, and T cell responses. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13120–4.

Angel T. E., Jacobs J. M., Spudich S. S., Gritsenko M. A., Fuchs D.,

Liegler T., Zetterberg H., Camp D. G., Price R. W., Smith R. D. (2012) The cerebrospinal fluid proteome in HIV infection: change associated with disease severity. *Clin. Proteomics* **9**, 3.

Anthonypillai C., Sanderson R. N., Gibbs J. E., Thomas S. A. (2004) The Distribution of the HIV Protease Inhibitor, Ritonavir, to the Brain, Cerebrospinal Fluid, and Choroid Plexuses of the Guinea Pig. *J. Pharmacol. Exp. Ther.* **308**.

Antinori A., Arendt G., Becker J. T., Brew B. J., Byrd D. A., Cherner M., Clifford D. B., et al. (2007) Updated research nosology for HIV-associated neurocognitive disorders. *Neurology* **69**, 1789–1799.

Apostolova N., Blas-García A., Esplugues J. V (2011) Mitochondrial toxicity in HAART: an overview of in vitro evidence. *Curr. Pharm. Des.* **17**, 2130–44.

Aquaro S., Caliò R., Balzarini J., Bellocchi M. C., Garaci E., Perno C. F. (2002) Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. *Antiviral Res.* **55**, 209–25.

Arendt G., Nocker D. de, Giesen H.-J. von, Nolting T. (2007) Neuropsychiatric side effects of efavirenz therapy. *Expert Opin. Drug Saf.* **6**, 147–154.

Asensio V. C., Campbell I. L. (1999) Chemokines in the CNS: plurifunctional mediators in diverse states. *Trends Neurosci.* **22**,

504–12.

Avery L. B., Sacktor N., McArthur J. C., Hendrix C. W. (2013) Protein-free efavirenz concentrations in cerebrospinal fluid and blood plasma are equivalent: applying the law of mass action to predict protein-free drug concentration. *Antimicrob. Agents Chemother.* **57**, 1409–14.

Baburamani A. A., Ek C. J., Walker D. W., Castillo-Melendez M. (2012) Vulnerability of the developing brain to hypoxic-ischemic damage: contribution of the cerebral vasculature to injury and repair? *Front. Physiol.* **3**, 424.

Bading H. (2017) Therapeutic targeting of the pathological triad of extrasynaptic NMDA receptor signaling in neurodegenerations. *J. Exp. Med.*

Bae M., Patel N., Xu H., Lee M., Tominaga-Yamanaka K., Nath A., Geiger J., Gorospe M., Mattson M. P., Haughey N. J. (2014) Activation of TRPML1 clears intraneuronal Aβ in preclinical models of HIV infection. *J. Neurosci.* **34**, 11485–503.

Bernstein H.-G., Bukowska A., Krell D., Bogerts B., Ansorge S., Lendeckel U. (2003) Comparative localization of ADAMs 10 and 15 in human cerebral cortex normal aging, Alzheimer disease and Down syndrome. *J. Neurocytol.* **32**, 153–60.

Bertrand L., Nair M., Toborek M. (2016) Solving the Blood-Brain Barrier Challenge for the Effective Treatment of HIV Replication in the Central Nervous System. *Curr. Pharm. Des.* **22**, 5477–5486.

Bertrand L., Toborek M. (2015) Dysregulation of Endoplasmic Reticulum Stress and Autophagic Responses by the Antiretroviral Drug Efavirenz. *Mol. Pharmacol.* **88**, 304–315.

Best B. M., Letendre S. L., Brigid E., Clifford D. B., Collier A. C., Gelman B. B., McArthur J. C., et al. (2009) Low atazanavir concentrations in cerebrospinal fluid. *AIDS* **23**, 83–87.

Best B. M., Letendre S. L., Koopmans P., Rossi S. S., Clifford D. B., Collier A. C., Gelman B. B., et al. (2012) Low Cerebrospinal Fluid Concentrations of the Nucleotide HIV Reverse Transcriptase Inhibitor, Tenofovir. *JAIDS J. Acquir. Immune Defic. Syndr.* **59**, 376–381.

Bhatti A. B., Usman M., Kandi V. (2016) Current Scenario of HIV/AIDS, Treatment Options, and Major Challenges with Compliance to Antiretroviral Therapy. *Cureus* **8**, e515.

Blas-Garcia A., Polo M., Alegre F., Funes H. A., Martinez E., Apostolova N., Esplugues J. V. (2014) Lack of mitochondrial toxicity of darunavir, raltegravir and rilpivirine in neurons and hepatocytes: a comparison with efavirenz. *J. Antimicrob. Chemother.* **69**, 2995–

3000.

Blennow K., Mattsson N., Schöll M., Hansson O., Zetterberg H. (2015) Amyloid biomarkers in Alzheimer's disease. *Trends Pharmacol. Sci.* **36**, 297–309.

Boer M. G. J. de, Berk G. E. L. van den, Holten N. van, Oryszcyn J. E., Dorama W., Moha D. ait, Brinkman K. (2016) Intolerance of dolutegravir-containing combination antiretroviral therapy regimens in real-life clinical practice. *AIDS* **30**, 2831–2834.

Borjabad A., Volsky D. J. (2012) Common Transcriptional Signatures in Brain Tissue from Patients with HIV-Associated Neurocognitive Disorders, Alzheimer's Disease, and Multiple Sclerosis. *J. Neuroimmune Pharmacol.* **7**, 914–926.

Borsa M., Ferreira P. L. C., Petry A., Ferreira L. G. E., Camargo M. M., Bou-Habib D. C., Pinto A. R. (2015) HIV infection and antiretroviral therapy lead to unfolded protein response activation. *Virol. J.* **12**, 77.

Brenneman D. E., Westbrook G. L., Fitzgerald S. P., Ennist D. L., Elkins K. L., Ruff M. R., Pert C. B. (1988) Neuronal cell killing by the envelope protein of HIV and its prevention by vasoactive intestinal peptide. *Nature* **335**, 639–642.

Brew B. J., Pemberton L., Blennow K., Wallin a, Hagberg L. (2005) CSF

amyloid beta42 and tau levels correlate with AIDS dementia complex. *Neurology* **65**, 1490–1492.

Brodt H. R., Kamps B. S., Gute P., Knupp B., Staszewski S., Helm E. B. (1997) Changing incidence of AIDS-defining illnesses in the era of antiretroviral combination therapy. *AIDS* **11**, 1731–8.

Brown A. (2015) Understanding the MIND phenotype: macrophage/microglia inflammation in neurocognitive disorders related to human immunodeficiency virus infection. *Clin. Transl. Med.* **4**, 7.

Brown L. a M., Jin J., Ferrell D., Sadic E., Obregon D., Smith A. J., Tan J., Giunta B. (2014) Efavirenz promotes β-secretase expression and increased Aβ1-40,42 via oxidative stress and reduced microglial phagocytosis: Implications for HIV Associated Neurocognitive Disorders (HAND). *PLoS One* **9**, 1–9.

Canestri A., Lescure F.-X., Jaureguiberry S., Moulignier A., Amiel C., Marcelin A. G., Peytavin G., Tubiana R., Pialoux G., Katlama C. (2010) Discordance between cerebral spinal fluid and plasma HIV replication in patients with neurological symptoms who are receiving suppressive antiretroviral therapy. *Clin. Infect. Dis.* **50**, 773–8.

Caniglia E. C., Cain L. E., Justice A., Tate J., Logan R., Sabin C., Winston A., et al. (2014) Antiretroviral penetration into the CNS and

incidence of AIDS-defining neurologic conditions. *Neurology* **83**, 134–41.

Capetti A., Cossu M. V., Rizzardini G. (2015) Darunavir/cobicistat for the treatment of HIV-1: a new era for compact drugs with high genetic barrier to resistance. *Expert Opin. Pharmacother.* **16**, 2689–2702.

Carvajal F. J., Mattison H. A., Cerpa W. (2016) Role of NMDA Receptor-Mediated Glutamatergic Signaling in Chronic and Acute Neuropathologies. *Neural Plast.* **2016**, 1–20.

Carvalhal A., Gill M. J., Letendre S. L., Rachlis A., Bekele T., Raboud J., Burchell A., Rourke S. B., HIV/AIDS and the C. for B. H. in (2016) Central nervous system penetration effectiveness of antiretroviral drugs and neuropsychological impairment in the Ontario HIV Treatment Network Cohort Study. *J. Neurovirol.* **22**, 349–357.

Castello M. A., Jeppson J. D., Soriano S. (2014) Moving beyond anti-amyloid therapy for the prevention and treatment of Alzheimer's disease. *BMC Neurol.* **14**, 169.

Centers for Disease Control and Prevention (2016) *HIV Surveillance Report, 2015*.

Chang W.-P., Huang X., Downs D., Cirrito J. R., Koelsch G., Holtzman D. M., Ghosh A. K., Tang J. (2011) Beta-Secretase inhibitor GRL-8234 rescues age-related cognitive decline in APP transgenic mice.

FASEB J. **25**, 775–784.

Chen J. (2014) Heme oxygenase in neuroprotection: from mechanisms to therapeutic implications. *Rev. Neurosci.* **25**, 269–80.

Chen M. F., Gill A. J., Kolson D. L. (2014) Neuropathogenesis of HIV-associated neurocognitive disorders. *Curr. Opin. HIV AIDS* **9**, 559–564.

Chen W., Sulcove J., Frank I., Jaffer S., Ozdener H., Kolson D. L. (2002) Development of a human neuronal cell model for human immunodeficiency virus (HIV)-infected macrophage-induced neurotoxicity: apoptosis induced by HIV type 1 primary isolates and evidence for involvement of the Bcl-2/Bcl-xL-sensitive intrinsic apoptosis pa. *J. Virol.* **76**, 9407–19.

Chen X., Hui L., Geiger N. H., Haughey N. J., Geiger J. D. (2013) Endolysosome involvement in HIV-1 transactivator protein-induced neuronal amyloid beta production. *Neurobiol. Aging* **34**, 2370–2378.

Citron M., Oltersdorf T., Haass C., McConlogue L., Hung A. Y., Seubert P., Vigo-Pelfrey C., Lieberburg I., Selkoe D. J. (1992) Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production. *Nature* **360**, 672–674.

Clifford D. B., Evans S., Yang Y., Acosta E. P., Ribaudo H., Gulick R. M., A5097s Study Team (2009a) Long-term impact of efavirenz on

neuropsychological performance and symptoms in HIV-infected individuals (ACTG 5097s). *HIV Clin. Trials* **10**, 343–55.

Clifford D. B., Fagan A. M., Holtzman D. M., Morris J. C., Teshome M., Shah A. R., Kauwe J. S. K. (2009b) CSF biomarkers of Alzheimer disease in HIV-associated neurologic disease. *Neurology* **73**, 1982–7.

Cohen R. A., Seider T. R., Navia B. (2015) HIV effects on age-associated neurocognitive dysfunction: premature cognitive aging or neurodegenerative disease? *Alzheimers. Res. Ther.* **7**, 37.

Colciaghi F., Borroni B., Pastorino L., Marcello E., Zimmermann M., Cattabeni F., Padovani A., Luca M. Di (2002) [alpha]-Secretase ADAM10 as well as [alpha]APPs is reduced in platelets and CSF of Alzheimer disease patients. *Mol. Med.* **8**, 67–74.

Colebunders R., Hilbrands R., Roo A. De, Pelgrom J. (2002) Neuropsychiatric reaction induced by abacavir. *Am. J. Med.* **113**, 616.

Côté H. C. F., Brumme Z. L., Craib K. J. P., Alexander C. S., Wynhoven B., Ting L., Wong H., et al. (2002) Changes in Mitochondrial DNA as a Marker of Nucleoside Toxicity in HIV-Infected Patients. *N. Engl. J. Med.* **346**, 811–820.

Cottrell M. L., Hadzic T., Kashuba A. D. M. (2013) Clinical

pharmacokinetic, pharmacodynamic and drug-interaction profile of the integrase inhibitor dolutegravir. *Clin. Pharmacokinet.* **52**, 981–94.

Cousins S. L., Dai W., Stephenson F. A. (2015) APLP1 and APLP2, members of the APP family of proteins, behave similarly to APP in that they associate with NMDA receptors and enhance NMDA receptor surface expression. *J. Neurochem.* **133**, 879–885.

Cousins S. L., Hoey S. E. A., Anne Stephenson F., Perkinton M. S. (2009) Amyloid precursor protein 695 associates with assembled NR2A- and NR2B-containing NMDA receptors to result in the enhancement of their cell surface delivery. *J. Neurochem.* **111**, 1501–13.

Cross S. a, Cook D. R., Chi A. W. S., Vance P. J., Kolson L. L., Wong B. J., Jordan-Sciutto K. L., Kolson D. L. (2011) Dimethyl fumarate, an immune modulator and inducer of the antioxidant response, suppresses HIV replication and macrophage-mediated neurotoxicity: a novel candidate for HIV neuroprotection. *J. Immunol.* **187**, 5015–25.

Cunha J. da, Ferreira Maselli L. M., Treitinger A., Monteiro A. M., Gidlund M., Maranhão R. C., Spada C., Bydlowski S. P. (2013) Serum levels of IgG antibodies against oxidized LDL and atherogenic indices in HIV-1-infected patients treated with protease inhibitors. *Clin. Chem.*

Lab. Med. **51**, 371–8.

Cunningham P. H., Smith D. G., Satchell C., Cooper D. A., Brew B. (2000) Evidence for independent development of resistance to HIV-1 reverse transcriptase inhibitors in the cerebrospinal fluid. *AIDS* **14**, 1949–54.

Dalakas M. C. (2001) Peripheral neuropathy and antiretroviral drugs. *J. Peripher. Nerv. Syst.* **6**, 14–20.

Deeks E. D. (2014) Darunavir: A Review of Its Use in the Management of HIV-1 Infection. *Drugs* **74**, 99–125.

Devi L., Tang J., Ohno M. (2015) Beneficial effects of the β-secretase inhibitor GRL-8234 in 5XFAD Alzheimer's transgenic mice lessen during disease progression. *Curr. Alzheimer Res.* **12**, 13–21.

DHHS (2016a) Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents.

DHHS (2016b) Guidelines for the use of antiretroviral agents in pediatric infection.

Dickerson B., Wolk D. A., Alzheimer's Disease Neuroimaging Initiative (2013) Biomarker-based prediction of progression in MCI: comparison of AD signature and hippocampal volume with spinal fluid amyloid-β and tau. *Front. Aging Neurosci.* **5**, 55.

Dong Y., Tan J., Cui M.-Z., Zhao G., Mao G., Singh N., Xu X. (2005)

Calpain inhibitor MDL28170 modulates Aβ formation by inhibiting the formation of intermediate Aβ 46 and protecting Aβ from degradation.

Dore G. J., Correll P. K., Li Y., Kaldor J. M., Cooper D. A., Brew B. J. (1999) Changes to AIDS dementia complex in the era of highly active antiretroviral therapy. *AIDS* **13**, 1249–53.

Dreyer E. B., Kaiser P. K., Offermann J. T., Lipton S. A. (1990) HIV-1 coat protein neurotoxicity prevented by calcium channel antagonists. *Science* **248**, 364–7.

Edén A., Fuchs D., Hagberg L., Nilsson S., Spudich S., Svennerholm B., Price R. W., Gisslén M. (2010) HIV-1 Viral Escape in Cerebrospinal Fluid of Subjects on Suppressive Antiretroviral Treatment. *J. Infect. Dis.* **202**, 1819–1825.

Eiden C., Peyriere H., Peytavin G., Reynes J. (2011) Severe insomnia related to high concentrations of raltegravir. *AIDS* **25**, 725–7.

Eisfeld C., Reichelt D., Evers S., Husstedt I. (2013) CSF penetration by antiretroviral drugs. *CNS Drugs* **27**, 31–55.

Elias A., Nelson B., Oputiri D., Geoffrey O.-B. P. (2013) Antiretroviral toxicity and oxidative stress. *Am. J. Pharmacol. Toxicol.* **8**, 187–196.

Ellis R. J., Letendre S., Vaida F., Haubrich R., Heaton R. K., Sacktor N., Clifford D. B., et al. (2014) Randomized trial of central nervous

system-targeted antiretrovirals for HIV-associated neurocognitive disorder. *Clin. Infect. Dis.* **58**, 1015–22.

Ellis R., Letendre S. L. (2016) Update and New Directions in Therapeutics for Neurological Complications of HIV Infections. *Neurotherapeutics* **13**, 471–476.

Endres K., Deller T. (2017) Regulation of Alpha-Secretase ADAM10 In vitro and In vivo: Genetic, Epigenetic, and Protein-Based Mechanisms. *Front. Mol. Neurosci.* **10**, 56.

Endsley M. A., Somasunderam A. D., Li G., Oezguen N., Thiviyanathan V., Murray J. L., Rubin D. H., et al. (2014) Nuclear trafficking of the HIV-1 pre-integration complex depends on the ADAM10 intracellular domain. *Virology* **454**–**455**, 60–66.

Esiri M. M., Biddolph S. C., Morris C. S. (1998) Prevalence of Alzheimer plaques in AIDS. *J. Neurol. Neurosurg. Psychiatry* **65**, 29–33.

Etherton M. R., Lyons J. L., Ard K. L. (2015) HIV-associated Neurocognitive Disorders and Antiretroviral Therapy: Current Concepts and Controversies. *Curr. Infect. Dis. Rep.* **17**, 28.

Everall I. P., Heaton R. K., Marcotte T. D., Ellis R. J., McCutchan J. A., Atkinson J. H., Grant I., Mallory M., Masliah E. (1999) Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV

Neurobehavioral Research Center. *Brain Pathol.* **9**, 209–17.

Fagan A. M., Mintun M. A., Mach R. H., Lee S.-Y., Dence C. S., Shah A. R., LaRossa G. N., et al. (2006) Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid $A\beta_{42}$ in humans. *Ann. Neurol.* **59**, 512–519.

Fellgiebel A., Kojro E., Muller M. J., Scheurich A., Schmidt L. G., Fahrenholz F. (2009) CSF APPsα and Phosphorylated Tau Protein Levels in Mild Cognitive Impairment and Dementia of Alzheimer's Type. *J. Geriatr. Psychiatry Neurol.* **22**, 3–9.

Ferrando S., Gorp W. van, McElhiney M., Goggin K., Sewell M., Rabkin J. (1998) Highly active antiretroviral treatment in HIV infection: benefits for neuropsychological function. *AIDS* **12**, F65-70.

Ferrarese C., Aliprandi A., Tremolizzo L., Stanzani L., Micheli A. De, Dolara A., Frattola L. (2001) Increased glutamate in CSF and plasma of patients with HIV dementia. *Neurology* **57**, 671–5.

Ferrarese C., Riva R., Dolara A., Micheli A. De, Frattola L. (1997) Elevated Glutamate in the Cerebrospinal Fluid of Patients With HIV Dementia. *JAMA J. Am. Med. Assoc.* **277**, 630.

Festa L., Gutoskey C. J., Graziano A., Waterhouse B. D., Meucci O. (2015) Induction of Interleukin-1{beta} by Human Immunodeficiency Virus-1 Viral Proteins Leads to Increased Levels

of Neuronal Ferritin Heavy Chain, Synaptic Injury, and Deficits in Flexible Attention. *J. Neurosci.* **35**, 10550–10561.

Fischer-Smith T., Bell C., Croul S., Lewis M., Rappaport J. (2008) Monocyte/macrophage trafficking in acquired immunodeficiency syndrome encephalitis: lessons from human and nonhuman primate studies. *J. Neurovirol.* **14**, 318–26.

Fischl M. A., Richman D. D., Grieco M. H., Gottlieb M. S., Volberding P. A., Laskin O. L., Leedom J. M., et al. (1987) The Efficacy of Azidothymidine (AZT) in the Treatment of Patients with AIDS and AIDS-Related Complex. *N. Engl. J. Med.* **317**, 185–191.

Fontana G., Valenti L., Raiteri M. (1997) Gp120 can revert antagonism at the glycine site of NMDA receptors mediating GABA release from cultured hippocampal neurons. *J. Neurosci. Res.* **49**, 732–8.

Foster R., Taylor C., Everall I. P. (2004) More on abacavir-induced neuropsychiatric reactions. *AIDS* **18**, 2449.

Fukumoto H., Cheung B. S., Hyman B. T., Irizarry M. C. (2002) Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. *Arch. Neurol.* **59**, 1381–9.

Fukumoto H., Takahashi H., Tarui N., Matsui J., Tomita T., Hirode M., Sagayama M., et al. (2010) A Noncompetitive BACE1 Inhibitor TAK-070 Ameliorates A Pathology and Behavioral Deficits in a Mouse

Model of Alzheimer's Disease. *J. Neurosci.* **30**, 11157–11166.

Furukawa K., Mattson M. P. (1998) Secreted amyloid precursor protein alpha selectively suppresses N-methyl-D-aspartate currents in hippocampal neurons: involvement of cyclic GMP. *Neuroscience* **83**, 429–38.

Gannon P. J., Akay-Espinoza C., Yee A. C., Briand L. A., Erickson M. A., Gelman B. B., Gao Y., et al. (2017) HIV Protease Inhibitors Alter Amyloid Precursor Protein Processing via β-Site Amyloid Precursor Protein Cleaving Enzyme-1 Translational Up-Regulation. *Am. J. Pathol.* **187**, 91–109.

Gassart A. De, Bujisic B., Zaffalon L., Decosterd L. A., Micco A. Di, Frera G., Tallant R., Martinon F. (2016) An inhibitor of HIV-1 protease modulates constitutive eIF2α dephosphorylation to trigger a specific integrated stress response. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E117-26.

Gelman B. B. (2015) Neuropathology of HAND With Suppressive Antiretroviral Therapy: Encephalitis and Neurodegeneration Reconsidered. *Curr. HIV/AIDS Rep.* **12**, 272–279.

Gelman B. B., Schuenke K. (2004) Brain aging in acquired immunodeficiency syndrome: increased ubiquitin-protein conjugate is correlated with decreased synaptic protein but not amyloid plaque

accumulation. *J. Neurovirol.* **10**, 98–108.

Genis P., Jett M., Bernton E. W., Boyle T., Gelbard H. A., Dzenko K., Keane R. W., Resnick L., Mizrachi Y., Volsky D. J. (1992) Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interactions: implications for the neuropathogenesis of HIV disease. *J. Exp. Med.* **176**, 1703–18.

Gerson T., Makarov E., Senanayake T. H., Gorantla S., Poluektova L. Y., Vinogradov S. V. (2014) Nano-NRTIs demonstrate low neurotoxicity and high antiviral activity against HIV infection in the brain. *Nanomedicine Nanotechnology, Biol. Med.* **10**, 177–185.

Ghosh A. K., Tang J. (2015) Prospects of β-Secretase Inhibitors for the Treatment of Alzheimer's Disease. *ChemMedChem*.

Giacobini E., Gold G. (2013) Alzheimer disease therapy--moving from amyloid-β to tau. *Nat. Rev. Neurol.* **9**, 677–86.

Gill A. J., Kovacsics C. E., Cross S. a, Vance P. J., Kolson L. L., Jordan-sciutto K. L., Gelman B. B., Kolson D. L. (2014) Heme oxygenase-1 deficiency accompanies neuropathogenesis of HIV-associated neurocognitive disorders. **124**, 4459–4472.

Gisslén M., Price R. W., Nilsson S. (2011) The definition of HIV-associated neurocognitive disorders: are we overestimating the real

prevalence? *BMC Infect. Dis.* **11**, 356.

Giulian D., Vaca K., Noonan C. A. (1990) Secretion of neurotoxins by mononuclear phagocytes infected with HIV-1. *Science* **250**, 1593–6.

Giulian D., Wendt E., Vaca K., Noonan C. A. (1993) The envelope glycoprotein of human immunodeficiency virus type 1 stimulates release of neurotoxins from monocytes. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2769–73.

Grant I., Franklin D. R., Deutsch R., Woods S. P., Vaida F., Ellis R. J., Letendre S. L., et al. (2014a) Asymptomatic HIV-associated neurocognitive impairment increases risk for symptomatic decline. *Neurology* **82**, 2055–2062.

Grant I., Franklin D. R., Deutsch R., Woods S. P., Vaida F., Ellis R. J., Letendre S. L., et al. (2014b) Asymptomatic HIV-associated neurocognitive impairment increases risk for symptomatic decline. *Neurology* **82**, 2055–62.

Gray J., Young B. (2009) Acute onset insomnia associated with the initiation of raltegravir: a report of two cases and literature review. *AIDS Patient Care STDS* **23**, 689–90.

Green D. a, Masliah E., Vinters H. V, Beizai P., Moore D. J., Achim C. L. (2005) Brain deposition of beta-amyloid is a common pathologic

feature in HIV positive patients. *AIDS* **19**, 407–411.

Günthard H. F., Saag M. S., Benson C. A., Rio C. del, Eron J. J., Gallant J. E., Hoy J. F., et al. (2016) Antiretroviral Drugs for Treatment and Prevention of HIV Infection in Adults. *JAMA* **316**, 191.

Haass C., Selkoe D. J. (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide. *Nat. Rev. Mol. Cell Biol.* **8**, 101–112.

Habib A., Sawmiller D., Tan J. (2017) Restoring Soluble Amyloid Precursor Protein α Functions as a Potential Treatment for Alzheimer's Disease. *J. Neurosci. Res.* **95**, 973–991.

Hamel F. G., Fawcett J., Tsui B. T., Bennett R. G., Duckworth W. C. (2006) Effect of nelfinavir on insulin metabolism, proteasome activity and protein degradation in HepG2 cells. *Diabetes, Obes. Metab.* **8**, 661–668.

Han P., Dou F., Li F., Zhang X., Zhang Y.-W., Zheng H., Lipton S. A., Xu H., Liao F.-F. (2005) Suppression of cyclin-dependent kinase 5 activation by amyloid precursor protein: a novel excitoprotective mechanism involving modulation of tau phosphorylation. *J. Neurosci.* **25**, 11542–52.

Harper S. J., Bilsland J. G., Shearman M. S., Zheng H., Ploeg L. Van der, Sirinathsinghji D. J. (1998) Mouse cortical neurones lacking APP

show normal neurite outgrowth and survival responses in vitro. *Neuroreport* **9**, 3053–8.

Harris M., Larsen G., Montaner J. S. G. (2008) Exacerbation of depression associated with starting raltegravir: a report of four cases. *AIDS* **22**, 1890–2.

Heaton R. K., Franklin D. R., Ellis R. J., McCutchan J. A., Letendre S. L., Leblanc S., Corkran S. H., et al. (2011) HIV-associated neurocognitive disorders before and during the era of combination antiretroviral therapy: differences in rates, nature, and predictors. *J. Neurovirol.* **17**, 3–16.

Hefter D., Draguhn A. (2017) APP as a Protective Factor in Acute Neuronal Insults. *Front. Mol. Neurosci.* **10**, 22.

Hellmuth J., Milanini B., Valcour V. (2014) Interactions between ageing and NeuroAIDS. *Curr. Opin. HIV AIDS* **9**, 527–532.

Hesselgesser J., Taub D., Baskar P., Greenberg M., Hoxie J., Kolson D. L., Horuk R. (1998) Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4. *Curr. Biol.* **8**, 595–8.

Ho D. D., Rota T. R., Schooley R. T., Kaplan J. C., Allan J. D., Groopman J. E., Resnick L., Felsenstein D., Andrews C. A., Hirsch M. S. (1985) Isolation of HTLV-III from Cerebrospinal Fluid and Neural Tissues of

Patients with Neurologic Syndromes Related to the Acquired Immunodeficiency Syndrome. *N. Engl. J. Med.* **313**, 1493–1497.

Hoe H.-S., Fu Z., Makarova A., Lee J.-Y., Lu C., Feng L., Pajoohesh-Ganji A., et al. (2009) The Effects of Amyloid Precursor Protein on Postsynaptic Composition and Activity. *J. Biol. Chem.* **284**, 8495–8506.

Hoffmann C., Welz T., Sabranski M., Kolb M., Wolf E., Stellbrink H.-J., Wyen C. (2017) Higher rates of neuropsychiatric adverse events leading to dolutegravir discontinuation in women and older patients. *HIV Med.* **18**, 56–63.

Holsinger R. M. D., McLean C. A., Beyreuther K., Masters C. L., Evin G. (2002) Increased expression of the amyloid precursor ?-secretase in Alzheimer's disease. *Ann. Neurol.* **51**, 783–786.

Honeycutt J. B., Sheridan P. A., Matsushima G. K., Garcia J. V. (2015) Humanized mouse models for HIV-1 infection of the CNS. *J. Neurovirol.* **21**, 301–309.

Hoozemans J. J. M., Haastert E. S. van, Nijholt D. a T., Rozemuller A. J. M., Scheper W. (2012) Activation of the unfolded protein response is an early event in Alzheimer's and Parkinson's disease. *Neurodegener. Dis.* **10**, 212–5.

Innocent N., Cousins S. L., Stephenson F. A. (2012) *NMDA*

receptor/amyloid precursor protein interactions: A comparison between wild-type and amyloid precursor protein mutations associated with familial Alzheimer's disease.

Ivanov A. V., Valuev-Elliston V. T., Ivanova O. N., Kochetkov S. N., Starodubova E. S., Bartosch B., Isaguliants M. G. (2016) Oxidative Stress during HIV Infection: Mechanisms and Consequences. *Oxid. Med. Cell. Longev.* **2016**, 1–18.

Izycka-Swieszewska E., Zółtowska A., Rzepko R., Gross M., Borowska-Lehman J. (2000) Vasculopathy and amyloid beta reactivity in brains of patients with acquired immune deficiency (AIDS). *Folia Neuropathol.* **38**, 175–82.

Jiang Z. G., Piggee C., Heyes M. P., Murphy C., Quearry B., Bauer M., Zheng J., Gendelman H. E., Markey S. P. (2001) Glutamate is a mediator of neurotoxicity in secretions of activated HIV-1-infected macrophages. *J. Neuroimmunol.* **117**, 97–107.

Johnston J. A., Liu W. W., Todd S. A., Coulson D. T. R., Murphy S., Irvine G. B., Passmore A. P. (2005) Expression and activity of β-site amyloid precursor protein cleaving enzyme in Alzheimer's disease. *Biochem. Soc. Trans.* **33**.

Kamat A., Lyons J. L., Misra V., Uno H., Morgello S., Singer E. J., Gabuzda D. (2012) Monocyte Activation Markers in Cerebrospinal

Fluid Associated With Impaired Neurocognitive Testing in Advanced HIV Infection. *JAIDS J. Acquir. Immune Defic. Syndr.* **60**, 234–243.

Kamminga J., Bloch M., Vincent T., Carberry A., Brew B. J., Cysique L. A. (2017a) Determining optimal impairment rating methodology for a new HIV-associated neurocognitive disorder screening procedure. *J. Clin. Exp. Neuropsychol.*, 1–15.

Kamminga J., Cysique L. A., Lu G., Batchelor J., Brew B. J. (2013) Validity of Cognitive Screens for HIV-Associated Neurocognitive Disorder: A Systematic Review and an Informed Screen Selection Guide. *Curr. HIV/AIDS Rep.* **10**, 342–355.

Kamminga J., Lal L., Wright E. J., Bloch M., Brew B. J., Cysique L. A. (2017b) Monitoring HIV-Associated Neurocognitive Disorder Using Screenings: a Critical Review Including Guidelines for Clinical and Research Use. *Curr. HIV/AIDS Rep.*, 1–10.

Kandalepas P. C., Vassar R. (2014) The normal and pathologic roles of the Alzheimer's β-secretase, BACE1. *Curr. Alzheimer Res.* **11**, 441–9.

Kanters S., Vitoria M., Doherty M., Socias M. E., Ford N., Forrest J. I., Popoff E., et al. (2016) Comparative efficacy and safety of first-line antiretroviral therapy for the treatment of HIV infection: a systematic review and network meta-analysis. *Lancet HIV* **3**, e510–

e520.

Kaul M. (2008) HIV's double strike at the brain: neuronal toxicity and compromised neurogenesis. *Front. Biosci.* **13**, 2484–94.

Kaul M., Garden G. A., Lipton S. A. (2001) Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* **410**, 988–994.

Kaul M., Lipton S. A. (1999) Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8212–6.

Kaul M., Lipton S. A. (2006) Mechanisms of Neuroimmunity and Neurodegeneration Associated with HIV-1 Infection and AIDS. *J. Neuroimmune Pharmacol.* **1**, 138–151.

Kaul M., Zheng J., Okamoto S., Gendelman H. E., Lipton S. a (2005) HIV-1 infection and AIDS: consequences for the central nervous system. *Cell Death Differ.* **12 Suppl 1**, 878–892.

Kayed R., Lasagna-Reeves C. A. (2013) Molecular mechanisms of amyloid oligomers toxicity. *J. Alzheimers. Dis.* **33 Suppl 1**, S67-78.

Kheloufi F., Allemand J., Mokhtari S., Default A. (2015) Psychiatric disorders after starting dolutegravir. *AIDS* **29**, 1723–1725.

Kim D. Y., Carey B. W., Wang H., Ingano L. A. M., Binshtok A. M., Wertz M. H., Pettingell W. H., et al. (2007) BACE1 regulates voltage-gated sodium channels and neuronal activity. *Nat. Cell Biol.* **9**, 755–764.

Kim J., Yoon J.-H., Kim Y.-S. (2013) HIV-1 Tat Interacts with and Regulates the Localization and Processing of Amyloid Precursor Protein. *PLoS One* **8**, e77972.

Kocahan S., Doğan Z. (2017) Mechanisms of Alzheimer's Disease Pathogenesis and Prevention: The Brain, Neural Pathology, N-methyl-D-aspartate Receptors, Tau Protein and Other Risk Factors. *Clin. Psychopharmacol. Neurosci.* **15**, 1–8.

Koedel U., Kohleisen B., Sporer B., Lahrtz F., Ovod V., Fontana A., Erfle V., Pfister H. W. (1999) HIV type 1 Nef protein is a viral factor for leukocyte recruitment into the central nervous system. *J. Immunol.* **163**, 1237–45.

Koenig S., Gendelman H. E., Orenstein J. M., Dal Canto M. C., Pezeshkpour G. H., Yungbluth M., Janotta F., Aksamit A., Martin M. A., Fauci A. S. (1986) Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233**, 1089–93.

Kovalevich J., Langford D. (2012) Neuronal toxicity in HIV CNS disease. *Future Virol.* **7**, 687–698.

Krut J. J., Zetterberg H., Blennow K., Cinque P., Hagberg L., Price R. W., Studahl M., Gisslén M. (2013) Cerebrospinal fluid Alzheimer's biomarker profiles in CNS infections. *J. Neurol.* **260**, 620–626.

Lai D. J., Tarwater P. M., Hardy R. J. (2006) Measuring the impact of HIV/AIDS, heart disease and malignant neoplasms on life expectancy in the USA from 1987 to 2000. *Public Health* **120**, 486–92.

Lammich S., Kojro E., Postina R., Gilbert S., Pfeiffer R., Jasionowski M., Haass C., Fahrenholz F. (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3922–7.

Lannfelt L., Basun H., Wahlund L. O., Rowe B. A., Wagner S. L. (1995) Decreased alpha-secretase-cleaved amyloid precursor protein as a diagnostic marker for Alzheimer's disease. *Nat. Med.* **1**, 829–32.

Lannuzel A., Lledo P. M., Lamghitnia H. O., Vincent J. D., Tardieu M. (1995) HIV-1 envelope proteins gp120 and gp160 potentiate NMDA-induced [Ca2+]i increase, alter [Ca2+]i homeostasis and induce neurotoxicity in human embryonic neurons. *Eur. J. Neurosci.* **7**, 2285–93.

Larder B. A., Kemp S. D. (1989) Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155–8.

Lesné S., Ali C., Gabriel C., Croci N., MacKenzie E. T., Glabe C. G.,

Plotkine M., Marchand-Verrecchia C., Vivien D., Buisson A. (2005) NMDA Receptor Activation Inhibits α-Secretase and Promotes Neuronal Amyloid-β Production. *J. Neurosci.* **25**, 9367–9377.

Letendre S. L., Mills A. M., Tashima K. T., Thomas D. A., Min S. S., Chen S., Song I. H., Piscitelli S. C., extended ING116070 study team (2014) ING116070: A Study of the Pharmacokinetics and Antiviral Activity of Dolutegravir in Cerebrospinal Fluid in HIV-1-Infected, Antiretroviral Therapy-Naive Subjects. *Clin. Infect. Dis.* **59**, 1032–1037.

Letendre S., Marquie-Beck J., Capparelli E., Best B., Clifford D., Collier A. C., Gelman B. B., et al. (2008) Validation of the CNS Penetration-Effectiveness Rank for Quantifying Antiretroviral Penetration Into the Central Nervous System. *Arch. Neurol.* **65**, 65.

Levine A. J., Miller J. A., Shapshak P., Gelman B., Singer E. J., Hinkin C. H., Commins D., Morgello S., Grant I., Horvath S. (2013) Systems analysis of human brain gene expression: mechanisms for HIV-associated neurocognitive impairment and common pathways with Alzheimer's disease. *BMC Med. Genomics* **6**, 4.

Levine A. J., Soontornniyomkij V., Achim C. L., Masliah E., Gelman B. B., Sinsheimer J. S., Singer E. J., Moore D. J. (2016) Multilevel analysis of neuropathogenesis of neurocognitive impairment in HIV.

J. Neurovirol. **22**, 431–441.

Lewis W., Dalakas M. C. (1995) Mitochondrial toxicity of antiviral drugs. *Nat. Med.* **1**, 417–22.

Liang B., Duan B.-Y., Zhou X.-P., Gong J.-X., Luo Z.-G. (2010) Calpain activation promotes BACE1 expression, amyloid precursor protein processing, and amyloid plaque formation in a transgenic mouse model of Alzheimer disease. *J. Biol. Chem.* **285**, 27737–44.

Lindl K. A., Akay C., Wang Y., White M. G., Jordan-Sciutto K. L. (2007) Expression of the endoplasmic reticulum stress response marker, BiP, in the central nervous system of HIV-positive individuals. *Neuropathol. Appl. Neurobiol.* **33**, 658–69.

Lindl K. A., Marks D. R., Kolson D. L., Jordan-Sciutto K. L. (2010) HIV-Associated Neurocognitive Disorder: Pathogenesis and Therapeutic Opportunities. *J. Neuroimmune Pharmacol.* **5**, 294–309.

Lipton S. A. (2004) Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx* **1**, 101–10.

Lipton S. A., Rosenberg P. A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* **330**, 613–22.

Lu M.-C., Ji J.-A., Jiang Z.-Y., You Q.-D. (2016) The Keap1-Nrf2-ARE Pathway As a Potential Preventive and Therapeutic Target: An Update. *Med. Res. Rev.* **36**, 924–963.

Lyons J. L., Uno H., Ancuta P., Kamat A., Moore D. J., Singer E. J., Morgello S., Gabuzda D. (2011) Plasma sCD14 Is a Biomarker Associated With Impaired Neurocognitive Test Performance in Attention and Learning Domains in HIV Infection. *JAIDS J. Acquir. Immune Defic. Syndr.* **57**, 371–379.

Ma Q., Vaida F., Wong J., Sanders C. A., Kao Y., Croteau D., Clifford D. B., et al. (2016) Long-term efavirenz use is associated with worse neurocognitive functioning in HIV-infected patients. *J. Neurovirol.* **22**, 170–8.

MacLeod R., Hillert E.-K., Cameron R. T., Baillie G. S. (2015) The role and therapeutic targeting of α-, β- and γ-secretase in Alzheimer's disease. *Futur. Sci. OA* **1**, fso.15.9.

Mäkitalo S., Mellgren Å., Borgh E., Kilander L., Skillbäck T., Zetterberg H., Gisslén M. (2015) The cerebrospinal fluid biomarker profile in an HIV-infected subject with Alzheimer's disease. *AIDS Res. Ther.* **12**, 23.

Mamada N., Tanokashira D., Hosaka A., Kametani F., Tamaoka A., Araki W. (2015) Amyloid β-protein oligomers upregulate the β-secretase,

BACE1, through a post-translational mechanism involving its altered subcellular distribution in neurons. *Mol. Brain* **8**, 73.

Markowitz A. J. B., White M. G., Kolson D. L., Jordan-Sciutto K. L. (2007) Cellular interplay between neurons and glia: toward a comprehensive mechanism for excitotoxic neuronal loss in neurodegeneration. *Cellscience* **4**, 111–146.

Marra C. M., Zhao Y., Clifford D. B., Letendre S., Evans S., Henry K., Ellis R. J., et al. (2009) Impact of combination antiretroviral therapy on cerebrospinal fluid HIV RNA and neurocognitive performance. *AIDS* **23**, 1359–1366.

Masliah E., Roberts E. S., Langford D., Everall I., Crews L., Adame A., Rockenstein E., Fox H. S. (2004) Patterns of gene dysregulation in the frontal cortex of patients with HIV encephalitis. *J. Neuroimmunol.* **157**, 163–175.

McArthur J. C., Haughey N., Gartner S., Conant K., Pardo C., Nath A., Sacktor N. (2003) Human immunodeficiency virus-associated dementia: an evolving disease. *J. Neurovirol.* **9**, 205–21.

McConlogue L., Buttini M., Anderson J. P., Brigham E. F., Chen K. S., Freedman S. B., Games D., et al. (2007) Partial reduction of BACE1 has dramatic effects on Alzheimer plaque and synaptic pathology in APP Transgenic Mice. *J. Biol. Chem.* **282**, 26326–34.

Medders K. E., Sejbuk N. E., Maung R., Desai M. K., Kaul M. (2010) Activation of p38 MAPK Is Required in Monocytic and Neuronal Cells for HIV Glycoprotein 120-Induced Neurotoxicity. *J. Immunol.* **185**, 4883–4895.

Meehan R. A., Brush J. A. (2001) An overview of AIDS dementia complex. *Am. J. Alzheimers. Dis. Other Demen.* **16**, 225–229.

Mehta A., Prabhakar M., Kumar P., Deshmukh R., Sharma P. L. (2013) Excitotoxicity: Bridge to various triggers in neurodegenerative disorders. *Eur. J. Pharmacol.* **698**, 6–18.

Melkova Z., Shankaran P., Madlenakova M., Bodor J. (2017) Current views on HIV-1 latency, persistence, and cure. *Folia Microbiol. (Praha).* **62**, 73–87.

Meucci O., Fatatis A., Simen A. A., Bushell T. J., Gray P. W., Miller R. J. (1998) Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14500–5.

Milanini B., Catella S., Perkovich B., Esmaeili-Firidouni P., Wendelken L., Paul R., Greene M., Ketelle R., Valcour V. (2017) Psychiatric symptom burden in older people living with HIV with and without cognitive impairment: the UCSF HIV over 60 cohort study. *AIDS Care*, 1–8.

Mills A. M., Nelson M., Jayaweera D., Ruxrungtham K., Cassetti I., Girard

P.-M., Workman C., et al. (2009) Once-daily darunavir/ritonavir vs. lopinavir/ritonavir in treatment-naive, HIV-1-infected patients: 96-week analysis. *AIDS* **23**, 1679–1688.

Milosch N., Tanriöver G., Kundu A., Rami A., François J.-C., Baumkötter F., Weyer S. W., et al. (2014) Holo-APP and G-protein-mediated signaling are required for sAPPα-induced activation of the Akt survival pathway. *Cell Death Dis.* **5**, e1391.

Moidunny S., Matos M., Wesseling E., Banerjee S., Volsky D. J., Cunha R. A., Agostinho P., Boddeke H. W., Roy S. (2016) Oncostatin M promotes excitotoxicity by inhibiting glutamate uptake in astrocytes: implications in HIV-associated neurotoxicity. *J. Neuroinflammation* **13**, 144.

Mollace V., Muscoli C., Nottet H. S. L. M., Turco M. C., Salvemini D., Perno C. F. (2001) Oxidative stress and neuroAIDS: triggers, modulators, and novel antioxidants. *TRENDS Neurosci.* **24**.

Mollan K. R., Smurzynski M., Eron J. J., Daar E. S., Campbell T. B., Sax P. E., Gulick R. M., et al. (2014) Association between efavirenz as initial therapy for HIV-1 infection and increased risk for suicidal ideation or attempted or completed suicide: an analysis of trial data. *Ann. Intern. Med.* **161**, 1–10.

Morlese J. F., Qazi N. A., Gazzard B. G., Nelson M. R. (2002) Nevirapine-

induced neuropsychiatric complications, a class effect of non-nucleoside reverse transcriptase inhibitors? *AIDS* **16**, 1840–1.

Mouton-Liger F., Paquet C., Dumurgier J., Bouras C., Pradier L., Gray F., Hugon J. (2012) Oxidative stress increases BACE1 protein levels through activation of the PKR-eIF2α pathway. *Biochim. Biophys. Acta* **1822**, 885–96.

Mucke L., Abraham C. R., Ruppe M. D., Rockenstein E. M., Toggas S. M., Mallory M., Alford M., Masliah E. (1995) Protection against HIV-1 gp120-induced brain damage by neuronal expression of human amyloid precursor protein. *J. Exp. Med.* **181**, 1551–1556.

Müller U. C., Deller T., Korte M. (2017) Not just amyloid: physiological functions of the amyloid precursor protein family. *Nat. Rev. Neurosci.*

Munro K. M., Nash A., Pigoni M., Lichtenthaler S. F., Gunnersen J. M. (2016) Functions of the Alzheimer's Disease Protease BACE1 at the Synapse in the Central Nervous System. *J. Mol. Neurosci.* **60**, 305–315.

Mzingwane M. L., Tiemessen C. T. (2017) Mechanisms of HIV persistence in HIV reservoirs. *Rev. Med. Virol.* **27**, e1924.

Nakagawa S., Castro V., Toborek M. (2012) Infection of human pericytes by HIV-1 disrupts the integrity of the blood-brain barrier.

J. Cell. Mol. Med. **16**, 2950–2957.

Nakanishi N., Kang Y.-J., Tu S., McKercher S. R., Masliah E., Lipton S. A. (2016) Differential Effects of Pharmacologic and Genetic Modulation of NMDA Receptor Activity on HIV/gp120-Induced Neuronal Damage in an In Vivo Mouse Model. *J. Mol. Neurosci.* **58**, 59–65.

Navia B. A., Cho E.-S., Petito C. K., Price R. W. (1986a) The AIDS dementia complex: II. Neuropathology. *Ann. Neurol.* **19**, 525–535.

Navia B. A., Jordan B. D., Price R. W. (1986b) The AIDS dementia complex: I. Clinical features. *Ann. Neurol.* **19**, 517–524.

New D. R., Ma M., Epstein L. G., Nath A., Gelbard H. A. (1997) Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures. *J. Neurovirol.* **3**, 168–73.

Ngondi J. L., Oben J., Etame L. H., Forkah D. M., Mbanya D. (2006) The effect of different combination therapies on oxidative stress markers in HIV infected patients in Cameroon. *AIDS Res. Ther.* **3**, 19.

Nicoletti F., Bockaert J., Collingridge G. L., Conn P. J., Ferraguti F., Schoepp D. D., Wroblewski J. T., Pin J. P. (2011) Metabotropic glutamate receptors: from the workbench to the bedside. *Neuropharmacology* **60**, 1017–41.

Noguera-Julian A., Morén C., Rovira N., Garrabou G., Catalán M., Sánchez E., Cardellach F., Miró Ó., Fortuny C. (2015) Decreased Mitochondrial Function Among Healthy Infants Exposed to Antiretrovirals During Gestation, Delivery and the Neonatal Period. *Pediatr. Infect. Dis. J.* **34**, 1349–1354.

Nottet H. S., Asbeck B. S. van, Graaf L. de, Vos N. M. de, Visser M. R., Verhoef J. (1994) Role for oxygen radicals in self-sustained HIV-1 replication in monocyte-derived macrophages: enhanced HIV-1 replication by N-acetyl-L-cysteine. *J. Leukoc. Biol.* **56**, 702–7.

O'Connor T., Sadleir K. R., Maus E., Velliquette R. A., Zhao J., Cole S. L., Eimer W. A., et al. (2008) Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. *Neuron* **60**, 988–1009.

O'Donnell L. a, Agrawal A., Jordan-Sciutto K. L., Dichter M. a, Lynch D. R., Kolson D. L. (2006) Human immunodeficiency virus (HIV)-induced neurotoxicity: roles for the NMDA receptor subtypes. *J. Neurosci.* **26**, 981–990.

Obregon D., Hou H., Deng J., Giunta B., Tian J., Darlington D., Shahaduzzaman M., et al. (2012) Soluble amyloid precursor protein-α modulates β-secretase activity and amyloid-β generation. *Nat. Commun.* **3**, 777.

Ohno M., Sametsky E. A., Younkin L. H., Oakley H., Younkin S. G., Citron M., Vassar R., Disterhoft J. F. (2004) BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron* **41**, 27–33.

Olivetta E., Mallozzi C., Ruggieri V., Pietraforte D., Federico M., Sanchez M. (2009) HIV-1 Nef induces p47 ^{phox} phosphorylation leading to a rapid superoxide anion release from the U937 human monoblastic cell line. *J. Cell. Biochem.* **106**, 812–822.

Olivetta E., Pietraforte D., Schiavoni I., Minetti M., Federico M., Sanchez M. (2005) HIV-1 Nef regulates the release of superoxide anions from human macrophages. *Biochem. J.* **390**, 591–602.

Olsson A., Höglund K., Sjögren M., Andreasen N., Minthon L., Lannfelt L., Buerger K., et al. (2003) Measurement of alpha- and beta-secretase cleaved amyloid precursor protein in cerebrospinal fluid from Alzheimer patients. *Exp. Neurol.* **183**, 74–80.

Ortega M., Ances B. M. (2014) Role of HIV in Amyloid Metabolism. *J. Neuroimmune Pharmacol.* **9**, 483–491.

Pajonk F., Himmelsbach J., Riess K., Sommer A., McBride W. H. (2002) The human immunodeficiency virus (HIV)-1 protease inhibitor saquinavir inhibits proteasome function and causes apoptosis and radiosensitization in non-HIV-associated human cancer cells. *Cancer*

Res. **62**, 5230–5.

Palacin P. S., Aramburo A., Moraga F. A., Caba??as M. J., Figueras C. (2006) Neuropsychiatric Reaction Induced by Abacavir in a Pediatric Human Immunodeficiency Virus-Infected Patient. *Pediatr. Infect. Dis. J.* **25**, 382.

Panos S., Hinkin C. H., Singer E., Thames A., Patel S. M., Sinsheimer J. S., Re A. C. Del, et al. (2013) Apolipoprotein-E genotype and human immunodeficiency virus-associated neurocognitive disorder: the modulating effects of older age and disease severity. *Neurobehav. HIV Med.* **5**, 11.

Pegu A., Hessell A. J., Mascola J. R., Haigwood N. L. (2017) Use of broadly neutralizing antibodies for HIV-1 prevention. *Immunol. Rev.* **275**, 296–312.

Peluso M. J., Ferretti F., Peterson J., Lee E., Fuchs D., Boschini A., Gisslén M., et al. (2012) Cerebrospinal fluid HIV escape associated with progressive neurologic dysfunction in patients on antiretroviral therapy with well controlled plasma viral load. *AIDS* **26**, 1765–1774.

Peluso M. J., Meyerhoff D. J., Price R. W., Peterson J., Lee E., Young A. C., Walter R., et al. (2013) Cerebrospinal fluid and neuroimaging biomarker abnormalities suggest early neurological injury in a subset of individuals during primary HIV infection. *J. Infect. Dis.*

207, 1703–12.

Perez R. G., Zheng H., Ploeg L. H. T. Van der, Koo E. H. (1997) The β-Amyloid Precursor Protein of Alzheimer's Disease Enhances Neuron Viability and Modulates Neuronal Polarity. *J. Neurosci.* **17**.

Perrin R. J., Fagan A. M., Holtzman D. M. (2009) Multimodal techniques for diagnosis and prognosis of Alzheimer's disease. *Nature* **461**, 916–922.

Petito C. K., Cho E. S., Lemann W., Navia B. A., Price R. W. (1986) Neuropathology of acquired immunodeficiency syndrome (AIDS): an autopsy review. *J. Neuropathol. Exp. Neurol.* **45**, 635–46.

Podany A. T., Scarsi K. K., Fletcher C. V (2017) Comparative Clinical Pharmacokinetics and Pharmacodynamics of HIV-1 Integrase Strand Transfer Inhibitors. *Clin. Pharmacokinet.* **56**, 25–40.

Postina R., Schroeder A., Dewachter I., Bohl J., Schmitt U., Kojro E., Prinzen C., et al. (2004) A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. *J. Clin. Invest.* **113**, 1456–64.

Prentice H., Modi J. P., Wu J. (2015) Mechanisms of Neuronal Protection against Excitotoxicity , Endoplasmic Reticulum Stress , and Mitochondrial Dysfunction in Stroke and Neurodegenerative Diseases. *Oxid. Med. Cell. Longev.* **2015**.

Price R. W. (1996) Neurological complications of HIV infection. *Lancet* **348**, 445–452.

Price R. W., Yiannoutsos C. T., Clifford D. B., Zaborski L., Tselis A., Sidtis J. J., Cohen B., Hall C. D., Erice A., Henry K. (1999) Neurological outcomes in late HIV infection: adverse impact of neurological impairment on survival and protective effect of antiviral therapy. AIDS Clinical Trial Group and Neurological AIDS Research Consortium study team. *AIDS* **13**, 1677–85.

Price T. O., Uras F., Banks W. A., Ercal N. (2006) A novel antioxidant N-acetylcysteine amide prevents gp120-and Tat-induced oxidative stress in brain endothelial cells.

Purnell P. R., Fox H. S. (2014) Efavirenz Induces Neuronal Autophagy and Mitochondrial Alterations. *J. Pharmacol. Exp. Ther.* **351**, 250–258.

Raja F., Sherriff F. E., Morris C. S., Bridges L. R., Esiri M. M. (1997) Cerebral white matter damage in HIV infection demonstrated using beta-amyloid precursor protein immunoreactivity. *Acta Neuropathol.* **93**, 184–189.

Ramanathan S., Mathias A. A., German P., Kearney B. P. (2011) Clinical Pharmacokinetic and Pharmacodynamic Profile of the HIV Integrase Inhibitor Elvitegravir. *Clin. Pharmacokinet.* **50**, 229–244.

Rappaport J., Volsky D. J. (2015) Role of the macrophage in HIV-associated neurocognitive disorders and other comorbidities in patients on effective antiretroviral treatment. *J. Neurovirol.* **21**, 235–241.

Reinhardt S., Schuck F., Grosgen S., Riemenschneider M., Hartmann T., Postina R., Grimm M., Endres K. (2014) Unfolded protein response signaling by transcription factor XBP-1 regulates ADAM10 and is affected in Alzheimer's disease. *FASEB J.* **28**, 978–997.

Rempel H. C., Pulliam L. (2005) HIV-1 Tat inhibits neprilysin and elevates amyloid beta. *AIDS* **19**, 127–35.

Robertson K., Jiang H., Kumwenda J., Supparatpinyo K., Evans S., Campbell T. B., Price R., et al. (2012a) Improved neuropsychological and neurological functioning across three antiretroviral regimens in diverse resource-limited settings: AIDS Clinical Trials Group study a5199, the International Neurological Study. *Clin. Infect. Dis.* **55**, 868–76.

Robertson K., Liner J., Meeker R. B. (2012b) Antiretroviral neurotoxicity. *J. Neurovirol.* **18**, 388–399.

Robertson K. R., Su Z., Margolis D. M., Krambrink A., Havlir D. V., Evans S., Skiest D. J., A5170 Study Team (2010) Neurocognitive effects of treatment interruption in stable HIV-positive patients in an

observational cohort. *Neurology* **74**, 1260–1266.

Roe C. M., Fagan A. M., Grant E. A., Hassenstab J., Moulder K. L., Maue Dreyfus D., Sutphen C. L., et al. (2013) Amyloid imaging and CSF biomarkers in predicting cognitive impairment up to 7.5 years later. *Neurology* **80**, 1784–1791.

Rosca E. C., Rosca O., Simu M., Chirileanu R. D. (2012) HIV-associated Neurocognitive Disorders: a historical review. *Neurologist* **18**, 64–67.

Rush T., Buisson A. (2014) Reciprocal disruption of neuronal signaling and Aβ production mediated by extrasynaptic NMDA receptors: a downward spiral. *Cell Tissue Res.* **356**, 279–86.

Sacktor N., Lyles R. H., Skolasky R., Kleeberger C., Selnes O. a, Miller E. N., Becker J. T., Cohen B., McArthur J. C. (2001) HIV-associated neurologic disease incidence changes:: Multicenter AIDS Cohort Study, 1990-1998. *Neurology* **56**, 257–260.

Sacktor N., McDermott M. P., Marder K., Schifitto G., Selnes O. A., McArthur J. C., Stern Y., et al. (2002) HIV-associated cognitive impairment before and after the advent of combination therapy. *J. Neurovirol.* **8**, 136–142.

Sacktor N., Robertson K. (2014) Evolving clinical phenotypes in HIV-associated neurocognitive disorders. *Curr. Opin. HIV AIDS* **9**, 517–

520.

Sacktor N., Skolasky R. L., Seaberg E., Munro C., Becker J. T., Martin E., Ragin A., Levine A., Miller E. (2016) Prevalence of HIV-associated neurocognitive disorders in the Multicenter AIDS Cohort Study. *Neurology* **86**, 334–340.

Sadleir K. R., Vassar R. (2012) Cdk5 Protein Inhibition and A-beta42 Increase BACE1 Protein Level in Primary Neurons by a Post-transcriptional Mechanism: IMPLICATIONS OF CDK5 AS A THERAPEUTIC TARGET FOR ALZHEIMER DISEASE. *J. Biol. Chem.* **287**, 7224–7235.

Sanchez A., Kaul M. (2017) Neuronal Stress and Injury Caused by HIV-1, cART and Drug Abuse: Converging Contributions to HAND. *Brain Sci.* **7**, 25.

Saunders N. R., Liddelow S. A., Dziegielewska K. M. (2012) Barrier mechanisms in the developing brain. *Front. Pharmacol.* **3**, 46.

Sawada M. (2009) Neuroprotective and toxic changes in microglia in neurodegenerative disease. *Parkinsonism Relat. Disord.* **15**, S39–S41.

Saylor D., Dickens A. M., Sacktor N., Haughey N., Slusher B., Pletnikov M., Mankowski J. L., Brown A., Volsky D. J., McArthur J. C. (2016) HIV-associated neurocognitive disorder--pathogenesis and

prospects for treatment. *Nat. Rev. Neurol.* **12**, 234–48.

Schifitto G., Navia B. A., Yiannoutsos C. T., Marra C. M., Chang L., Ernst T., Jarvik J. G., et al. (2007) Memantine and HIV-associated cognitive impairment: a neuropsychological and proton magnetic resonance spectroscopy study. *AIDS* **21**, 1877–86.

Schmit J. C., Weber B. (1997) Recent advances in antiretroviral therapy and HIV infection monitoring. *Intervirology* **40**, 304–21.

Schmitt F. A., Bigley J. W., McKinnis R., Logue P. E., Evans R. W., Drucker J. L. (1988) Neuropsychological outcome of zidovudine (AZT) treatment of patients with AIDS and AIDS-related complex. *N. Engl. J. Med.* **319**, 1573–8.

Schmitt U., Hiemke C., Fahrenholz F., Schroeder A. (2006) Over-expression of two different forms of the α-secretase ADAM10 affects learning and memory in mice. *Behav. Brain Res.* **175**, 278–284.

Selkoe D. J., Wolfe M. S. (2000) In search of gamma-secretase: presenilin at the cutting edge. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5690–2.

Sengupta U., Nilson A. N., Kayed R. (2016) The Role of Amyloid-β Oligomers in Toxicity, Propagation, and Immunotherapy. *EBioMedicine* **6**, 42–9.

Shah A., Gangwani M. R., Chaudhari N. S., Glazyrin A. (2016)

Neurotoxicity in the Post-HAART Era : Caution for the Antiretroviral Therapeutics. *Neurotox. Res.*

Shin A., Kim H., Thayer S. (2012) Subtype selective NMDA receptor antagonists induce recovery of synapses lost following exposure to HIV-1 Tat. *Br. J. Pharmacol.* **166**, 1002–1017.

Singer O., Marr R. a, Rockenstein E., Crews L., Coufal N. G., Gage F. H., Verma I. M., Masliah E. (2005) Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. *Nat. Neurosci.* **8**, 1343–1349.

Singh H., Kaur M., Kakkar A. K., Kumar H. (2016a) The Promise of Dolutegravir: A Novel Second Generation Integrase Strand Transfer Inhibitor. *Curr. Clin. Pharmacol.* **11**, 88–94.

Singh M. V., Davidson D. C., Jackson J. W., Singh V. B., Silva J., Ramirez S. H., Maggirwar S. B. (2014) Characterization of Platelet–Monocyte Complexes in HIV-1–Infected Individuals: Possible Role in HIV-Associated Neuroinflammation. *J. Immunol.* **192**.

Singh V. B., Singh M. V., Gorantla S., Poluektova L. Y., Maggirwar S. B., Heaton R. K., McArthur J. C., et al. (2016b) Smoothened Agonist Reduces Human Immunodeficiency Virus Type-1-Induced Blood-Brain Barrier Breakdown in Humanized Mice. *Sci. Rep.* **6**, 26876.

Smurzynski M., Wu K., Letendre S., Robertson K., Bosch R. J., Clifford

D. B., Evans S., Collier A. C., Taylor M., Ellis R. (2011) Effects of central nervous system antiretroviral penetration on cognitive functioning in the ALLRT cohort. *AIDS* **25**, 357–365.

Song X., Hu Z., Zhang H. Acute dystonia induced by lamivudine. *Clin. Neuropharmacol.* **28**, 193–4.

Soontornniyomkij V., Moore D. J., Gouaux B., Soontornniyomkij B., Tatro E. T., Umlauf A., Masliah E., et al. (2012) Cerebral β-amyloid deposition predicts HIV-associated neurocognitive disorders in APOE ε4 carriers. *AIDS* **26**, 2327–2335.

Steinbrink F., Evers S., Buerke B., Young P., Arendt G., Koutsilieri E., Reichelt D., Lohmann H., Husstedt I.-W. (2013) Cognitive impairment in HIV infection is associated with MRI and CSF pattern of neurodegeneration. *Eur. J. Neurol.* **20**, 420–8.

Sutton J. S., Weiss L. (1966) Transformation of monocytes in tissue culture into macrophages, epithelioid cells, and multinucleated giant cells. An electron microscope study. *J. Cell Biol.* **28**, 303–32.

Szirony T. A. (1999) Infection with HIV in the elderly population. *J. Gerontol. Nurs.* **25**, 25–31.

Tamagno E., Bardini P., Obbili A., Vitali A., Borghi R., Zaccheo D., Pronzato M. a, et al. (2002) Oxidative stress increases expression and activity of BACE in NT2 neurons. *Neurobiol. Dis.* **10**, 279–288.

Tamagno E., Guglielmotto M., Aragno M., Borghi R., Autelli R., Giliberto L., Muraca G., et al. (2008) Oxidative stress activates a positive feedback between the γ- and β-secretase cleavages of the β-amyloid precursor protein. *J. Neurochem.* **104**, 683–695.

Tamagno E., Guglielmotto M., Monteleone D., Tabaton M. (2012a) Amyloid-β Production: Major Link Between Oxidative Stress and BACE1. *Neurotox. Res.* **22**, 208–219.

Tamagno E., Guglielmotto M., Monteleone D., Vercelli A., Tabaton M. (2012b) Transcriptional and post-transcriptional regulation of beta-secretase. *IUBMB Life* **64**, 943–950.

Tan I. L., Smith B. R., Hammond E., Vornbrock-Roosa H., Creighton J., Selnes O., McArthur J. C., Sacktor N. (2013) Older individuals with HIV infection have greater memory deficits than younger individuals. *J. Neurovirol.* **19**, 531–536.

Teeraananchai S., Kerr S., Amin J., Ruxrungtham K., Law M. (2016) Life expectancy of HIV-positive people after starting combination antiretroviral therapy: a meta-analysis. *HIV Med.*

Tiraboschi J. M., Knobel H., Imaz A., Villar J., Ferrer E., Saumoy M., González A., et al. (2015) Cerebrospinal fluid and plasma lopinavir concentrations and viral response in virologically suppressed patients switching to lopinavir/ritonavir monotherapy once daily.

Antivir. Ther. **21**, 359–363.

Toborek M., Lee Y. W., Pu H., Malecki A., Flora G., Garrido R., Hennig B., Bauer H.-C., Nath A. (2003) HIV-Tat protein induces oxidative and inflammatory pathways in brain endothelium. *J. Neurochem.* **84**, 169–79.

Tong Y., Zhou W., Fung V., Christensen M. A., Qing H., Sun X., Song W. (2004) Oxidative stress potentiates BACE1 gene expression and A? generation. *J. Neural Transm.* **112**, 455–469.

Tozzi V., Balestra P., Salvatori M. F., Vlassi C., Liuzzi G., Giancola M. L., Giulianelli M., Narciso P., Antinori A. (2009) Changes in cognition during antiretroviral therapy: comparison of 2 different ranking systems to measure antiretroviral drug efficacy on HIV-associated neurocognitive disorders. *J. Acquir. Immune Defic. Syndr.* **52**, 56–63.

Traynelis S. F., Wollmuth L. P., McBain C. J., Menniti F. S., Vance K. M., Ogden K. K., Hansen K. B., Yuan H., Myers S. J., Dingledine R. (2010) Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol. Rev.* **62**, 405–96.

Treisman G. J., Soudry O. (2016) Neuropsychiatric Effects of HIV Antiviral Medications. *Drug Saf.* **39**, 945–957.

Tricarico P. M., Oliveira Franca R. F. de, Pacor S., Ceglia V., Crovella S.,

Celsi F. (2016) HIV Protease Inhibitors Apoptotic Effect in SH-SY5Y Neuronal Cell Line. *Cell. Physiol. Biochem.* **39**, 1463–1470.

Trojanowski J. Q., Shin R. W., Schmidt M. L., Lee V. M. (1995) Relationship between plaques, tangles, and dystrophic processes in Alzheimer's disease. *Neurobiol. Aging* **16**, 335-40–5.

Troya J., Bascuñana J. (2016) Safety and Tolerability: Current Challenges to Antiretroviral Therapy for the Long-Term Management of HIV Infection. *AIDS Rev.* **18**, 127–137.

UNAIDS (2015) *AIDS by the numbers*.

Valcour V. G. (2013) HIV, aging, and cognition: emerging issues. *Top. Antivir. Med.* **21**, 119–23.

Vance D. E., Rubin L. H., Valcour V., Waldrop-Valverde D., Maki P. M. (2016) Aging and Neurocognitive Functioning in HIV-Infected Women: a Review of the Literature Involving the Women's Interagency HIV Study. *Curr. HIV/AIDS Rep.* **13**, 399–411.

Vázquez-Santiago F. J., Noel R. J., Porter J. T., Rivera-Amill V., Rivera-Amill V. (2014) Glutamate metabolism and HIV-associated neurocognitive disorders. *J. Neurovirol.* **20**, 315–31.

Verges D. K., Restivo J. L., Goebel W. D., Holtzman D. M., Cirrito J. R. (2011) Opposing synaptic regulation of amyloid-β metabolism by NMDA receptors in vivo. *J. Neurosci.* **31**, 11328–37.

Viviani B., Corsini E., Binaglia M., Galli C. ., Marinovich M. (2001) Reactive oxygen species generated by glia are responsible for neuron death induced by human immunodeficiency virus-glycoprotein 120 in vitro. *Neuroscience* **107**, 51–58.

Vivithanaporn P., Asahchop E. L., Acharjee S., Baker G. B., Power C. (2016) HIV protease inhibitors disrupt astrocytic glutamate transporter function and neurobehavioral performance. *AIDS* **30**, 543–552.

Wan X.-Z., Li B., Li Y.-C., Yang X.-L., Zhang W., Zhong L., Tang S.-J. (2012) Activation of NMDA Receptors Upregulates A Disintegrin and Metalloproteinase 10 via a Wnt/MAPK Signaling Pathway. *J. Neurosci.* **32**, 3910–3916.

Watson R. E., DeSesso J. M., Hurtt M. E., Cappon G. D. (2006) Postnatal growth and morphological development of the brain: a species comparison. *Birth Defects Res. Part B Dev. Reprod. Toxicol.* **77**, 471–484.

Weiß M., Kost B., Renner-Müller I., Wolf E., Mylonas I., Brüning A. (2016) Efavirenz Causes Oxidative Stress, Endoplasmic Reticulum Stress, and Autophagy in Endothelial Cells. *Cardiovasc. Toxicol.* **16**, 90–99.

Wendelken L. A., Jahanshad N., Rosen H. J., Busovaca E., Allen I.,

Coppola G., Adams C., et al. (2016) ApoE ε4 Is Associated With Cognition, Brain Integrity, and Atrophy in HIV Over Age 60. *JAIDS J. Acquir. Immune Defic. Syndr.* **73**, 426–432.

Wensing A. M. J., Maarseveen N. M. van, Nijhuis M. (2010) Fifteen years of HIV Protease Inhibitors: raising the barrier to resistance. *Antiviral Res.* **85**, 59–74.

White A. R., Multhaup G., Maher F., Bellingham S., Camakaris J., Zheng H., Bush A. I., Beyreuther K., Masters C. L., Cappai R. (1999) The Alzheimer's disease amyloid precursor protein modulates copper-induced toxicity and oxidative stress in primary neuronal cultures. *J. Neurosci.* **19**, 9170–9.

White A. R., Zheng H., Galatis D., Maher F., Hesse L., Multhaup G., Beyreuther K., Masters C. L., Cappai R. (1998) Survival of Cultured Neurons from Amyloid Precursor Protein Knock-Out Mice against Alzheimer's Amyloid-β Toxicity and Oxidative Stress. *J. Neurosci.* **18**.

WHO (2016a) Clinical Guidelines: Antiretroviral Therapy. Second Edition, 129.

WHO (2016b) Progress Report 2016. Prevent HIV,Test and Treat All. *Prog. Rep. 2016. Prev. HIV,Test Treat All*.

Wilcox K. S., Buchhalter J., Dichter M. A. (1994) Properties of inhibitory

and excitatory synapses between hippocampal neurons in very low density cultures. *Synapse* **18**, 128–151.

Wise M. E. J., Mistry K., Reid S. (2002) Drug points: Neuropsychiatric complications of nevirapine treatment. *BMJ* **324**, 879.

Xu J., Ikezu T. (2008) The Comorbidity of HIV-Associated Neurocognitive Disorders and Alzheimer's Disease: A Foreseeable Medical Challenge in Post-HAART Era. *J. Neuroimmune Pharmacol.* **4**, 200–212.

Yan R., Vassar R. (2014) Targeting the β secretase BACE1 for Alzheimer's disease therapy. *Lancet Neurol.* **13**, 319–329.

Yang L.-B., Lindholm K., Yan R., Citron M., Xia W., Yang X.-L., Beach T., et al. (2003) Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat. Med.* **9**, 3–4.

Yarchoan R., Berg G., Brouwers P., Fischl M. A., Spitzer A. R., Wichman A., Grafman J., Thomas R. V, Safai B., Brunetti A. (1987) Response of human-immunodeficiency-virus-associated neurological disease to 3'-azido-3'-deoxythymidine. *Lancet (London, England)* **1**, 132–5.

Yarchoan R., Klecker R. W., Weinhold K. J., Markham P. D., Lyerly H. K., Durack D. T., Gelmann E., Lehrman S. N., Blum R. M., Barry D. W. (1986) Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-

related complex. *Lancet (London, England)* **1**, 575–80.

Yates M. S., Tauchi M., Katsuoka F., Flanders K. C., Liby K. T., Honda T., Gribble G. W., et al. (2007) Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol. Cancer Ther.* **6**.

Yilmaz A., Gisslén M., Spudich S., Lee E., Jayewardene A., Aweeka F., Price R. W. (2009a) Raltegravir Cerebrospinal Fluid Concentrations in HIV-1 Infection. *PLoS One* **4**, e6877.

Yilmaz A., Izadkhashti A., Price R. W., Mallon P. W., Meulder M. De, Timmerman P., Gisslén M. (2009b) Darunavir Concentrations in Cerebrospinal Fluid and Blood in HIV-1–Infected Individuals. *AIDS Res. Hum. Retroviruses* **25**, 457–461.

Zhao Y., Navia B. A., Marra C. M., Singer E. J., Chang L., Berger J., Ellis R. J., et al. (2010) Memantine for AIDS Dementia Complex: Open-Label Report of ACTG 301. *HIV Clin. Trials* **11**, 59–67.

Zhou Y., Liu J., Xiong H. (2016) HIV-1 Glycoprotein 120 Enhancement of N-Methyl-D-Aspartate NMDA Receptor-Mediated Excitatory Postsynaptic Currents: Implications for HIV-1-Associated Neural Injury. *J. Neuroimmune Pharmacol.*

Zink W. E., Anderson E., Boyle J., Hock L., Rodriguez-Sierra J., Xiong H., Gendelman H. E., Persidsky Y. (2002) Impaired spatial cognition

and synaptic potentiation in a murine model of human immunodeficiency virus type 1 encephalitis. *J. Neurosci.* **22**, 2096–105.