Persistent "Hijacking" of Brain Proteasomes in HIV-Associated Dementia

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

October, 2008

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Publication No._____

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The University of Texas Medical Branch, 2008

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Human immunodeficiency virus-1 (HIV) infection of the central nervous system results in a syndrome of neuropsychological impairment, motor deficits, and behavioral changes diagnosed as HIV-associated dementia. Findings of increased ubiquitin-stained deposits and high molecular weight ubiquitin-protein conjugates in brains of HIV-positive subjects suggest impaired protein turnover by the ubiquitin-proteasome system analogous to neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. HIV infection of the brain and the consequent inflammatory response was hypothesized to alter the ubiquitin-proteasome system, leading to altered brain protein turnover and neuronal function. Investigations into the ubiquitin-proteasome system in HIV-infected brains were performed using the resources of the Texas NeuroAIDS Research Center and the National NeuroAIDS Tissue Consortium, including clinical data and frontal cortex brain tissue from 153 autopsy cases. Assessment of frontal cortex proteasome proteolytic activity revealed an abnormal catalytic profile that was more severe in those with HIV-associated neuropsychological impairment or HIV encephalitis.

Proteasome subunit composition analysis by immunoblotting showed increases in immunoproteasome subunits LMP7 and PA28 alpha and decreases in constitutive proteasome regulatory subunit Rpn2 in the frontal cortex of HIV-positive subjects that correlated with the abnormal proteasome proteinase profile and were associated with neuropsychological impairment and HIV encephalitis. Immunoproteasome increases correlated with lower performance on neurocognitive tests specific for frontal lobe functioning domains, providing indications of regional specificity. Immunoproteasome increases also correlated with increases in frontal cortex tissue HIV loads. Immunoproteasomes were localized by immunofluorescence to the perikarya and distal processes of neurons, as well as to oligodendrocytes, astrocytes, and microglia. The potential consequence of immunoproteasomes in neurons was investigated with the analysis of synaptic proteins. Decreases in synaptophysin correlated with immunoproteasome increases, indicating the potential for synaptic protein alterations associated with immunoproteasomes. Analysis of isolated nerve endings, or synaptosomes, revealed immunoproteasome increases in synapses of HIV-positive subjects that correlated with increases in 14-3-3 zeta and decreases in synapsin 1. These findings suggest "hijacking" of constitutive proteasomes by immunoproteasomes with the persistent inflammatory response in HIV-infected brains. Ubiquitin-mediated protein turnover by constitutive proteasomes is consequently impaired, resulting in the dysregulation of neuronal and synaptic protein composition that leads to neuronal dysfunction.

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Chapter 1: Introduction

1.1 HIV AND AIDS

The Human Immunodeficiency Virus (HIV) is a retrovirus that can destroy the cell-mediated immune response in those infected, leading to the development of Acquired Immunodeficiency Syndrome (AIDS). There are two genetically different, but related, versions of the virus: HIV-1, responsible for the largest number of cases worldwide, including the United States, Europe, and sub-Saharan Africa; and HIV-2, found primarily in western Africa. HIV is usually transmitted through sexual contact, parenteral inoculation, or from mother to child during birth.

HIV is roughly spherical in shape and consists of a phospholipid envelope and viral protein matrix surrounding a capsid containing two copies of positive-strand RNA, reverse transcriptase, integrase, and protease enzymes. HIV primarily infects monocytes, macrophages and T-cells through the binding of the viral surface glycoprotein gp120 to CD4 surface receptor proteins of the host cell and either the CCR5 (macrophage) or CXCR4 (T-cell) coreceptors, followed by a conformational change in glycoprotein gp41 triggering fusion of the viral envelope with the cellular plasma membrane. Complementary viral DNA is synthesized by the reverse transcriptase, transferred to the nucleus, and spliced into the host DNA via the integrase enzyme. The viral genome codes for three polyproteins: gag (group-specific antigen, coding for capsid proteins), pol (coding for polymerase, protease, and integrase), and env (envelope, coding for glycoproteins). Additional viral proteins (tat, rev, nef, vif, vpu, and vpr) serve to regulate viral synthesis and maturation. Binding of viral proteins and the viral genome to the plasma membrane with integrated viral glycoproteins triggers the budding of the membrane and release of the replicated virion.

The course of HIV infection generally occurs in three phases. Initially, there is a period of high levels of viral production, viremia, widespread seeding of lymphoid tissue and an abrupt drop in CD4+ T-cells accompanied by the development of an antiviral immune response and symptoms similar to influenza or mononucleosis infections. This subsides in two to three weeks, followed by a latent, asymptomatic period where blood viral loads decrease. However, viral replication continues in the lymph nodes, and a generalized lymphadenopathy may develop. Without treatment, the latent period may last for seven to ten years, after which there is an increase in viral loads, decreased CD4+ T-cell counts, and an overall deterioration of the immune response leading to susceptibility to opportunistic pathogens. The onset of AIDS occurs once the CD4+ T-cell count drops below 200/uL. Opportunistic infections commonly associated with AIDS include *Pneumocystis carinii* pneumonia, *Mycobacterium avium-intracellulare* complex, severe *Cytomegalovirus* disease and Kaposi's sarcoma.

Recent data (Hariri and McKenna 2007) show that approximately 1.2 million people in the United States are currently infected with HIV, 34 % of which have AIDS. Some 40,000 new cases of HIV infection are reported each year. With the development and implementation of antiretroviral therapies in the mid-1990s, death rates decreased from a peak of 17 per 100,000 in 1995 to 5 per 100,000 in 1998, where it has since stabilized.

1.2 HIV INFECTION OF THE BRAIN AND HIV-ASSOCIATED DEMENTIA

HIV enters the central nervous system soon after the initial systemic infection via infected peripheral monocytes that migrate across the blood brain barrier to replenish the perivascular macrophage population (Davis, Hjelle et al. 1992; An, Groves et al. 1999; Kaul, Garden et al. 2001; Gonzalez-Scarano and Martin-Garcia 2005). The infected monocytes provide the seed for further infection of brain macrophages and microglia, the

main reservoirs for HIV replication in the brain. HIV also infects a small proportion of astrocytes, but the infection is generally non-productive. Regulatory proteins nef and rev are expressed in infected astrocytes, but the lack of viral structural protein expression restricts viral assembly (Gorry, Ong et al. 2003). Research suggests that HIV can infect cultures of oligodendrocytes (Albright, Strizki et al. 1996) and neuronal cell lines (Mizrachi, Rodriguez et al. 1994; Ensoli, Cafaro et al. 1995) but evidence of infection *in vivo* has been contradictory and inconclusive (Kramer-Hammerle, Rothenaigner et al. 2005).

The neuropathophysiology underlying the cognitive decline in HIV-positive people is not completely understood. Research suggests both a "direct injury" hypothesis and an "indirect" hypothesis (Kaul and Lipton 2006). Experiments using neuroblastoma cell lines and neuronal cultures free of nonneuronal cells have shown that viral proteins such as gp120, Tat, and Vpr, are directly neurotoxic through interactions with chemokine or neurotransmitter receptors and dysregulation of excitatory amino acids and intracellular calcium concentrations (Nath and Geiger 1998). In addition, activated macrophages and microglial cells release inflammatory cytokines that may disrupt neuronal function by inducing apoptosis, myelin damage, reactive oxygen species formation or intracellular calcium dysregulation (Nath and Geiger 1998).

The resulting neurological syndrome, HIV-Associated Dementia (HAD), includes symptoms of impaired short term memory, reduced concentration, motor disabilities, and alterations in behavior (Navia and Price 1987; Janssen 1991). Those with less severe impairment are diagnosed with Minor Cognitive Motor Disorder (MCMD). The incidence of dementia has decreased since the introduction of Highly Active Antiretroviral Therapy (HAART), but the prevalence of neuropsychological impairment has actually increased as more people are living longer with HIV. Recent estimates for

HAD and MCMD prevalence were 10 % and 30 %, respectively (Kaul, Garden et al. 2001; McArthur 2004; Gonzalez-Scarano and Martin-Garcia 2005). Pathologically, severe HIV involvement in the brain is diagnosed as HIV encephalitis and includes histological findings of multinucleated giant cells, astrocytosis, myelin pallor, and microglial nodules (Nath and Geiger 1998; Kaul, Garden et al. 2001; Gonzalez-Scarano and Martin-Garcia 2005). The hallmark of HIV encephalitis is the multinucleated giant cell (Budka 1986), a syncytium of HIV-infected macrophages resulting from the fusion activity of the transmembrane glycoprotein gp41 (Perez, O'Donnell et al. 1992).

1.3 THE UBIQUITIN PROTEASOME SYSTEM

Adenosine triphosphate (ATP)-dependent protein degradation by the ubiquitin-proteasome system is responsible for the degradation of a majority of cellular proteins, including those required for regulation of normal cellular function (e.g., rate-limiting enzymes, transcriptional regulators, regulatory proteins) as well as damaged or misfolded proteins. Ubiquitin-mediated proteasomal protein degradation involves the formation of polyubiquitin chains attached to the protein substrate and proteolysis by the 26S proteasome complex, which contains a 20S proteasome core and 19S regulatory complexes at either end (Coux, Tanaka et al. 1996; Hochstrasser 1996; Hershko and Ciechanover 1998; Voges, Zwickl et al. 1999). Substrate ubiquitinylation is a multi-step process where ubiquitin is activated by an ATP-dependent activating enzyme E1, transferred to an ubiquitin-carrier protein E2, and ligated to specific protein substrates by an ubiquitin-protein ligase, which catalyzes the formation of an isopeptide bond between the C-terminus of ubiquitin and a lysine residue of the target protein (Hochstrasser 1996; Hershko and Ciechanover 1998). Repeated isopeptide linkages of ubiquitin molecules form a poly-ubiquitin chain that signals for 26S proteasome proteolysis of the target protein. The 19S regulatory complex of the 26S proteasome, composed of several

ATPase and other component subunits, recognizes and binds to the poly-ubiquitin chain (Coux, Tanaka et al. 1996; Voges, Zwickl et al. 1999). The 19S complex then unfolds and guides the protein through the 20S proteasome complex while cleaving off the poly-ubiquitin chain. The 20S proteasome consists of four seven-subunit rings forming a cylindrical core. The two outer rings are each composed of seven alpha subunits. They bind to the 19S regulatory complex and form the opening pores of the 20S proteasome complex. The two inner rings are each composed of seven beta subunits. Three beta subunits, X, Y, and Z, have post-glutamyl peptide hydrolyzing (cleaving after acidic residues), trypsin-like (cleaving after basic residues), and chymotrypsin-like (cleaving after hydrophobic residues) proteasome active sites, respectively. These active sites are contained within the 20S proteasome core, limiting proteolysis to only those substrates that enter through the alpha subunit ring pore via the 19S regulatory complex. Protein substrate hydrolysis yields short peptides and a polyubiquitin chain, which is dismantled by the ubiquitin c-terminal hydrolase to form free ubiquitin molecules for continued protein substrate targeting.

1.4 IMMUNOPROTEASOMES

The immunoproteasome is a variant of the constitutively expressed proteasome that is induced by interferon-gamma or tumor necrosis factor-alpha for heightened major histocompatibility complex (MHC) class I antigen presentation. The inflammatory cytokines increase the expression of inducible immunoproteasome subunits LMP7, LMP2 and MECL-1, which are then incorporated into newly synthesized immunoproteasome 20S core complexes to replace the constitutive subunits X, Y, and Z, respectively. The replacement of constitutive proteasome subunits with immunoproteasome subunits results in increases in chymotrypsin-like and trypsin-like activities and a decrease in post-glutamyl peptide hydrolyzing activity that optimizes the

production of appropriate antigenic peptides for MHC class I antigen presentation (Gaczynska, Rock et al. 1994; Groettrup, Khan et al. 2001). There is also a concomitant induction of the immunoproteasome 11S regulator composed of three PA28 alpha subunits and four PA28 beta subunits arranged in a heptameric ring that associates preferentially with immunoproteasomes in order to activate and enhance antigenic peptide production (Groettrup, Khan et al. 2001). Interferon-gamma induction of the immunoproteasome complex also results in a decrease in constitutive 26S proteasome complex (Bose, Brooks et al. 2001), suggesting that the induced immune response is "borrowing" the constitutive cellular proteolytic machinery to enhance antigen processing. Immunoproteasome induction is designed to be a temporary response, however, because the immunoproteasome complex lacks the full functionality of constitutive proteasomes necessary for cellular proteolytic requirements (Groettrup, Khan et al. 2001; Heink, Ludwig et al. 2005; Strehl, Seifert et al. 2005).

1.5 HIV AND THE UBIQUITIN PROTEASOME SYSTEM

Recent studies have analyzed the interaction between HIV, particularly the viral transactivator of transcription protein Tat, and proteasomal function. Tat was shown to inhibit both proteasome peptidase activity and the binding of the 11S regulatory complex to the 20S proteasome core in isolated human proteasome components (Seeger, Ferrell et al. 1997). The binding of Tat to numerous 20S proteasome subunits suggests that a direct interference of active sites and complex binding sites is possible (Huang, Seifert et al. 2002; Apcher, Heink et al. 2003). However, in lymphoblastoid cell lines designed to express Tat, immunoproteasome subunits MECL-1 and LMP7 were upregulated, and LMP2 was downregulated, resulting in an increase in all three proteinase activities (Gavioli, Gallerani et al. 2004). Further experimentation revealed that Tat repressed transcription of LMP2 by competing with the signal transducer and activator of

transcription 1 (STAT1) for binding to the interferon-regulatory factor-1 (IRF-1) in the interferon-gamma signaling pathway (Remoli, Marsili et al. 2006). However, this effect could not overcome interferon-gamma-induced expression of LMP2. In addition, the same study revealed that extracellular Tat actually induces LMP2 expression. In another study, HIV infection of monocyte-derived macrophages resulted in both decreased proteasome activity and proteasome and immunoproteasome content (Haorah, Heilman et al. 2004). Treatment with interferon-gamma was only able to partially restore proteasome content and activity. Together, these results demonstrate the potential for HIV-induced proteasomal alterations in infected cells. What effect HIV has *in vivo* on proteasomes in brain cells has yet to be determined.

1.6 THE UBIQUITIN PROTEASOME SYSTEM IN NEURODEGENERATIVE DISORDERS

Defective neuronal protein turnover by the ubiquitin-proteasome system is an underlying component of neurodegenerative disorders. The selective neurodegeneration in disease-specific brain regions is often associated with neuropathological finding of insoluble aggregates or inclusions that consist of abnormal or misfolded proteins (resulting from missense mutations, post-translational modifications, or damage), or amino acid repeat expansions that resist proteolytic degradation (Petrucelli and Dawson 2004; Ardley, Hung et al. 2005). Ubiquitin immunopositivity of the inclusions suggests that expression of aggregate-prone proteins exceeds the neuronal proteasome proteolytic capacity, leading to accumulation and aggregation of these proteins. Decreases in proteasome proteolytic activity, which could lead to aggregate formation, have been observed in several neurodegenerative diseases and in brain aging. Proteasome activity decreases could be due to interactions between proteasomes and the abnormal proteins and aggregates associated with neurodegenerative diseases. Loss of proteasome

effectivity, while inducing pathological protein aggregates, is also detrimental for cellular survival, considering the tight regulation of protein degradation necessary for proper cellular function (Keller and Markesbery 2000; Qiu, Asai et al. 2000). The common theme of protein aggregation and proteasome dysfunction in several neurodegenerative disorders suggests that (1) the ubiquitin-proteasome system is integral to neuronal homeostasis, and (2) impairment or alteration of the ubiquitin proteasome system can result in neurodegeneration and neurocognitive impairment. The evidence for involvement of the ubiquitin-proteasome system in neurodegenerative diseases is quite extensive and is summarized in more detail below.

1.6.1 Alzheimer's Disease

Alzheimer's disease is the most common neurodegenerative disorder and cause of dementia. Pathological signs of Alzheimer's disease include the loss of neurons and synapses, extracellular senile plaques containing beta-amyloid, and intracellular neurofibrillary tangles consisting of paired helical filaments of hyperphosphorylated tau protein. Analyses of the neurofibrillary tangles and senile plaques revealed the incorporation of ubiquitin (Mori, Kondo et al. 1987; Perry, Friedman et al. 1987; He, Delaere et al. 1993; Morishima-Kawashima, Hasegawa et al. 1993), which suggests a decreased capacity to effectively degrade these proteins by the ubiquitin-proteasome system. This is supported by findings of both decreased proteasome proteolytic activity (Keller, Hanni et al. 2000; Keck, Nitsch et al. 2003) and impairment of ubiquitin activating and conjugating enzymes to form ubiquitin-protein conjugates (Lopez Salon, Morelli et al. 2000) in Alzheimer's disease brain tissue. One potential factor in Alzheimer's disease that could contribute to the dysfunction of the ubiquitin proteasome system is the paired helical filament composed of polymerized hyperphosphorylated tau, which has been shown to inhibit proteasome activity (Keck, Nitsch et al. 2003). As well,

synthetic soluble peptide monomers of beta-amyloid has been shown to inhibit proteasome proteolytic activity (Gregori, Fuchs et al. 1995; Oh, Hong et al. 2005) and produce significant increases in ubiquitin-protein conjugates (Lopez Salon, Pasquini et al. 2003). Interestingly, studies have also discovered a frameshift mutant form of ubiquitin, UBB+1, in Alzheimer's disease. UBB+1 functions similarly to normal ubiquitin in forming polyubiquitin chains to mark substrates for degradation, but is refractory to disassembly by deubiquitinating enzymes, which limits degradation of the substrate and inhibits the ubiquitin-proteasome system (Lam, Pickart et al. 2000; Lindsten, de Vrij et al. 2002).

1.6.2 Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease and is characterized clinically by major motor difficulties such as bradykinesia, rigidity, tremors, postural instability and gait dysfunction. Key neuropathological findings in Parkinson's disease include a loss of dopaminergic neurons in substantia nigra pars compacta and the presence of eosinophilic intracytoplasmic inclusions known as Lewy bodies. Evidence of proteasomal inhibition in Parkinson's disease include reduced proteasome enzymatic activities in substantia nigra pars compacta (McNaught and Jenner 2001; McNaught, Belizaire et al. 2003), and decreased expression of proteasome alpha subunits and regulatory subunits that could alter proteasome assembly and stability (McNaught, Belizaire et al. 2002; McNaught, Belizaire et al. 2003). In animal studies, exposure to exogenous proteasome inhibitors by injections into either the substantia nigra or systemically resulted in parkinsonism, dopaminergic neuron degeneration and inclusion body formation (McNaught, Bjorklund et al. 2002; McNaught, Perl et al. 2004). Abnormal proteins associated with familial forms of Parkinson's disease, including alpha-synuclein, parkin, and ubiquitin carboxy-

terminal hydrolase L1 (UCH-L1), are all involved with proteasome inhibition. Alpha-synuclein is a presynaptic terminal protein of unknown function that accumulates in Lewy bodies along with ubiquitin. Alpha-synuclein have been shown to bind to proteasome subunits and decrease proteasomal activity (Snyder, Mensah et al. 2003; Lindersson, Beedholm et al. 2004), which would promote buildup of alpha-synuclein and perpetuate its effects on proteasome inhibition. Ubiquitinylation of alpha-synuclein is not required for inclusion formation, but may represent attempts by the impaired ubiquitin-proteasome system to clear the inclusions (Sampathu, Giasson et al. 2003). Parkin can also be found in Lewy bodies of both sporadic and familial Parkinson's disease (Schlossmacher, Frosch et al. 2002). Parkin is an E2-dependent ubiquitin-protein ligase (E3) that is mutated in autosomal-recessive juvenile Parkinson's disease, resulting in the loss of ubiquitinylation activity (Shimura, Hattori et al. 2000). Glycosylated alpha-synuclein was identified as a parkin substrate that accumulates in juvenile Parkinson's disease due to the parkin loss-of-function mutation and the resultant decrease in ubiquitin-dependent degradation (Shimura, Schlossmacher et al. 2001). UCH-L1, the major neuronal deubiquitinylating enzyme, is found in Lewy bodies as well (Lowe, McDermott et al. 1990). Two genetic species are of interest. The I93M UCH-L1 mutation results in decreased hydrolase activity and produces autosomal dominant Parkinson's disease. The S18Y UCH-L1 polymorphism is associated with decreased susceptibility to Parkinson's disease. I93M transfection resulted in an increase in the number of cells with aggresome-like inclusions containing UCH-L1, ubiquitinated proteins, and alpha-synuclein. This was enhanced by proteasome inhibition. Inclusion formation was reduced with S18Y cotransfection, supporting the hypotheses of the protective nature of the S18Y polymorphism in decreasing susceptibility to Parkinson's disease (Ardley, Scott et al. 2004).

1.6.3 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis is a paralytic neurodegenerative disease with selective degeneration of motor neurons in the spinal cord, brainstem, and motor cortex. The majority of cases are considered sporadic with an unknown etiology, while a subset of cases are familial and linked to a genetic mutation in copper/zinc superoxide dismutase (SOD1). Common to both sporadic and familial cases of Amyotrophic Lateral Sclerosis are proteinaceous cytosolic aggregates containing ubiquitinylated proteins. These ubiquitinylated protein aggregates are found in the surviving motor neurons, suggesting an association with dysfunction of the neuronal ubiquitin proteasome system (Leigh, Whitwell et al. 1991; Watanabe, Dykes-Hoberg et al. 2001; Cheroni, Peviani et al. 2005; Mendonca, Chimelli et al. 2006). The animal models of Amyotrophic Lateral Sclerosis based on the familial SOD1 mutations contain ubiquitinated inclusion bodies that are immunopositive for SOD1 (Tu, Raju et al. 1996; Kato, Horiuchi et al. 2000), which is the major component of these aggregates, and a copper chaperone for SOD1 (CSS) (Watanabe, Dykes-Hoberg et al. 2001). The findings in familial Amyotrophic Lateral Sclerosis animal models that describe decreases in proteasome enzymatic activity (Urushitani, Kurisu et al. 2002; Kabashi, Agar et al. 2004) and show that proteasome inhibition coincides with mutant SOD1 accumulation and aggregation (Johnston, Dalton et al. 2000; Urushitani, Kurisu et al. 2002; Puttaparthi, Wojcik et al. 2003) implicate the dynamic involvement of proteasome function in Amyotrophic Lateral Sclerosis.

1.6.4 Huntington's Disease

Huntington's disease is an autosomal dominant neurodegenerative disorder linked to the abnormal expansion of an N-terminal polyglutamine region in the huntingtin gene. Pathological findings include atrophy and loss of neurons in the striatum and, in later stages, the cerebral cortex. Within the surviving neurons are neuronal intranuclear

inclusions containing fragments of the mutant huntingtin protein. These huntingtin aggregates have been shown to be immunopositive for ubiquitin (DiFiglia, Sapp et al. 1997; Sieradzan, Mechan et al. 1999), and are present even in presymptomatic cases of gene carriers (Gomez-Tortosa, MacDonald et al. 2001). Researchers have shown that proteasome components are incorporated into these inclusions as well (Jana, Zemskov et al. 2001). These researchers also demonstrated impaired proteasome degradation of huntingtin with increased polyglutamine length, which they suggested would hinder the ability of the protease to degrade other protein substrates (Jana, Zemskov et al. 2001). Indeed, studies have shown that ubiquitinated huntingtin extracted from neuronal intranuclear inclusions can interact with and inhibit proteasomes (Diaz-Hernandez, Valera et al. 2006), which could produce the inhibited proteasome activity observed in post-mortem brain tissue from both early and late stage Huntington's disease (Seo, Sonntag et al. 2004). The mutant ubiquitin UBB+1 described in Alzheimer's disease has also been found in the neuronal intranuclear inclusions and cytoplasm of neurons in Huntington's disease (de Pril, Fischer et al. 2004), suggesting a common mechanism of inhibition of the ubiquitin-proteasome system in these neurodegenerative diseases.

1.6.5 Brain Aging

Alterations in central nervous system proteasome function and expression have been shown to occur with aging and may contribute to the aging process (Keller, Gee et al. 2002; Keller, Dimayuga et al. 2004). In animal studies, proteasome activity decreases with increasing age in various organs and tissue types, and is more prominent in the central nervous system, especially in the spinal cord, hippocampus, and cerebral cortex (Keller, Hanni et al. 2000). Oxidative stress plays a pivotal role in the aging process and may also account for the detrimental modification of proteasome activity (Keller, Hanni et al. 2000). As well, decreased proteasome expression in the central nervous system is

seen with aging, which would also impair proteolytic activity (Keller, Huang et al. 2000). The potential consequences of proteasomal dysfunction were observed in studies analyzing brain tissue sections of aged subjects. These studies have consistently shown increases in ubiquitinylated deposits compared to younger subjects (Pappolla, Omar et al. 1989; Migheli, Attanasio et al. 1992).

1.7 ALTERED PROTEASOMAL FUNCTION IN THE HIV-INFECTED BRAIN

Increases in ubiquitin-protein conjugates were observed in the brains of HIV/AIDS patients (Gelman and Schuenke 2004). Significant accumulations of ubiquitin-stained deposits were detected in AIDS brains via immunohistochemistry. These deposits were increased compared to age-matched HIV-negative subjects, and were similar to those that appear during brain aging. Confirmatory immunoblotting revealed increased high molecular weight ubiquitin conjugates in AIDS brain samples. These changes coincided with increased markers of inflammation and decreased synaptic proteins synaptophysin and growth associated protein-43. These findings suggested that neuronal protein turnover and the ubiquitin-proteasome system are impaired in AIDS brains, possibly due to HIV infection and viral protein release and/or the resulting chronic inflammatory stress induced by the viral infection. The effect of HIV infection in the brain may also act in conjunction with the effect of aging on proteasome proteolytic capacity or with the pathophysiology of other neurodegenerative disorders, leading to further inhibition of protein degradation and accumulation of ubiquitin-protein aggregates.

1.8 HYPOTHESIS AND EXPERIMENTAL DESIGN

The hypothesis is that HIV infection of the brain and the consequent persistent inflammatory response results in alterations to the ubiquitin-proteasome system that

impair brain protein turnover necessary for cellular function. This would lead to the accumulation of ubiquitinated proteins and ultimately to the neuronal dysfunction underlying HIV-associated neuropsychological impairment.

To test this hypothesis, I analyzed the proteasome in frozen human frontal cortex brain tissue specimens from HIV-negative and HIV-positive subjects using the resources of the National NeuroAIDS Tissue Consortium. The analysis addressed four specific aims:

Specific Aim 1: Proteasome enzymatic activity and subunit composition. To characterize the differences in proteolytic activities found with HIV infection of the brain, I first measured the proteasome chymotrypsin-like, post-glutamyl peptide hydrolyzing, and trypsin-like activities. Those results revealed an altered proteolytic profile suggestive of a difference in proteasome subunit active sites that was associated with neuropsychological impairment and neuropathology. I then analyzed specific subunits using immunoblotting to screen for changes in proteasome complex composition that could account for changes in proteasome enzymatic activity, and discovered an increase in immunoproteasome subunit expression in HIV-positive subjects. The subunit expression levels were assessed across the study group to determine significance and association with neuropsychological impairment and neuropathology.

Specific Aim 2: Clinicopathologic correlations. To refine the analysis detailing the association between immunoproteasome induction and neuropsychological impairment, I assessed the correlation between immunoproteasome subunit expression and neurocognitive performance scores that represent specific cognitive functioning domains. As well, I compared immunoproteasome subunit expression to clinical laboratory measurements of HIV infection, including viral loads in brain tissue, cerebrospinal fluid, and blood, and CD4+ lymphocyte counts in blood to determine the

relationship between immunoproteasome induction, HIV infection, and immunodeficiency.

Specific Aim 3: Microanatomical interrelationships. I performed indirect immunoperoxidase histochemistry to determine localization of immunoproteasomes in brain cell types. To expand those findings, I performed laser confocal microscopy and double-labeled immunofluorescence staining with antibodies directed against immunoproteasome subunits and cell markers for neurons, astrocytes, oligodendrocytes, and microglia.

Specific Aim 4: Associations with abnormal synaptic proteins. To assess possible consequences of immunoproteasome induction in neurons, I studied its association with synaptic proteins known to be altered in brain HIV infection. To identify additional synaptic proteins with altered expression in the presence of immunoproteasome induction, I then performed a proteomic analysis of isolated nerve endings (synaptosomes) using two-dimensional gel electrophoresis and mass spectroscopy. I also demonstrated by using immunofluorescence that immunoproteasomes are present in synapses.

Chapter 2: Materials and Methods

2.1 THE NATIONAL NEUROAIDS TISSUE CONSORTIUM

Central to my analysis of HIV neuropathophysiology was the resource of human post-mortem brain tissue from fully characterized HIV-positive subjects collected by the National NeuroAIDS Tissue Consortium (NNTC). Four academic institutions participate in this HIV brain banking project, including the Mount Sinai Medical Center (MSMC, New York, NY), The University of California, Los Angeles (UCLA), The University of California, San Diego (UCSD), and the University of Texas Medical Branch (UTMB, Galveston, TX). Since 1998, the NNTC has collected, stored, and distributed samples of nervous tissue, cerebrospinal fluid, blood, and other tissues from HIV-infected individuals. HIV-positive patients are continually recruited for a longitudinal observational study where biannual neuropsychological evaluations are performed and blood and cerebral spinal fluids are analyzed to document the development and progression of HIV-induced neurocognitive decline.

2.1.1 Neurocognitive Testing

The battery of neurocognitive performance tests administered to the HIV-positive subjects upon enrollment in the study and at six-month intervals was designed by the NNTC to perform a concise evaluation of specific domains of cognitive functioning known to be compromised in MCMD and HAD (Morgello, Gelman et al. 2001). The Wisconsin Card Sorting Test – 64 (WCST-64) assesses abstract and executive functioning, which is primarily driven by frontal lobe circuitry. The Hopkins Verbal Learning Test – Revised (HVLT-R) and Brief Visuospatial Memory Test – Revised (BVMT-R) assesses learning and memory functioning. The Paced Auditory Serial

Addition Test (PASAT) assesses working memory function through a series of mental calculations. The Wechsler Adult Intelligence Scale – III assesses the speed of information processing, which requires the integration of visual information to perform certain tasks. The F-A-S Test measures verbal fluency. The Wide Range Assessment Test (WRAT3) is a basic reading skills assessment that determines intellectual level. The battery of tests is administered by a neuropsychologist, who then calculates the testing scores and formulates a neuropsychological diagnosis based on the subject's performance.

2.1.2 Clinical Virology

In the NNTC protocol, the laboratory values of plasma CD4+ lymphocyte count, plasma viral load, and CSF viral load are obtained for each HIV-positive subject from blood and CSF drawn at six-month intervals. The university hospital clinical laboratory measures blood CD4+ lymphocyte counts as an indication of the progression of HIV-infection to AIDS. Blood and CSF are either analyzed in house (UCLA and UCSD) or sent to Johns Hopkins University (MSMC and UTMB) for measurement of viral load using the Roche AMPLICOR HIV-1 Monitor Test.

2.1.3 Handling and Storage of Brain Tissue

In the NNTC protocol, the subject's brain is recovered postmortem during the autopsy procedure. One hemisphere is cut in approximately one centimeter coronal slices. The slices are then placed in a plastic zip lock bag and frozen flat on a copper plate at -80 °C. The bagged slices are turned over after an initial five minutes of freezing so that both cut surfaces are frozen flat. The following day, slices are placed in new zip lock bags and collected into a plastic container for long term storage at -80 °C. The remaining hemisphere is fixed in neutral buffered formalin (10 % formaldehyde in phosphate

buffered saline) for approximately 72 h and then sliced into coronal sections. Tissue samples from predetermined regions of the brain are cut and paraffin embedded for future sectioning and immunohistochemical procedures. The remaining fixed tissue is placed in a plastic zip lock bag with formalin and stored in a plastic container for long-term storage at room temperature. For comparison, HIV-negative subjects are identified through the autopsy services at participating institutions. After obtaining consent, brain specimens are recovered in the same manner.

2.1.4 Brain Tissue Viral Load

Brain HIV RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). Single copy detection of the HIV-1 RNA in RNA extracts from brain tissue was performed using a modification of the protocol described by Palmer (Palmer, Wiegand et al. 2003). One μg of brain RNA and 1 μmol/L of anti-sense primer 84R were used in a 20 μl reaction (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA). Four μl of cDNA was used for a 25 μl real-time PCR. JumpStart Taq ReadyMix for Quantitative PCR (Sigma Aldrich, Saint Louis, MO) and SmartCycler (Cepheid, Sunnyvale, CA) were used in PCR. The results were standardized against a known brain secondary standard and expressed relative to wet weight of brain tissue.

2.1.5 Study Subjects

The subjects selected for analysis consisted of 65 HIV-negative subjects and 88 HIV-positive subjects, summarized in **Table 2.1**. A summary of the neurocognitive status of the selected HIV subjects is shown in **Table 2.2**. HIV-positive subjects were selected to span the broadest age range possible to allow for analyses of potential synergy between aging and HIV-infection on alterations of the proteasome. Case selection placed emphasis on HIV-positive subjects with signs of neuropsychological impairment (HAD

and MCMD). Also included for the analyses were HIV-positive subjects without neurocognitive impairment (Subsyndromic or Normal) and subjects classified as Neuropsychological Impairment-Other (NPI-O). NPI-O indicates that neuropsychological impairment was present, but it could not be definitively attributed to HIV-infection due to the presence of comorbid factors. To include illustrative neuropathological cases, samples from ten HIV-positive subjects without neurocognitive testing data were included. Of the 88 HIV-positive subjects selected, 20 had neuropathological findings indicative of HIV encephalitis as determined by a neuropathologist. HIV-negative subjects were selected to best match the selected HIV-positive group according to age and gender. A subset of HIV-negative subjects over the age of 65 (n = 11) was also included to provide a wider age range with which to analyze potential aging effects on proteasome structure and functioning.

Table 2.1 Demographics of study subjects

		Age		Gender	
Group	***n***	**Range**	**Mean**	**Male**	**Female**
HIV-	65	23-90	50	49	16
HIV+	88	28-66	45	71	17

Table 2.2 Neurocognitive and neuropathological diagnoses of HIV-positive subjects

Diagnosis	*n*
Neuropsychological Impairment (NPI)	47
HIV-Associated Dementia (HAD)	23
Minor Cognitive Motor Disorder (MCMD)	24
No Neuropsychological Impairment (No NPI)	11
Subsyndromic	6
Normal	5
Neuropsychological Impairment - Other (NPI-O)	20
No Neuropychological Data	10
HIV Encephalitis (HIVE)	20

2.2 BRAIN TISSUE PREPARATION FOR BIOCHEMICAL ANALYSIS

Tissue samples from the dorsolateral prefrontal cortex (Brodmann area 8 or 9) and adjacent subcortical white matter were dissected from the fresh-frozen brain slices stored at -80 °C. 100-500 mg of tissue were sampled from the frozen brain slices over dry ice and placed in pre-weighed 2 mL conical tubes. Sample masses were determined and recorded. The tissue samples were homogenized in buffer (10 mM Tris-HCl, 0.5 mM Dithiothreitol, 0.03 % Triton X-100, 5 mM $MgCl_2$, pH 7.8) by silica bead beating for 20 s and sonication over ice for 20 s. The Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the method of Bradford, was used according to the supplied protocol to determine total protein concentration using bovine serum albumin as a standard. The protein assays were run in 96-well clear flat bottom assay plates and read using the VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.3 PROTEASOME ENZYMATIC ACTIVITY ASSAY

Homogenized samples were centrifuged at 13,000 x g in 4 °C for 5 min to remove cellular debris. Protein concentration of the resulting supernatant was adjusted to 0.5 mg/mL by diluting with homogenizing buffer. Ten microliters of sample was added 190

μL of reaction buffer (25 mM HEPES, 0.5 mM EDTA, 0.03 % SDS) in triplicate in 96-well opaque flat bottom assay plates. Artificial substrates Z-LLL-AMC (1.0 μM), Z-LLE-AMC (2.5 μM), or Suc-LLVY-AMC (2.5 μM) (Boston Biochem, Cambridge, MA) were then added. Hydrolysis of these substrates by proteasomes releases fluorescent 7-amino-4-methylcoumarin (AMC) that corresponds to proteasomal trypsin-like, post-glutamyl peptide hydrolyzing, and chymotrypsin-like activities, respectively. Background fluorescence was determined by incubating a subset of samples for 30 min with 1 μM MG132 proteasome inhibitor (Boston Biochem) prior to adding artificial substrate. Samples were incubated for 5 h at 37 °C and then analyzed by the Gemini XPS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) at 345 nM excitation/ 445 nm emission. The amount of AMC fluorescence accumulated over time was used to calculate specific proteasome activity (pmol AMC/hour/mg Protein) using an AMC titration curve.

2.4 WESTERN BLOTTING

10-30 μg of total protein in 15 μL volume was added to 15 μL of 2X Laemmli Sample Buffer (Bio-Rad Laboratories) with 5 % beta-mercaptoethanol in 1.5 mL microcentrifuge tubes and boiled for 5 min. Samples were then loaded into Criterion Precast 18-well 5 %, 15 %, or 4-20 % gradient Tris-HCL gels (Bio-Rad Laboratories) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to run for approximately 1 h at 180 V. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ) for 3 h at 60 V in 4 °C. Membranes were blocked with 5 % nonfat dry milk in TBST (0.05 M Trizma-HCl, 0.15 M NaCl, 0.1 % Tween 20) for 1 h, and then incubated overnight with primary antibody diluted in TBST according to the antibody supplier data sheet. **Table 2.3** lists the primary antibodies used. Membranes were then washed 3 times for 5 min with TBST and

incubated with the appropriate anti-rabbit or anti-mouse secondary antibody diluted 1:5000 for 1 h (Amersham Biosciences). After a second series of washes, Enhanced Chemiluminescence Detection Reagent (Amersham Biosciences) was applied to the membrane for 2 min. The membrane was then exposed to Kodak BioMAX XAR film (Rochester, NY) for 30 s to 3 min, depending upon signal intensity. The film was then developed and digitized by computer scanner. Quantification of band density was performed using densitometry software One-Dscan (BD Biosciences Bioimaging, Rockville, MD).

Table 2.3 Proteasome subunit primary antibodies

Subunit Antibody (Clone)	Source	Product ID	Species	Type	Dilution
20S α-type					
α4 (MCP79)	Biomol	PW9140	mouse	monoclonal	1:1000
α6 (MCP20)	Biomol	PW8100	mouse	monoclonal	1:1000
α7 (MCP72)	Biomol	PW8110	mouse	monoclonal	1:1000
20S β-type					
X / β5	Affinity Bioreagents	PA1-977	rabbit	polyclonal	1:1000
Y / β1	Affinity Bioreagents	PA1-978	rabbit	polyclonal	1:1000
Z / β2	Affinity Bioreagents	PA1-979	rabbit	polyclonal	1:1000
LMP7 / β5i (LMP7-1)	Biomol	PW8845	mouse	monoclonal	1:1000
LMP2 / β1i (LMP2-13)	Biomol	PW8840	mouse	monoclonal	1:1000
MECL-1 / β2i	Biomol	PW8150	rabbit	polyclonal	1:1000
19S regulator ATPase					
Rpt1	Biomol	PW8315	rabbit	polyclonal	1:1000
Rpt5	Affinity Bioreagents	PA1-967	rabbit	polyclonal	1:500
19S regulator non-ATPase					
Rpn2	Affinity Bioreagents	PA1-973	rabbit	polyclonal	1:1000
Rpn8	Affinity Bioreagents	PA1-1963	rabbit	polyclonal	1:1000
11S regulator					
PA28α	Biomol	PW8185	rabbit	polyclonal	1:1000

2.5 IMMUNOPEROXIDASE IMMUNOHISTOCHEMISTRY

Tissue sections of 6 micron thickness were cut from the archival formalin-fixed paraffin-embedded tissue blocks and placed onto pre-labeled Superfrost Plus Gold microscope slides (Fisher Scientific, Pittsburgh, PA) by the UTMB Histopathology Core Lab. The slides were baked at 60 °C for 30 min to promote adherence of the tissue section to the slides. Tissue sections were deparaffinized in three 3-minute washes of xylene and rehydrated in 2-minute washes of decreasing concentrations of ethanol, with two washes in 100 % ethanol, and one wash each in 95 %, 80 %, and 70 % ethanol. Endogenous peroxidase activity was inactivated with a 5-minute wash with 3 % hydrogen peroxide in methanol. Antigen retrieval was performed by microwaving slides in Copland jars containing sodium citrate buffer (10 mM sodium citrate, 0.05 % Tween-20, pH 6.0) for 10 min at 10 % power within a 2 L water bath. Slides were blocked in solution containing 0.1 % nonfat dry milk and 1 % goat serum and then incubated with primary antibody diluted in blocking solution overnight at 4 °C. Primary antibodies included rabbit polyclonal anti-LMP7 and anti-PA28 alpha (BIOMOL International, Inc, Plymouth Meeting, PA). Appropriate secondary antibody was applied for one hour, and then developed using the Vectastain ABC and Peroxidase substrate DAB kit (Vector Laboratories, Burlingame, CA). Slides were dehydrated in increasing ethanol and xylene baths. Coverslips (Fisher Scientific) were mounted using Permount mounting medium (Fisher Scientific).

2.6 IMMUNOFLUORESCENCE

The procedure for immunofluorescence was similar to that of immunoperoxidase immunohistochemistry with few substantial modifications up until secondary antibody application. Antigen retrieval was performed by placing slides in a plastic slide-holding container with preheated sodium citrate buffer. The container was covered and placed in

a preheated vegetable steamer for 20 min, after which it was removed, uncovered, and allowed to cool for an additional 20 min. Nonspecific staining was blocked using Image-iT FX signal enhancer (Invitrogen Molecular Probes, Eugene, Oregon) applied for 30 min prior to serum blocking (5 % bovine serum albumin, 5 % normal goat serum in TBST) applied for 1 h. Primary antibodies listed in **Table 2.4** were diluted in blocking solution and applied overnight at 4 °C. The slides were then incubated with the appropriate Alexa-Fluor fluorochrome-conjugated secondary antibody (Invitrogen Molecular Probes) diluted in blocking solution for 1 h. To block tissue autofluorescence caused by lipofuscin deposits and tissue fixation, sections are incubated with Sudan Black B (Sigma Aldrich) solution (1 % Sudan Black B in 70 % ethanol) for 10 min. The Sudan Black B solution was then removed by pipetting TBST over the tissue section and washing once for 5 min in TBST and twice for 5 min in water. Excess moisture was removed. Coverslips were mounted using Slow Fade Gold with DAPI (Invitrogen Molecular Probes) mounting medium. Clear nail polish was applied to seal the edges of the coverslip. Slides were then numbered and stored in slide holders at 4 °C.

Laser confocal microscopy was performed at the UTMB Infectious Disease and Toxicology Optical Imaging Core Laboratory. Images were acquired with a Zeiss LSM 510 UV META laser scanning confocal microscope consisting of an Axiovert 200M Inverted Microscope equipped with an oil-immersion 100X-resolution objective for fluorescence, Ar, dual HeNe, and UV lasers and fluorescence filters set for DAPI, FITC, TRITC, and far red, a scanning module with visible and UV acousto optical tunable filters, two independent fluorescence channels (2 PMTs), and a 32-PMT array (Carl Zeiss MicroImaging, Inc, Thornwood, NY).

Table 2.4 Primary antibodies for immunofluorescence analysis.

Antibody (Clone)	Source	Product ID	Species	Type	Dilution
Immunoproteasome					
LMP2 / β1i					
Neurons					
NeuN (A60)	Millipore	MAB377	mouse	monoclonal	1:100
MAP2 (AP20)	Millipore	MAB3418	mouse	monoclonal	1:200
Neurofilament (SPM204)	Abcam	ab17832	mouse	monoclonal	1:50
Astrocytes					
GFAP (GA5)	Millipore	MAB360	mouse	monoclonal	1:400
Oligodendrocytes					
OMG (4A9)	Lifespan	LS-C27282	mouse	monoclonal	1:300
Macrophage/Microglia					
CD68 (KP1)	Dako	M0814	mouse	monoclonal	1:100

2.7 SYNAPTOSOME FRACTIONATION

Synaptosomes are nerve endings isolated from brain homogenates and are enriched in certain neuronal proteins versus the homogenate. They were isolated in a standard fashion through the processes of differential centrifugation and density centrifugation using a discontinuous sucrose gradient (Gray and Whittaker 1962; Dodd, Hardy et al. 1981; Wood, MacMillan et al. 1996; Eshleman, Wolfrum et al. 2001; Mash, Pablo et al. 2002). About 500 mg of frozen frontal cortex brain tissue was used for each synaptosome isolation procedure. The tissue was thawed briefly at room temperature and added to 5 mL of ice cold buffered sucrose (0.32 M sucrose, 5 mM HEPES, 25 μL protease inhibitor cocktail (Sigma Aldrich), 50 μL phosphatase inhibitor cocktail (EMD Chemicals, Inc., Gibbstown, NJ), pH 7.4) in a 10 mL Potter-Elvehjem tube on ice. The tissue was homogenized with 8 up-and-down strokes of the Potter-Elvehjem tissue grinder with 0.1 mm to 0.5 mm clearance (Fisher Scientific) and a 30 s ice bath cooling halfway in between. The homogenate was transferred to 15 mL conical tube and

centrifuged at 1000 x g for 10 min at 4 °C to yield a crude pellet (P1a) and supernatant (S1a). P1a was resuspended in 5 mL buffered sucrose and centrifugation was repeated, yielding another pellet (P1b) and supernatant (S1b). S1a and S1b were combined to form S1 in an ultracentrifuge tube and brought to a volume of 10 mL by adding buffered sucrose. S1 was centrifuged at 10,000 x g for 20 min at 4 °C to yield a pellet (P2) and supernatant (S2). After S2 was removed, P2 was resuspended in 2.5 mL buffered sucrose and then layered on top of a discontinuous gradient with 2.5 mL each of buffered sucrose with concentrations of 0.8 M, 1.0 M, and 1.2 M prepared in another ultracentrifuge tube. The sucrose gradient with the resuspended P2 was centrifuged at 150,000 x g for 2 h at 4 °C. Synaptosomes (P3) were recovered between the layers of 1.0 M and 1.2 M buffered sucrose and transferred to a 15 mL conical tube. P3 was then diluted to 0.32 M buffered sucrose and centrifuged at 150,000 x g for 30 min at 4 °C. The supernatant was discarded, leaving behind the synaptosome pellet.

2.8 SYNAPTOSOME 2D GEL ELECTROPHORESIS

Proteomic analysis was performed using synaptosomes prepared from prefrontal cortex samples of 19 subjects. Synaptosomes isolated from 12 subjects were separated into the following groups based upon the diagnoses of HIV-associated neuropsychological impairment and HIV encephalitis: A, HIV-negative subjects (n = 3); B, HIV-positive subjects without neuropsychological impairment (n = 3); C, HIV-positive subjects with neuropsychological impairment but without HIV encephalitis (n = 3); D, HIV-positive subjects with neuropsychological impairment and HIV encephalitis (n = 3). Synaptosomes from an additional seven HIV-positive subjects were isolated, and in combination with the previously isolated synaptosomes from HIV-positive subjects, were separated into the following groups: E, low immunoproteasome expression (n = 4); F, high immunoproteasome expression without HIV encephalitis (n = 4); G, high

immunoproteasome expression and HIV encephalitis (n = 4). Synaptosome samples from each group were pooled with equal contribution from each subject. Utilizing the UTMB Proteomics Core Laboratory, the synaptosome sample pools were analyzed in triplicate by 2-dimensional gel electrophoresis with isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. Each gel was then stained with SYPRO Red and digitally photographed. The digital images of the gels were analyzed by Nonlinear Dynamics Progenesis SameSpots software (Nonlinear USA, Inc, Durham, NC). Computer spot analysis with comparison between groups produced a list of spots with at least a 2-fold difference in averaged optical density. The spot list was refined after manual analysis of the gel images to eliminate spots that had low intensity, were part of a streak, or were not present in at least two of the three gels. For each remaining spot, the gel with the highest spot intensity was selected and the area corresponding to the spot was manually removed by a large-bore pipette tip and sent to the UTMB Mass Spectrometry Core Laboratory for protein identification.

2.9 SAMPLE PREPARATION FOR MASS SPECTROMETRY

Gel samples were cut into 1 mm size pieces or smaller and placed into separate 0.5 mL polypropylene tubes. 100 µl of 50 mM ammonium bicarbonate buffer was added to each tube and the samples were incubated at 37 °C for 30 min. The buffer was removed and 100 µl of water was then added to each tube. The samples were incubated again at 37 °C for 30 min. After incubation, the water was removed and 100 µl of acetonitrile was added to each tube to dehydrate the gel pieces. The samples were vortexed, and after 5 min the acetonitrile was removed. 100 µl of acetonitrile was again added to the sample tubes, vortexed, and acetonitrile removed after 5 min. The samples were then placed in a speedvac for 45 min to remove any excess solvent. 2 mL of 25 mM ammonium bicarbonate at pH 8.0 was added to a 20 µg vial of lyophilized trypsin

(Promega Corporation, Madison, WI). The trypsin solution was vortexed and added to each sample tube in an amount (approximately 10 μL) to just cover the dried gel. The samples were then incubated at 37 °C for 6 h.

After digestion, the samples were removed from the oven and 1 μL of sample solution was spotted directly onto a MALDI target plate and allowed to dry. 1 μL of alpha-cyano-4-hydroxycinnamic acid (Sigma Aldrich) matrix solution (50:50 acetonitrile/water at 5 mg/mL) was then applied on the sample spot and allowed to dry. The dried MALDI spot was blown with compressed air (Decon Laboratories, Inc, King of Prussia, PA) before inserting into the mass spectrometer.

2.10 MASS SPECTROMETRY ANALYSIS

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI TOF-MS) was used to analyze the samples and determine protein identification. Data were acquired with an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA). Applied Biosystems software package included 4000 Series Explorer (v. 3.6 RC1) with Oracle Database Schema Version (v. 3.19.0), Data Version (3.80.0) to acquire both MS and MS/MS spectral data. The instrument was operated in positive ion reflectron mode, mass range was 850 – 3000 Da, and the focus mass was set at 1700 Da. For MS data, 1000-2000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using a peptide mixture with reference masses 904.468, 1296.685, 1570.677, and 2465.199.

Following MALDI MS analysis, MALDI MS/MS was performed on several (5-10) abundant ions from each sample spot. A 1 kV positive ion MS/MS method was used to acquire data under post-source decay (PSD) conditions. The instrument precursor

selection window was +/- 3 Da. For MS/MS data, 2000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 480.257, 684.347, 1056.475, and 1441.635 (from precursor mass 1570.700).

Applied Biosystems GPS Explorer™ (v. 3.6) software was used in conjunction with MASCOT to search the respective protein database using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or MASCOT protein scores. MS peak filtering included the following parameters: mass range 800 Da to 3000 Da, minimum S/N filter = 10, mass exclusion list tolerance = 0.5 Da, and mass exclusion list (for some trypsin and keratin-containing compounds) included masses 842.51, 870.45, 1045.56, 1179.60, 1277.71, 1475.79, and 2211.1. For MS/MS peak filtering, the minimum S/N filter = 10.

For protein identification, the human taxonomy was searched in the database. Other parameters included the following: selecting the enzyme as trypsin; maximum missed cleavages = 1; fixed modifications included carbamidomethyl (C) for 2-D gel analyses only; variable modifications included oxidation (M); precursor tolerance was set at 0.2 Da; MS/MS fragment tolerance was set at 0.3 Da; mass = monoisotopic; and peptide charges were only considered as +1. The significance of a protein match, based on both the peptide mass fingerprint (PMF) in the first MS and the MS/MS data from several precursor ions, is based on expectation values; each protein match is accompanied by an expectation value. The expectation value is the number of matches with equal or better scores that are expected to occur by chance alone. The default significance threshold is $p<0.05$, so an expectation value of 0.05 is considered to be on this threshold. A more stringent threshold of 10^{-3} for protein identification was used; the lower the expectation value, the more significant the score.

2.11 SYNAPTIC PROTEIN ANALYSIS

Western blotting analyses synaptic protein and immunoproteasome expression levels in frontal cortex brain homogenates and isolated synaptosomes were performed as previously described. Immunofluorescence analysis of immunoproteasomes and synaptic proteins in prefrontal cortex tissue sections were also performed as previously described. Antibodies used for these analyses are listed in **Table 2.5**.

Table 2.5 Primary antibodies used for the analysis of synaptic proteins.

Antibody (Clone)	Source	Product ID	Species	Type	Dilution
Immunoblotting					
Synaptophysin (SVP-38)	Sigma Aldrich	S5768	mouse	monoclonal	1:5000
GAP43 (GAP-7B10)	Sigma Aldrich	G9264	mouse	monoclonal	1:4000
LMP7 / β5i (LMP7-1)	Biomol	PW8845	mouse	monoclonal	1:1000
Dynamin 1 (D5)	Millipore	MAB5402	mouse	monoclonal	1:1000
SCAMP1 (22)	BD Biosciences	612087	mouse	monoclonal	1:500
SV2C	Santa Cruz Biotech	sc-28957	rabbit	polyclonal	1:500
VMAT2	Millipore	AB1598P	rabbit	polyclonal	1:1000
GluR2	Abcam	ab40878	rabbit	polyclonal	1:5000
Homer1B/C	Millipore	AB5877	rat	polyclonal	1:1000
NMDAR2B	Millipore	AB1557P	rabbit	polyclonal	1:1000
PSD95	Cell Signaling	2507	rabbit	polyclonal	1:1000
SynGAP	Sigma Aldrich	S5437	rabbit	polyclonal	1:1000
CD68 (E-11)	Santa Cruz Biotech	sc-17832	mouse	monoclonal	1:200
GFAP (2E1)	BD Biosciences	556330	mouse	monoclonal	1:500
14-3-3ζ	Millipore	AB9746	rabbit	polyclonal	1:3000
14-3-3ε (12)	BD Biosciences	610543	mouse	monoclonal	1:1000
Synapsin 1	Abcam	ab8	rabbit	polyclonal	1:1000
Annexin V	Abcam	ab14196	rabbit	polyclonal	1:2000
CRMP2	Abcam	ab36201	rabbit	polyclonal	1:2000
αB crystallin	Abcam	ab13497	rabbit	polyclonal	1:1000
αFodrin	Cell Signaling	2122	rabbit	polyclonal	1:1000
Stathmin	Abcam	ab47468	rabbit	polyclonal	1:1000
Immunofluorescence					
LMP2 / β1i	Abcam	ab3328	rabbit	polyclonal	1:1000
14-3-3ζ (8C3)	Abcam	ab36777	mouse	monoclonal	1:500
Synaptophysin (SVP-38)	Sigma Aldrich	S5768	mouse	monoclonal	1:200

2.12 STATISTICAL ANALYSIS

Data was compiled and analyzed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, Washington) and GraphPad InStat Version 3.06 (GraphPad Software, Inc., La Jolla, CA). Student's t-test was performed in Microsoft Excel 2003 to determine differences between two groups. Comparisons between three or more groups were performed by analysis of variance (ANOVA) with post-test analyses in GraphPad Instat. Data was tested for Gaussian distribution by the method of Kolmogorov and Smirnov. If the data was considered normal, One-Way ANOVA was performed, followed by the Tukey-Kramer Multiple Comparisons Test. Data that did not pass the normality test was analyzed by the Kruskal-Wallis Test (non-parametric ANOVA) followed by Dunn's Multiple Comparisons Test. Analysis of correlation and regression was performed in Microsoft Excel. Graphs were produced in Microsoft Excel 2003. Where applicable, error bars represent an approximation of the standard error of the mean by the 95 % confidence intervals.

Chapter 3: Altered Proteasome Activity in HIV-Infected Brain Specimens

3.1 INTRODUCTION

The recent findings of increased ubiquitinated-protein deposits and high molecular-weight ubiquitin-protein conjugates in postmortem brain samples of HIV/AIDS patients suggested an impairment of protein turnover by the ubiquitin-proteasome system. Research in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis has also implicated a possible slowing of the ubiquitin-proteasome system leading to a buildup of ubiquitinated protein deposits. Various reports also show decreases in proteasome enzymatic activities associated with these neurodegenerative diseases (Keller, Hanni et al. 2000; McNaught and Jenner 2001; Urushitani, Kurisu et al. 2002; Keck, Nitsch et al. 2003; McNaught, Belizaire et al. 2003; Kabashi, Agar et al. 2004; Seo, Sonntag et al. 2004). My initial investigation into alterations in the ubiquitin-proteasome system of HIV-infected brains was aimed at measuring the three different enzymatic activities of the 20S proteasome active sites to determine changes associated with HIV infection with possible similarities to other neurodegenerative diseases. The analysis was performed using samples of frontal lobe cortex and white matter collected from 88 HIV-positive subjects and 65 HIV-negative subjects of the National NeuroAIDS Tissue Consortium. The proteasome proteolytic activities were assayed by measuring the degradation of commercially available artificial substrates that are specific for each of the three proteasome active sites. Analyses were performed to determine the extent of changes in proteasome activity with regard to HIV infection, HIV-associated neuropsychological impairment and neuropathology.

3.2 RESULTS

3.2.1 Increased Chymotrypsin-Like Activity in HIV-Infected Brains

I measured the chymotrypsin-like, post-glutamyl peptide hydrolyzing, and trypsin-like activities of the proteasome. The comparison of proteasome enzymatic activities between HIV-positive subjects and HIV-negative subjects using Student's T-test revealed significant increases in mean chymotrypsin-like activity of 9.7 % in cortex ($p < 0.01$; **Figure 3.1A**) and 16.4 % in white matter ($p < 0.0001$; **Figure 3.1B**). Mean post-glutamyl peptide hydrolyzing activity was decreased in the white matter of HIV-positive brain samples but did not reach significance ($p = 0.056$). No difference in mean trypsin-like activity was detected.

3.2.2 Increased Chymotrypsin-Like Activity in HIV-Positive Subjects with Neuropsychological Impairment

To determine the relationship between the changes in proteasome activity and HIV-associated neuropsychological impairment, I compared the levels of proteasome enzymatic activities between groups based on the neuropsychological diagnosis using ANOVA and post-hoc tests comparing pairs of groups. Levels of chymotrypsin-like activity were significantly different in both cortex ($p < 0.05$) and white matter ($p < 0.0001$) samples, with increases of 12 % in cortex ($p < 0.05$; **Figure 3.1C**) and 21 % in white matter ($p < 0.001$; **Figure 3.1D**) found in HIV-positive subjects with HIV-associated neuropsychological impairment compared to HIV-negative subjects. No significant differences were detected between groups for other proteasome activities in either cortex or white matter samples.

3.2.3 Increased Chymotrypsin-Like Activity and Decreased Post-Glutamyl Peptide Hydrolyzing Activity with HIV-Encephalitis

The differences in proteasome enzymatic activities were also analyzed between the HIV-negative subjects and the HIV-positive subjects with and without HIV encephalitis (**Figure 3.1E, F**). In the analysis of frontal cortex samples (**Figure 3.1E**), mean chymotrypsin-like activity was significantly different between groups ($p < 0.05$), with a 9 % increase in HIV-positive subjects without HIV encephalitis compared to HIV-negative subjects ($p < 0.05$). A 12 % increase in mean chymotrypsin-like activity in those with HIV encephalitis compared to the HIV-negative subjects did not reach significance. No significant differences in proteasome enzymatic activities were detected between HIV-positive subjects with or without HIV encephalitis in frontal cortex samples.

Analysis of white matter proteasome enzymatic activity (**Figure 3.1F**) also revealed significant differences in mean chymotrypsin-like activity ($p < 0.05$), with increases of 14 % in HIV-positive subjects without HIV encephalitis ($p < 0.01$), and 27 % in HIV-positive subjects with HIV encephalitis ($p < 0.001$) compared to HIV-negative subjects. Average post-glutamyl peptide hydrolyzing activity was also found to be statistically different ($p < 0.05$), with an 18 % decrease in HIV-positive subjects with HIV encephalitis compared to HIV-negative subjects ($p < 0.05$). No significant differences were detected between groups for other proteasome activities in either cortex or white matter samples.

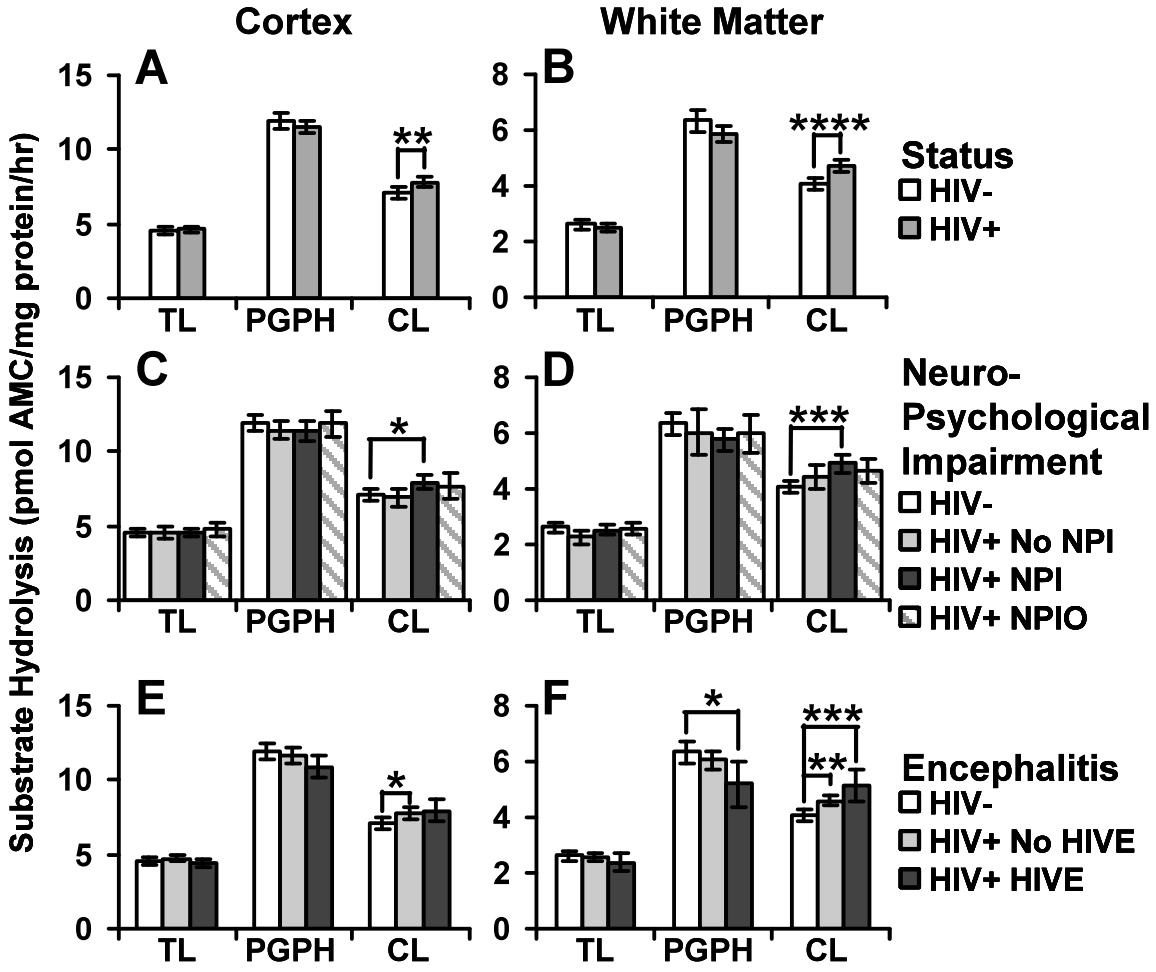

Cortex
White Matter
A
B
C
D
E
F
Substrate Hydrolysis (pmol AMC/mg protein/hr)
TL
PGPH
CL
**
****
*
***
Status
HIV-
HIV+
Neuro-Psychological Impairment
HIV-
HIV+ No NPI
HIV+ NPI
HIV+ NPIO
Encephalitis
HIV-
HIV+ No HIVE
HIV+ HIVE

Figure 3.1 Altered proteasome proteolytic activity in HIV-infected subjects. Proteasome enzymatic activities were assessed by measuring the degradation of artificial substrates in brain tissue homogenate samples. (A, B) Assays revealed significant increases in CL activity in HIV+ of (A) 9.7 % in cortex and (B) 16.4 % in white matter homogenate samples compared to HIV-. Decreases in PGPH activity were not significant. No differences in TL activity were noted. (C, D) Proteasome activities compared between groups according to neuropsychological diagnosis showed increased CL activity in HIV+ NPI compared to HIV- of (C) 12 % in cortex and (D) 21 % in white matter samples. (E, F) Proteasome activities were also compared between groups according to neuropathological diagnosis. CL activity was increased in HIV+ No HIVE compared to HIV- by (E) 9 % in cortex and (F) 14 % in white matter. In white matter, HIV+ HIVE showed an 18 % decrease in PGPH and a 27 % increase in CL activity. (TL, trypsin-like activity; PGPH, post-glutamyl peptide hydrolyzing activity; CL, chymotrypsin-like activity; NPI, HIV-associated neuropsychological impairment; NPIO, neuropsychological impairment with comorbid factors; HIVE, HIV encephalitis; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$)

3.2.4 Altered Proteasome Proteinase Profile in HIV-Positive Subjects with Neuropsychological Impairment and Encephalitis

Along with the significant increases in chymotrypsin-like activity in HIV-positive subjects, I observed a decreasing trend in post-glutamyl peptide hydrolyzing activity. To assess the significance of this reciprocal change in proteasome enzymatic activities, I analyzed the ratio of chymotrypsin-like activity to post-glutamyl peptide hydrolyzing activity (CL:PGPH) calculated for each study case. Initial comparisons of mean CL:PGPH between HIV-negative and HIV-positive cases revealed significant increases of 16 % in cortex samples ($p < 0.01$) and 28 % in white matter samples ($p < 0.0001$) in the HIV-positive group (**Figure 3.2A**).

ANOVA analysis of average CL:PGPH between groups in relation to neuropsychological impairment revealed significant differences in both cortex ($p < 0.001$) and white matter ($p < 0.0001$) samples. In cortex samples, the mean CL:PGPH of

HIV-positive subjects with neuropsychological impairment was 22 % higher than HIV-negative subjects ($p < 0.01$, **Figure 3.2B**). In white matter samples, the average CL:PGPH was 36 % higher in HIV-positive subjects with neuropsychological impairment ($p < 0.001$) compared to HIV-negative subjects. As well, HIV-positive subjects with neuropsychological impairment due to other causes had a 25 % increase in CL:PGPH compared to HIV-negative subjects ($p < 0.01$). Comparisons between other pairs of groups did not reveal any additional significant differences in either frontal cortex or white matter samples.

When comparing average CL:PGPH between those with and without HIV encephalitis, again, significant differences were detected between groups in both cortex ($p < 0.0001$) and white matter ($p < 0.0001$, **Figure 3.2C**). Compared to the HIV-negative subjects, there was a 14 % increase in the HIV-positive subjects without HIV encephalitis ($p < 0.01$), and a 23 % increase in those with HIV encephalitis ($p < 0.001$) in cortex samples. Analysis of white matter showed that in those with HIV encephalitis, the CL:PGPH average was 65 % higher than the HIV-negative subjects ($p < 0.001$). The HIV-positive subjects without HIV encephalitis also showed an 18 % increase in average CL:PGPH compared to HIV-negative subjects ($p < 0.01$). Comparisons between other pairs of groups did not reveal any additional significant differences in either frontal cortex or white matter samples.

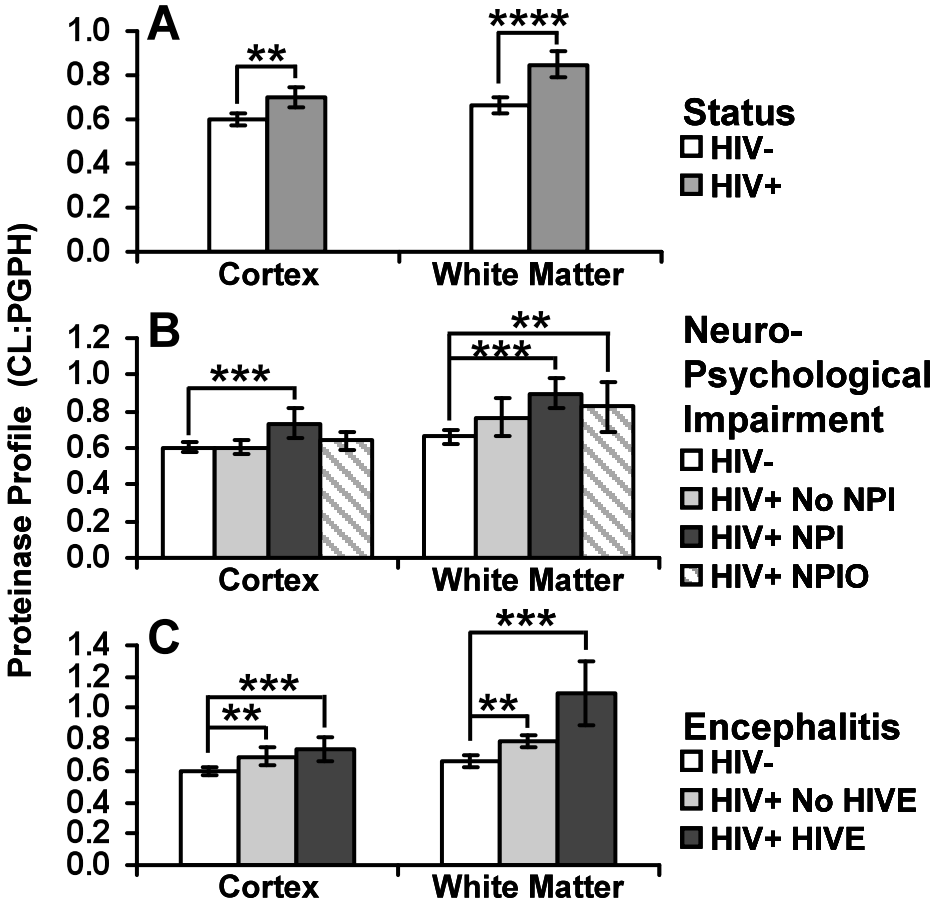


Figure 3.2 Altered proteinase profile in HIV-infected subjects associated with neuropsychological impairment and HIV encephalitis. The ratio of chymotrypsin-like activity to post-glutamyl peptide hydrolyzing activity (CL:PGPH) previously measured in brain homogenate samples was calculated for each subject to portray a "proteinase profile" in brain tissue homogenate samples. (A) HIV+ had significantly increased CL:PGPH compared to HIV- in both cortex (16 %) and white matter (28 %) samples. (B) HIV+ NPI had increased CL:PGPH in both cortex (22 %) and white matter (36 %) compared to HIV-. Increased CL:PGPH in HIV+ NPIO compared to HIV- was also detected in white matter (25 %). (C) HIV+ No HIVE and HIV+ HIVE had significantly increased CL:PGPH compared to HIV- in cortex (14 % and 23 %, respectively) and white matter samples (18 % and 65 %, respectively). No significant differences were found in other group comparisons. (NPI, HIV-associated neuropsychological impairment; NPIO, neuropsychological impairment with comorbid factors; HIVE, HIV encephalitis; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$)

3.3 DISCUSSION

These data represent the first analysis of the ubiquitin proteasome system in HIV-infected brain tissue through the assessment of proteasome proteolytic activity by enzymatic assays. The results revealed differences in proteasome activities associated with HIV infection. Increases in proteasome chymotrypsin-like activity were observed in brain tissue samples of HIV-infected subjects, especially in those with HIV-associated neuropsychological impairment and in those with HIV encephalitis. The proteasome post-glutamyl peptide hydrolyzing activity was also altered in the white matter of those with HIV encephalitis. There was little to no change in trypsin-like activity detected. The analysis of the ratio between chymotrypsin-like and post-glutamyl peptide hydrolyzing activities revealed similar changes between the compared groups, with heightened significance.

The increase in chymotrypsin-like activity and concomitant decrease in post-glutamyl peptide hydrolyzing activity is in marked contrast to the assessments of proteasome proteolytic activities in other neurodegenerative diseases, where reports indicated a generalized decrease in proteolytic activities. Analysis of proteasome activities in samples of Alzheimer's disease brains showed decreases in chymotrypsin-like activity and post-glutamyl peptide hydrolyzing activity ranging from 28 % to 60 % compared to control cases in regions with neurodegeneration (Keller, Hanni et al. 2000). Similarly, proteasome chymotrypsin-like and post-glutamyl peptide hydrolyzing activities in Huntington's disease subjects are also decreased in several brain regions analyzed, including striatum, cortex, cerebellum, and substantia nigra (Seo, Sonntag et al. 2004). Investigations in Parkinson's disease revealed 39 % to 44 % decrease in chymotrypsin-like activity, 42 % to 46 % decrease in trypsin-like activity, and 33 % to 44.6 % decrease in post-glutamyl peptide hydrolyzing activity in substantia nigra pars

compacta tissue samples of idiopathic and sporadic Parkinson's disease cases, but not in other regions analyzed (McNaught and Jenner 2001; McNaught, Belizaire et al. 2003). The decreases in proteasome enzymatic activities in these neurodegenerative diseases points towards a generalized decrease in proteasome proteolytic capacity due to either inhibition of the proteasome complex by disease-specific protein aggregations or a decrease in proteasome expression. The increased chymotrypsin-like activity and decreased post-glutamyl peptide hydrolyzing activity indicate that the mechanisms underlying the alterations in the ubiquitin-proteasome system in HIV-infected brains are different from age-associated neurodegenerative diseases.

The reciprocal changes in proteasome proteolytic activities observed in HIV-infected brain specimens yielded a proteolytic profile that suggests modifications to specific 20S beta subunits containing proteolytic active sites. This is in contrast to more common modes of proteasome activation and inhibition, where all three proteasome proteolytic activities are either increased or decreased. Activation of the 20S proteasome by the 11S proteasome regulatory complex, peptide-based activators, or low-concentration sodium dodecyl sulfate, increases all three proteolytic activities to varying degrees by facilitating substrate access to the active sites within the 20S proteasome core (Dubiel, Pratt et al. 1992; Ma, Slaughter et al. 1992; Shibatani and Ward 1995; Wilk and Chen 1997; Stohwasser, Salzmann et al. 2000). Pharmacological proteasome inhibitors like peptide aldehydes (MG132 and MG115), epoxomicin, lactacystin and the *clasto*-lactacystin-beta-lactone derivative selectively bind to and block the three proteasome proteolytic active sites (Fenteany, Standaert et al. 1995; Lee and Goldberg 1996; Craiu, Gaczynska et al. 1997; Lee and Goldberg 1998; Meng, Mohan et al. 1999). With regard to HIV infection, recombinant viral protein Tat was shown to bind to 20S proteasome subunits, including those containing active sites, and inhibit proteasome chymotrypsin-

like activity, though other proteasome activities were not assessed (Apcher, Heink et al. 2003). It is possible that additional proteasome effectors are present in HIV infection of the brain. These unidentified proteasome effectors could mimic pharmacological proteasome effectors such as betulinic acid, which preferentially activates chymotrypsin-like activity (Huang, Ho et al. 2007), or the HMG-CoA reductase inhibitors lovastatin and simvastatin, which have been shown to stimulate proteasome chymotrypsin-like activity and inhibit post-glutamyl peptide hydrolyzing activity (Wojcik, Bury et al. 2000).

Given the context of HIV infection of the brain and the associated inflammatory response, it is likely that brain proteasomes are modified by the inflammatory cytokine-mediated induction of immunoproteasome subunit expression and the subsequent replacement of constitutive subunits to form immunoproteasome complexes. Experiments on immunoproteasome subunit overexpression have shown that immunoproteasomes exhibit increased chymotrypsin-like and trypsin-like activity, and reduced post-glutamyl peptide hydrolyzing activity (Gaczynska, *et al*., 1994, Groettrup, *et al*., 2001). The changes in proteasome proteolytic activities seen with increased immunoproteasomes are similar to the proteolytic profile observed in HIV infection of the brain. The analysis of the proteasome proteolytic assay data with regard to the ratio of chymotrypsin-like activity to post-glutamyl peptide hydrolyzing activity highlighted this immunoproteasome proteolytic activity profile. Compared to the analysis of the individual proteasome enzymatic activities, the analysis of the enzymatic ratios showed differences between comparison groups with higher magnitude and statistical significance, which further implicated the presence of an increase in the immunoproteasome proteolytic activity profile and the induction of immunoproteasomes in subjects with HIV-associated neuropsychological impairment and encephalitis.

Chapter 4: Altered Proteasome Subunit Expression in HIV-Infected Brains

4.1 INTRODUCTION

The previous finding of an altered proteasome proteolytic profile in HIV-infected brains suggested that the anomaly was due to an alteration of 20S proteasome beta–subunits containing the proteolytic active sites. The most likely proteasome alteration was the incorporation of inducible immunoproteasome subunits into proteasome complexes in HIV-infected brain specimens. To test the hypothesis of immunoproteasome induction in HIV-infected brains, and to investigate the state of brain proteasome complex composition overall, I evaluated the protein expression levels of proteasome and immunoproteasome subunits in frontal cortex and white matter homogenate samples by immunoblotting. Initial analyses consisted of a proteasome subunit screening to detect changes in protein expression of the different classes of protease subunit including the alpha and beta subunits of the 20S proteasome core and subunits from the immunoproteasome 11S and constitutive proteasome 19S regulatory complexes. From this screening, the subunits identified as having altered protein expression levels in HIV-infected brain specimens were further analyzed across all study subjects in the selected cohort. I quantified the protein expression levels of these subunits from a series of immunoblots using optical densitometry and compared the results between groups according to HIV status to determine significance. I analyzed the correlation between immunoproteasome subunit levels and the previously characterized proteasome proteolytic profile to determine the relationship between immunoproteasome induction and the changes in proteasome enzymatic activities associated with HIV

infection. Additionally I analyzed the subunit levels with regard to neuropsychological impairment and diagnosis of HIV encephalitis.

4.2 RESULTS

4.2.1 Proteasome Subunit Screening

Immunoblotting analysis was used to compare frontal cortex and white matter tissue homogenate samples from eight HIV-negative subjects and eight HIV-positive subjects. Subjects were selected for the screening analysis based on the enzymatic activity profile previously analyzed to maximize the probability of detecting differences in proteasome subunit expression. The proteasome subunits analyzed included 20S proteasome core alpha and beta subunits, inducible immunoproteasome 11S regulatory complex subunit PA28 alpha, and constitutive proteasome 19S regulatory complex ATPase and non-ATPase subunits.

The analysis of the 20S proteasome core subunits revealed that the protein expression levels of inducible immunoproteasome subunits LMP7, LMP2, and MECL-1 were elevated in both cortical and white matter tissue samples of HIV-positive subjects compared to HIV-negative subjects (**Figure 4.1B**). Standard, constitutively expressed beta subunits X, Y, and Z showed no substantial differences between HIV-positive and HIV-negative subjects (**Figure 4.1A**). 20S proteasome alpha subunits were comparable between groups (**Figure 4.1E**).

The analysis of proteasome regulatory complex subunits showed increases in the inducible immunoproteasome 11S regulatory complex subunit PA28 alpha in HIV-positive subjects in both cortical and white matter tissue, similar to the protein expression of the immunoproteasome beta subunits (**Figure 4.1D**). Four constitutive proteasome 19S regulatory complex subunits were analyzed (**Figure 4.1C**). In frontal cortex tissue

homogenate samples, 19S non-ATPase subunit Rpn2 was decreased in HIV-positive subjects. 19S regulatory complex subunits Rpt1, Rpt5, and Rpn8 showed no appreciable differences. White matter 19S regulatory complex subunits showed similar protein expression levels between groups.

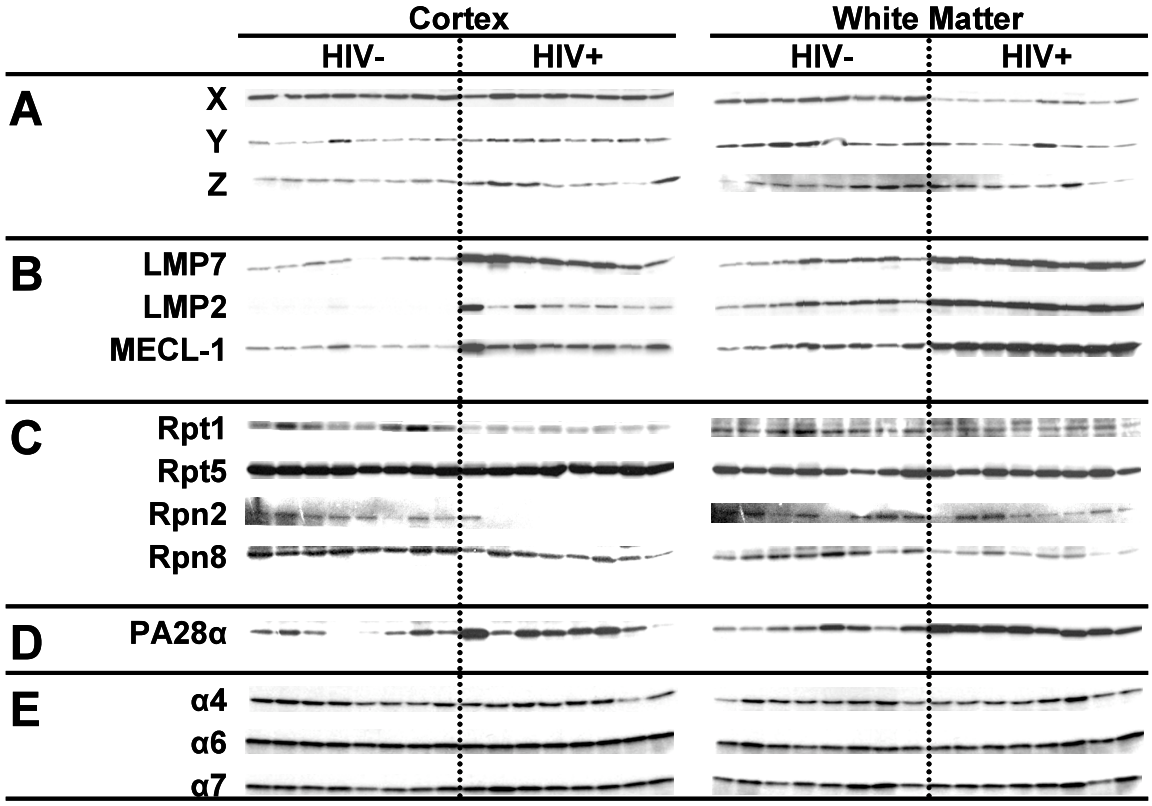


Figure 4.1 Proteasome subunit screening indicates increased protein expression levels of immunoproteasome subunits. Western blot analyses of frontal cortex and white matter samples from eight HIV-negative subjects and eight HIV positive subjects with altered proteinase profiles were performed to determine the subunit composition of brain proteasomes. (A) Analysis of constitutive 20S proteasome subunits that contain proteolytic active sites showed no difference between HIV-negative and HIV-positive subjects in either cortex or white matter samples. (B) All three inducible 20S immunoproteasome subunits were increased in HIV-positive subjects in both cortex and white matter samples compared to HIV-negative subjects. (C) Of the 19S proteasome regulatory complex subunits analyzed, only Rpn2 showed any difference between the compared groups, with a decrease in the frontal cortex samples of HIV-positive subjects. (D) Immunoproteasome 11S regulatory subunit PA28 alpha was increased in HIV-positive subjects in both cortex and white matter samples. (E) Proteasome 20S alpha subunits showed equal protein expression levels in both cortex and white matter samples.

4.2.2 Immunoproteasome Subunit Protein Expression Level Quantification

To confirm the differences in immunoproteasome subunit protein expression levels found in the previous screening, the levels of inducible 20S beta-subunit LMP7 and 11S subunit PA28 alpha were assessed in all subjects of the study cohort, consisting of the 88 HIV-positive subjects and 65 HIV-negative subjects previously analyzed for proteasome enzymatic activity. Immunoproteasome protein expression levels were measured in a series of immunoblots with randomized brain tissue homogenate samples. Detected bands were converted to digital images and quantified using densitometry software. Frontal cortex and white matter immunoproteasome levels were analyzed separately.

The protein expression levels of immunoproteasome subunits were significantly higher in HIV-positive subjects in both cortex and white matter compared to HIV-negative subjects. The mean LMP7 level in HIV-positive subjects was 99 % higher than that of HIV-negative subjects in frontal cortex ($p < 10^{-10}$, **Figure 4.3A**). Analysis of white matter revealed an LMP7 increase in HIV-positive subjects of 184 % ($p < 10^{-10}$**, Figure 4.3B**). Immunoproteasome 11S subunit PA28 alpha analysis showed higher magnitudes of increased protein expression levels. Mean PA28 alpha levels in HIV-positive subjects were 204 % ($p < 10^{-7}$**, Figure 4.3C**) and 233 % ($p < 10^{-7}$**, Figure 4.3D**) higher in frontal cortex and white matter, respectively.

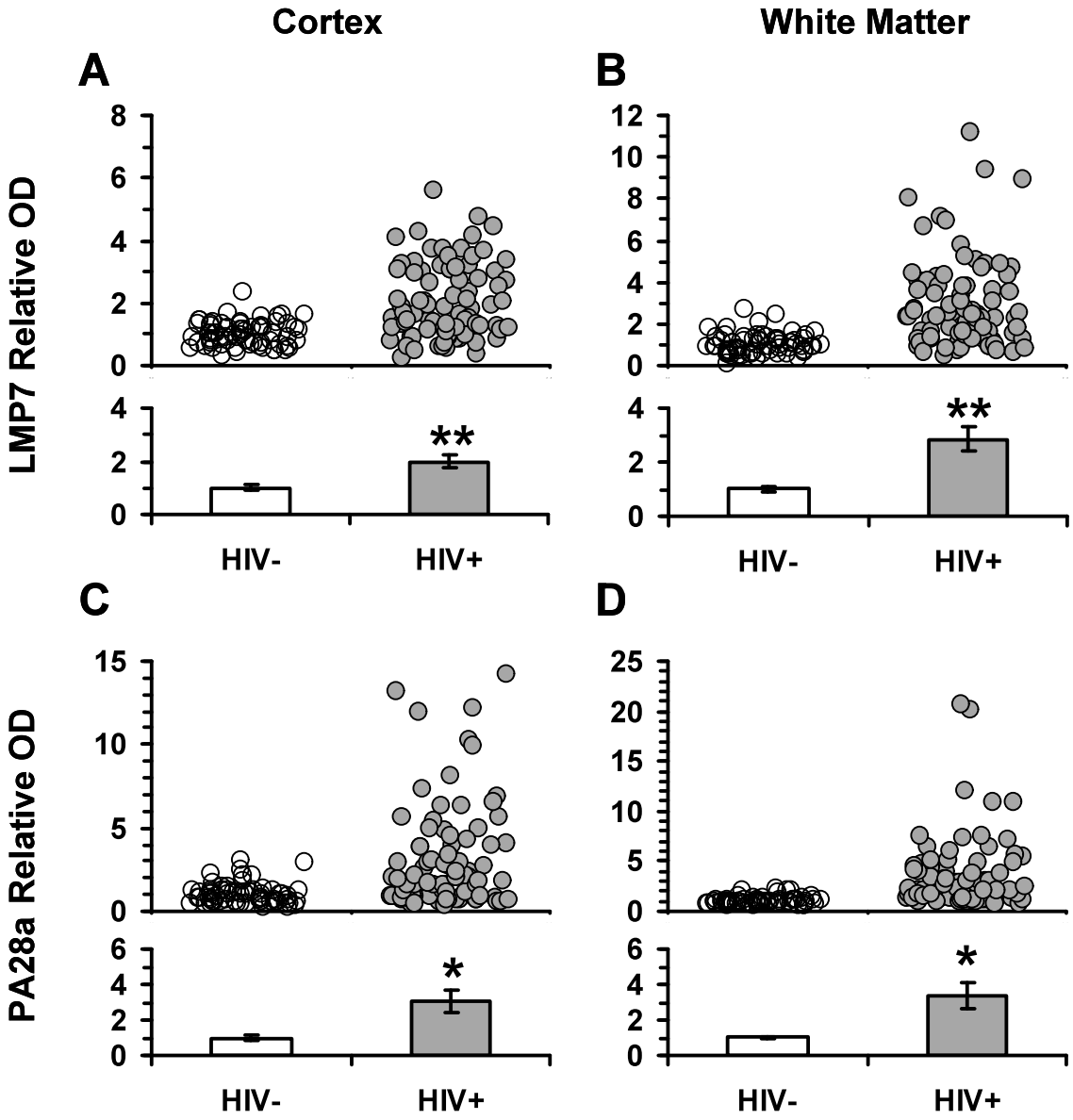


Figure 4.2 Increased immunoproteasome protein expression levels in HIV-positive subjects. Protein expression levels of immunoproteasome 20S beta subunit LMP7 and 11S subunit PA28 alpha were determined by densitometry of bands detected by immunoblotting. LMP7 levels in HIV-positive subjects were increased by (A) 99 % in cortex samples and (B) 184 % in white matter samples compared to HIV-negative subjects. PA28 alpha levels were increased by (C) 204 % in cortex samples and (D) 233 % in white matter samples (*, $p < 10^{-7}$, **, $p < 10^{-10}$).

4.2.3 Proteasome Proteolytic Profile Alteration Correlates with Immunoproteasome Subunit Protein Expression

The previous analysis of proteasome enzymatic activity revealed an alteration in the proteolytic profile suggestive of immunoproteasome subunit switching. To assess the relationship between proteolytic activity alteration and immunoproteasome induction associated with HIV infection of the brain, the proteasome proteolytic profile assessment data was compared to the protein expression levels of immunoproteasome beta subunit LMP7, which contains the proteasome active site putatively involved in the change in enzymatic activity. The comparison between the proteasome proteolytic profile and LMP7 expression showed highly significant positive correlations in both frontal cortex ($r = 0.609$; $p < 10^{-15}$; **Figure 4.3A**) and white matter ($r = 0.737$; $p < 10^{-26}$; **Figure 4.3B**).

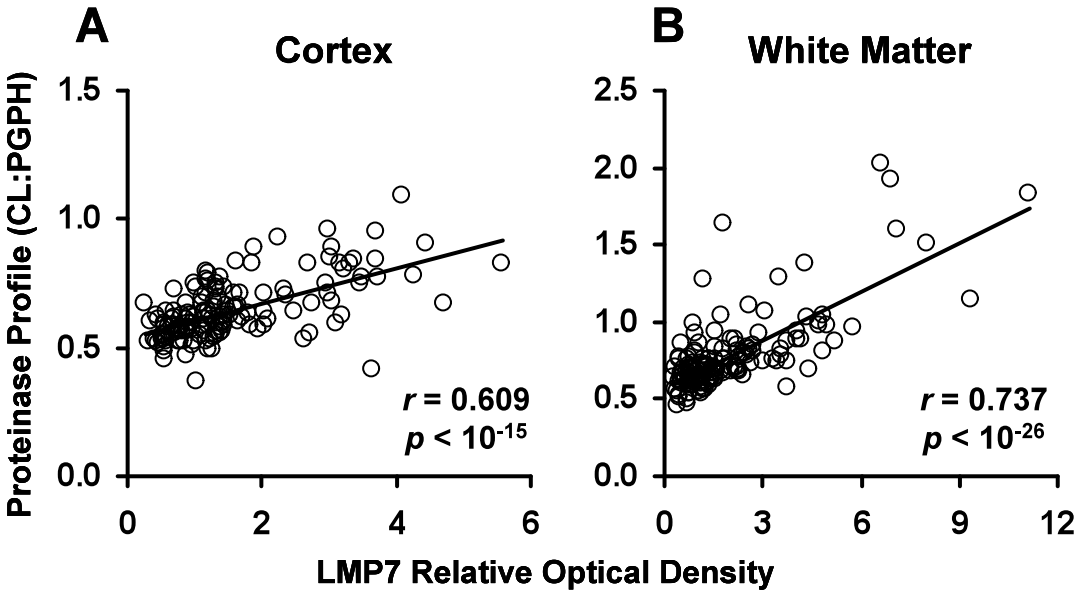


Figure 4.3 Proteasome proteolytic profile correlates with immunoproteasome subunit LMP7 protein expression. The proteasome proteolytic profile, which was suggestive of immunoproteasome induction in HIV-infected subjects, was compared to immunoproteasome subunit LMP7 protein expression. Significant correlations between the two measurements were found in both (A) cortex and (B) white matter, suggesting a causal relationship between immunoproteasome induction and changes in proteasome enzymatic activities associated with HIV infection.

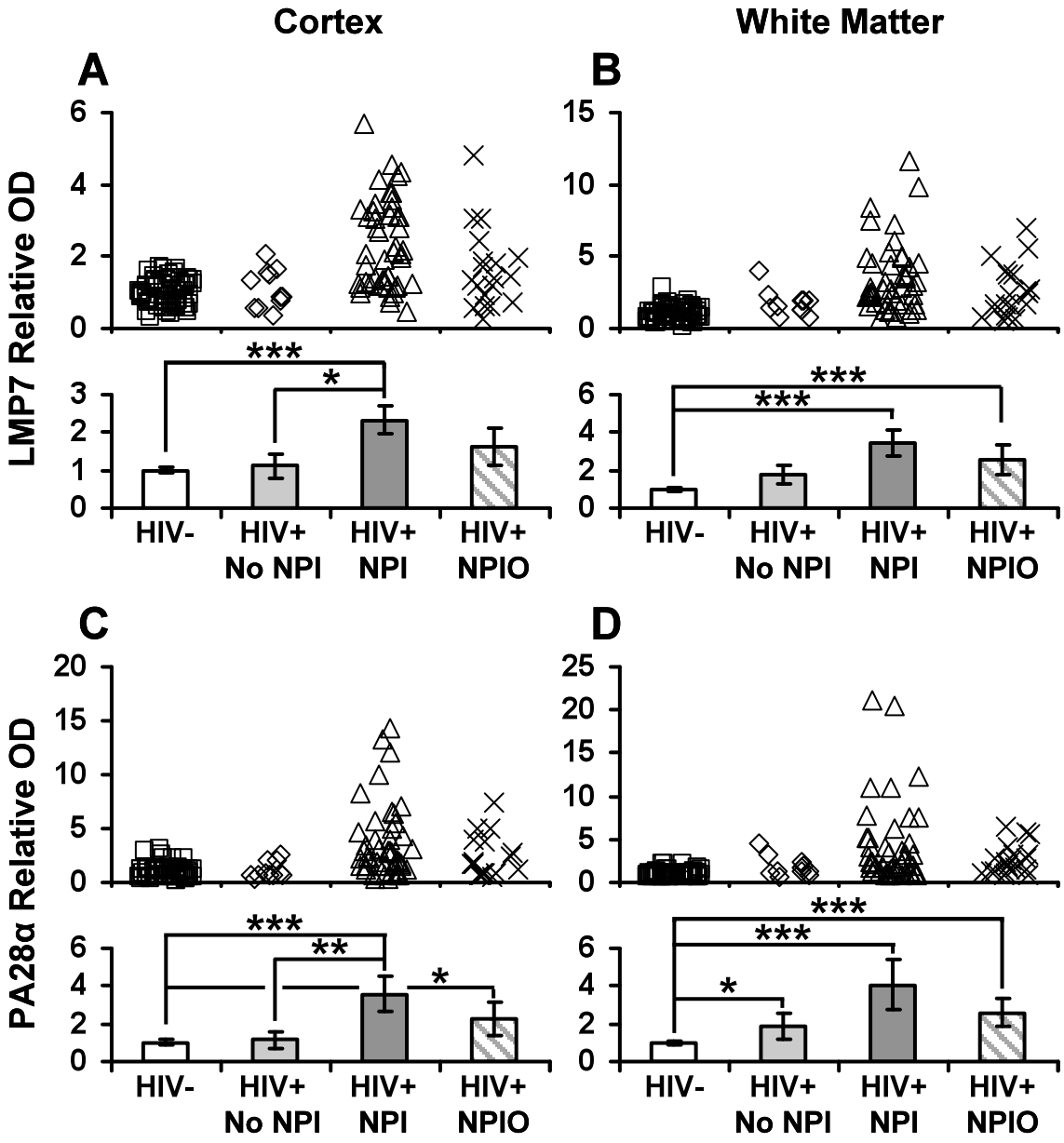
4.2.4 Increased Immunoproteasome Subunits in HIV-Positive Subjects with Neuropsychological Impairment

The protein expression levels of immunoproteasome subunits were analyzed to determine the relationship between increased immunoproteasomes and neuropsychological impairment. Protein expression levels of immunoproteasome 20S subunit LMP7 and 11S subunit PA28 alpha were analyzed by ANOVA and post-hoc tests comparing groups. Subject data was separated into groups defined as HIV-negative, HIV-positive without neuropsychological impairment, HIV-positive with HIV-associated neuropsychological impairment, and HIV-positive with neuropsychological impairment not specifically due to HIV infection.

Average LMP7 protein expression levels were significantly different between groups in both cortex ($p < 0.0001$; **Figure 4.4A**) and white matter ($p < 0.0001$; **Figure 4.4B**). HIV-positive subjects with HIV-associated neuropsychological impairment had increases in LMP7 protein expression levels of 133 % in cortex ($p < 0.001$) and 243 % in white matter ($p < 0.001$) compared to the levels of HIV-negative subjects, and increases of 110 % in cortex ($p < 0.05$) compared to the levels of HIV-positive subjects without neuropsychological impairment. Additionally, the HIV-positive subjects with neuropsychological impairment and other comorbid factors had increased LMP7 levels of 154 % ($p < 0.01$) in white matter compared to HIV-negative subjects.

Average protein expression levels of PA28 alpha were also significantly different between groups in cortex ($p < 0.0001$; **Figure 4.4C**) and white matter ($p < 0.0001$, **Figure 4.4D**). In frontal cortex, PA28 alpha levels in HIV-positive subjects with HIV-associated neuropsychological impairment were increased by 259 % compared to HIV-negative subjects ($p < 0.001$), and by 216 % compared to HIV-positive subjects without neuropsychological impairment ($p < 0.01$). HIV-positive subjects with

neuropsychological impairment and other comorbid factors had a 126 % increase in PA28 alpha levels compared to HIV-negative subjects as well ($p < 0.05$). In white matter, compared to the HIV-negative subjects, PA28 alpha protein expression levels were increased by 85 % in HIV-positive subjects without neuropsychological impairment ($p < 0.05$), 305 % in those with HIV-associated neuropsychological impairment ($p< 0.001$), and 159 % in those with neuropsychological impairment with other comorbid factors ($p < 0.001$).


Cortex
White Matter
A
B
C
D
LMP7 Relative OD
PA28α Relative OD
HIV-
HIV+ No NPI
HIV+ NPI
HIV+ NPIO
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*

Figure 4.4 Increased immunoproteasome subunit protein expression levels with HIV-associated neuropsychological impairment. Protein expression levels of immunoproteasome subunits (A, B) LMP7 and (C, D) PA28 alpha were compared between groups according to neuropsychological impairment. (A) Cortex sample LMP7 levels were increased in HIV+ NPI by 133 % compared to HIV- and by 110 % compared to HIV+ No NPI. (B) In white matter samples, LMP7 levels of HIV+ NPI and HIV+ NPIO were significantly higher than HIV- by 243 % and 154 %, respectively. (C) Cortex PA28 alpha levels of HIV+ NPI were 259 % higher than HIV- and 216 % higher than HIV+ No NPI. HIV+ NPIO PA28 alpha levels were also 126 % higher than HIV-. (D) In white matter samples, all three HIV+ groups had significantly higher PA28 alpha levels than HIV- (HIV+ No NPI, 85 %; HIV+ NPI, 305 %, HIV+ NPIO, 159 %). (NPI, HIV-associated neuropsychological impairment; NPIO, neuropsychological impairment with comorbid factors; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

4.2.5 Increased Immunoproteasome Subunit Expression in HIV-positive Subjects with and without HIV Encephalitis

Immunoproteasome subunit protein expression levels were analyzed to determine the relationship between increases in immunoproteasomes and the presence of neuropathological findings indicating HIV encephalitis. Similar to previous analyses, the protein expression levels of immunoproteasome subunits LMP7 and PA28 alpha were compared by ANOVA and post-hoc comparison tests. Subject data was divided into groups defined as HIV-negative, HIV-positive without HIV encephalitis, and HIV-positive with HIV encephalitis.

Analysis of average LMP7 protein expression levels revealed significant differences between groups compared in both cortex ($p < 0.0001$; **Figure 4.5A**) and white matter ($p < 0.0001$; **Figure 4.5B**). Cortex LMP7 levels in HIV-subjects both with and without HIV encephalitis were increased by 173 % ($p < 0.001$) and 81 % ($p < 0.001$), respectively, compared to the HIV-negative subjects. White matter LMP7 level in those with HIV encephalitis was increased by 414 % ($p < 0.001$) compared to HIV-negative

subjects and 114 % ($p < 0.05$) compared to HIV-positive subjects without HIV encephalitis. HIV-positive subjects without HIV encephalitis had an LMP7 level 140 % greater than the HIV-negative subjects ($p < 0.001$).

Average PA28 alpha protein expression levels were also significantly different in cortex ($p < 0.0001$; **Figure 4.5C**) and white matter ($p < 0.0001$; **Figure 4.5D**). In cortex samples, those with HIV encephalitis had a PA28 alpha expression level that was 349 % higher than HIV-negative subjects ($p < 0.001$). HIV-positive subjects without HIV encephalitis had a 166 % higher PA28 alpha level compared to HIV-negative subjects. In white matter, PA28 alpha levels were 443 % higher in HIV-positive subjects with HIV encephalitis ($p < 0.001$) and 185 % higher in the HIV-positive subjects without HIV encephalitis ($p < 0.001$) compared to the HIV-negative subjects.

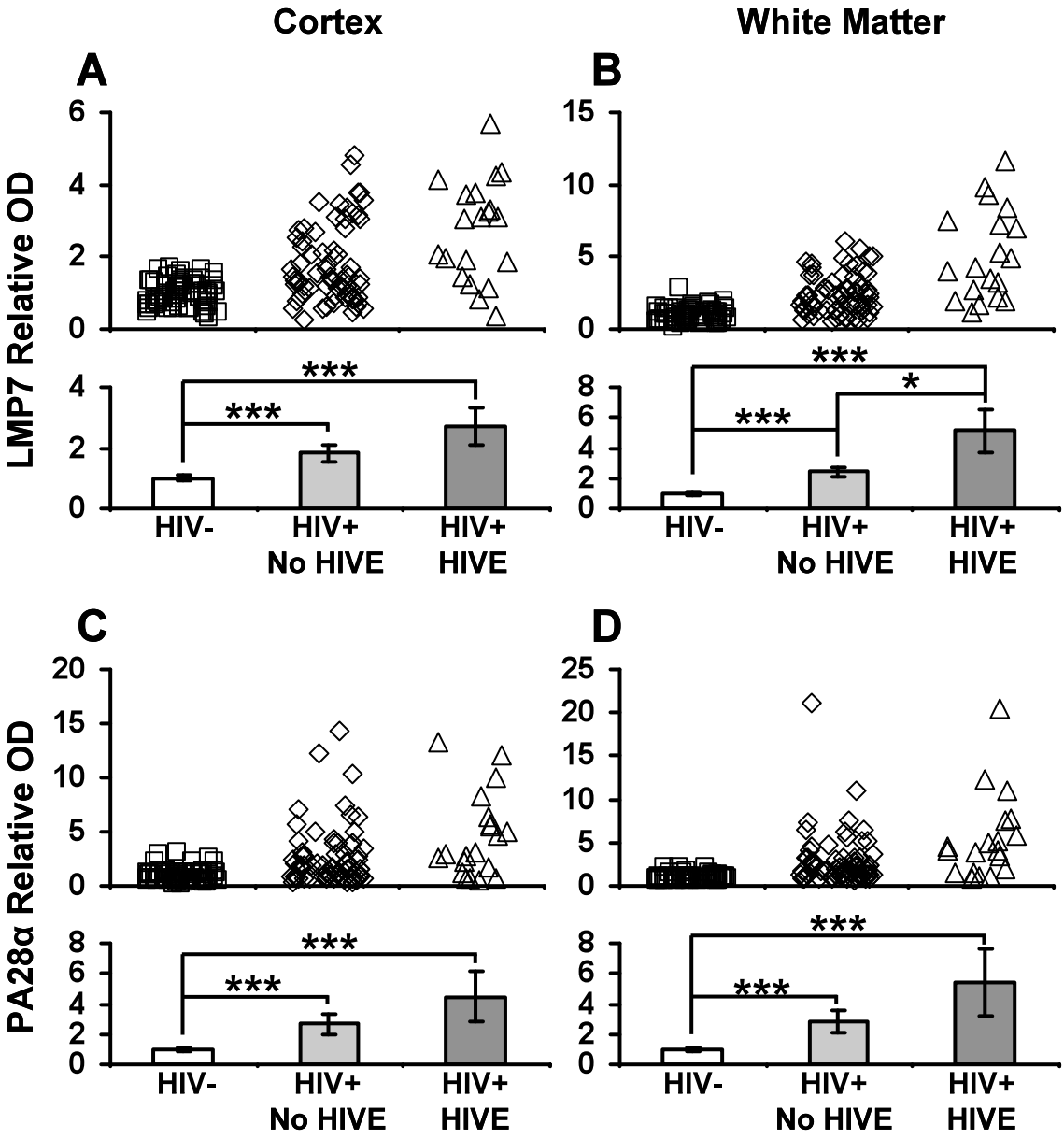
Cortex
White Matter
A
B
C
D
LMP7 Relative OD
PA28α Relative OD
HIV-
HIV+ No HIVE
HIV+ HIVE
***
*

Figure 4.5 Increased immunoproteasome subunit protein expression levels in HIV-positive subjects both with and without HIV encephalitis. Protein expression levels of immunoproteasome subunits (A, B) LMP7 and (C, D) PA28 alpha were compared between groups based on the pathological diagnosis of HIV encephalitis. (A, B) LMP7 levels in HIV+ HIVE were significantly greater than HIV- by 173 % in cortex and 414 % in white matter. Additionally, HIV+ HIVE had a 114 % higher LMP7 level than HIV+ No HIVE in white matter. LMP7 levels of HIV+ No HIVE were 81 % higher in cortex and 140 % higher in white matter, compared to HIV-. (C, D) Analysis of PA28 alpha protein expression levels showed that both HIV+ No HIVE and HIV+ HIVE had increased PA28 alpha levels compared to HIV- in cortex (349 % and 166 %, respectively) and white matter (443 % and 185 %, respectively), but were not significantly different from each other. (HIVE, HIV encephalitis; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

4.2.6 Decreased 19S Regulatory Subunit Rpn2 in HIV-Positive Subjects with Neuropsychological Impairment and HIV Encephalitis

The initial screening of proteasome subunits identified a substantial decrease in 19S regulatory complex non-ATPase subunit Rpn2 in frontal cortex samples. Analysis of 19S subunit Rpn2 protein expression levels was performed across all study subjects using immunoblotting in a similar manner to the analysis of immunoproteasome protein expression. 19S subunit Rpn2 levels were compared between HIV-negative and HIV-positive subjects and then analyzed between groups according to neuropsychological and neuropathological diagnoses (**Figure 4.6**). When the protein expression levels of 19S subunit Rpn2 were compared between HIV-negative subjects and HIV-positive subjects, a slight decrease was detected in the HIV-positive group (11 %), but it was not statistically significant ($p = 0.3889$; **Figure 4.6A**). However, Rpn2 protein expression analysis with HIV-positive groups separated into those with and without neuropsychological impairment showed a significant difference between groups ($p < 0.05$), with a 24 % decrease in Rpn2 in HIV-positive subjects with neuropsychological impairment compared to HIV-negative subjects ($p < 0.05$; **Figure 4.6B**). Further analysis

comparing groups with and without HIV encephalitis also showed a significant difference between groups ($p < 0.01$), with a Rpn2 level in HIV-positive subjects with HIV encephalitis that was 44 % lower than HIV-negative subjects ($p < 0.01$) and 43 % lower than HIV-positive subjects without HIV encephalitis ($p < 0.05$; **Figure 4.6C**).

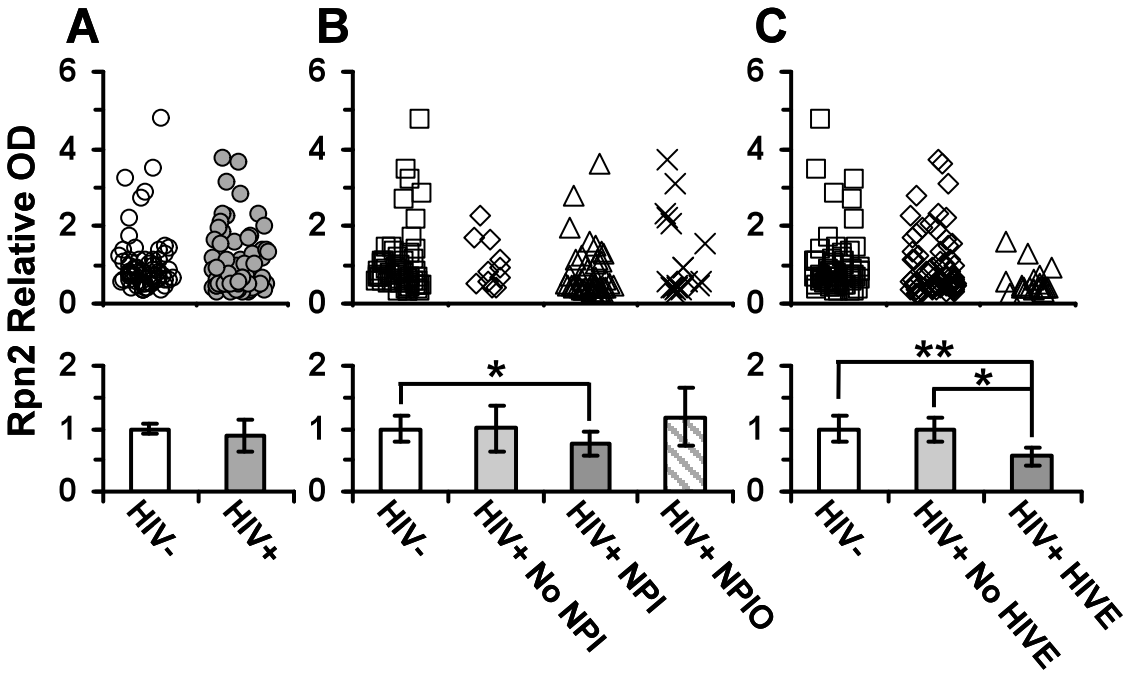

Figure 4.6 Decreased 19S regulatory non-ATPase subunit Rpn2 protein expression levels in those with neuropsychological impairment and those with HIV encephalitis. Analysis of 19S subunit Rpn2 protein expression levels was performed using frontal cortex tissue samples from all study subjects. (A) Comparison of Rpn2 levels between HIV- and HIV+ showed a slight decrease that was not significant. (B) When Rpn2 levels were compared between groups according to neuropsychological impairment, the level in HIV+ NPI was significantly lower than HIV- by 24 %. (C) Comparison between groups according to HIVE diagnosis showed that Rpn2 levels of HIV+ HIVE were significantly lower than both HIV+ No HIVE and HIV- by 43 % and 44 %, respectively. (NPI, HIV-associated neuropsychological impairment; NPIO, neuropsychological impairment with comorbid factors; HIVE, HIV encephalitis; *, $p < 0.05$; **, $p < 0.01$)

4.3 DISCUSSION

The investigation of proteasome subunit composition in brain specimens of HIV-infected subjects indicated significantly increased protein expression levels of inducible immunoproteasome subunits, especially in those with HIV-associated neuropsychological impairment and those with HIV encephalitis. These data support the hypothesis that an altered proteasome subunit composition modified the 20S proteasome active sites, which in turn resulted in changes to the proteasome proteolytic activities. The initial screening of proteasome units in a subset of study subjects showed substantially increased protein expression levels of the three inducible immunoproteasome 20S beta subunits LMP7, LMP2, and MECL-1 and the 11S regulatory complex subunit PA28 alpha in both frontal cortex and white matter samples in the HIV-positive subjects. Analysis of immunoproteasome subunits in all study subjects focused on LMP7 and PA28 alpha and revealed highly significant increases in protein expression levels of these subunits in HIV-positive subjects in both frontal cortex and white matter tissue samples. Comparisons between LMP7 protein levels and the previously assessed proteasome proteolytic activity profile showed highly significant correlations that supports the causal relationship between immunoproteasome induction and the alteration of proteasome proteolytic activity, namely the increase in chymotrypsin-like activity and concomitant decrease in post-glutamyl peptide hydrolyzing activity. Further refinement of the analysis indicated that the increase in immunoproteasome subunits was highest in those with HIV-associated neuropsychological impairment and in those with HIV encephalitis. The proteasome subunit screening also identified a decrease in 19S regulatory complex non-ATPase subunit Rpn2 in the cortex of HIV-positive subjects. When 19S subunit Rpn2 protein expression levels were assessed in all study subjects, no significant differences between HIV-negative and HIV-positive subjects were detected. However,

the additional analysis revealed slight, but statistically significant decreases in the Rpn2 protein expression levels of HIV-subjects with associated neuropsychological impairment and of HIV encephalitis subjects. A summary of the observed changes in proteasome subunit protein expression levels is provided in **Table 4.1**.

Table 4.1 Summary of changes in proteasome subunit protein expression

	LMP7		PA28α		Rpn2
	Cortex	White Matter	Cortex	White Matter	Cortex
HIV Status					
HIV- vs HIV+	99%[5]	184%[5]	204%[4]	233%[4]	ns
Neuropsychological Impairment					
HIV- vs HIV+ No NPI	ns	ns	ns	85%[1]	ns
HIV- vs HIV+ NPI	133%[3]	243%[3]	259%[3]	305%[3]	-24%[1]
HIV- vs HIV+ NPIO	ns	154%[3]	126%[1]	159%[3]	ns
HIV+ No NPI vs HIV+ NPI	110%[1]	ns	216%[2]	ns	ns
HIV+ No NPI vs HIV+ NPIO	ns	ns	ns	ns	ns
HIV+ NPI vs HIV+ NPIO	ns	ns	ns	ns	ns
Neuropathology					
HIV- vs HIV+ No HIVE	81%[3]	140%[3]	166%[3]	185%[3]	ns
HIV- vs HIV+ HIVE	173%[3]	414%[3]	349%[3]	443%[3]	-44%[2]
HIV+ No HIVE vs HIV+ HIVE	ns	114%[1]	ns	ns	-43%[1]

Note: differences between compared groups are noted as percent change relative to the first group. NPI, HIV-associated neuropsychological impairment; NPIO, neuropsychological impairment with comorbid factors; HIVE, HIV encephalitis; ns, no significant difference; [1], $p < 0.05$; [2], $p < 0.01$; [3], $p < 0.001$, [4], $p < 10^{-7}$; [5], $p < 10^{-10}$

The screening of proteasome subunits also provided insights into the status of constitutive proteasome subunits. Protein expression of constitutive proteasome 20S beta

subunits X, Y, and Z were no different between the HIV-negative and HIV-positive subjects analyzed. Protein expression levels of 20S proteasome alpha subunits also showed no difference between HIV-negative and HIV-positive subjects. Results of screening for differences in other proteasome 19S regulatory complex subunits showed no substantial changes in expression.

The dramatic increase in both immunoproteasome 20S beta and 11S regulatory complex subunits in the presence of decreased or unaltered constitutive proteasome subunits indicates an incorporation of inducible subunits to produce immunoproteasome complexes in HIV-infected brains well beyond the steady state levels of normal, non-infected brains. Analyses of proteasomes under the influence of inflammatory cytokine interferon-gamma have shown that incorporation of the inducible 20S subunits prevents the processing of constitutive subunit precursors, resulting in increased immunoproteasomes at the expense of constitutive proteasomes (Akiyama, Yokota et al. 1994; Fruh, Gossen et al. 1994). Interferon-gamma has also been shown to decrease and destabilize constitutive 26S proteasome complexes (Bose, Brooks et al. 2001; Bose, Stratford et al. 2004) and induce the expression of the 11S regulatory complex that preferentially associates with 20S proteasome containing immunoproteasome subunits (Groettrup, Khan et al. 2001). This induction of the immunoproteasome complex serves to enhance the generation of peptides for MHC class I antigen presentation, but at the same time may alter the ability to handle housekeeping proteasome-mediated proteolysis due to the inhibition of 19S regulatory complexes, which recognize and process ubiquitinated-protein substrates.

The central nervous system has long been considered to be an immunologically privileged organ with low constitutive expression levels of MHC molecules (Xiao and Link 1998), thus abrogating the need for immunoproteasomes, whose primary function is

processing peptides for MHC class I antigen presentation. When proteasome subunit tissue distributions were characterized in rats, immunoproteasome subunits were nearly absent in brain tissue, contrasting the abundance found in spleen, lung, and liver (Noda, Tanahashi et al. 2000). Even when endotoxemia was induced in rats by injections of lipopolysaccharide, immunoproteasome subunit protein expression was undetectable in brain tissue, but significantly increased in heart, kidney and lungs (Nelson, Loukissa et al. 2000). However, examination of proteasome subunits in a rat model of traumatic brain injury showed increased mRNA and protein expression of immunoproteasome subunits after lateral fluid perfusion injury (Yao, Liu et al. 2008), indicating that immunoproteasomes can be induced in response to stress and injury in the central nervous system.

The presence of immunoproteasome subunits in the human central nervous system has been documented, but increased protein expression of immunoproteasomes has only recently been observed in a handful of studies investigating neurodegenerative diseases. The analysis of purified proteasomes from normal human brain revealed that inducible immunoproteasome 20S beta subunits LMP2, LMP7, and MECL-1 are present in the central nervous system, but only at a third to a half of the level found in other organs such as the kidneys (Piccinini, Mostert et al. 2003). Induction of immunoproteasomes in a neurodegenerative disease was first described in Huntington's disease (Diaz-Hernandez, Hernandez et al. 2003). Initial investigations using the HD94 conditional mouse model of Huntington's disease showed increases in immunoproteasome subunits LMP2 and LMP7 in the cortex and striatum associated with neurodegeneration. Assessment of immunoproteasome subunits in the cortex and striatum of Huntington's disease subjects by immunoblotting confirmed the increase in immunoproteasome levels. The presence of immunoproteasomes was also investigated

in Alzheimer's disease (Mishto, Bellavista et al. 2006). Immunoblotting analysis showed that immunoproteasome subunits were present in the hippocampus and cerebellum of Alzheimer's disease subjects at higher levels compared to non-demented elderly subjects. Immunoproteasome levels were even lower in younger brain samples, suggesting that expression is upregulated during the aging process, especially when neurofibrillary tangles and senile plaques are present.

The induction of immunoproteasome expression was associated strongly with the infection of the central nervous system by HIV. The increase in immunoproteasome subunit protein expression was highly associated with the diagnoses of neuropsychological impairment and encephalitis associated with HIV infection. To further evaluate this relationship, I analyzed the levels of immunoproteasome subunits with regard to individual measurements of neurocognitive function and measurements of HIV virology, including viral loads and CD4+ T lymphocyte counts in blood plasma.

Chapter 5: Clinical and Virological Correlations with Immunoproteasome Induction

5.1 INTRODUCTION

Investigations of immunoproteasome induction in the brains of HIV-infected subjects indicated increased immunoproteasome subunit protein expression that was strongly associated with HIV-associated neuropsychological impairment. The neuropsychological diagnosis for each subject was based upon performance on a battery of neurocognitive functioning tests and the analyses of these results by neuropsychologists. Administration of these neurocognitive tests allowed for a concise evaluation of specific domains of cognitive functioning known to be compromised in HIV-associated dementia and minor cognitive motor disorder. To determine the influence of immunoproteasome induction on specific cognitive functioning domains, I compared the protein expression levels of immunoproteasome subunits to the results of the individual neurocognitive functioning tests for each HIV-positive subject.

Additionally, immunoproteasome induction in HIV-infected subjects needed to be examined with regard to virological measurements of HIV replication and disease progression. To that end, I compared the protein expression levels of the immunoproteasome subunits to the HIV loads measured in frontal cortex brain tissue and cerebrospinal fluid to ascertain the variation of immunoproteasome levels with the amount of HIV in the central nervous system. I also compared immunoproteasome subunit levels to HIV loads and CD4+ T-cell counts in blood plasma to assess the relationship with systemic virus replication and the progression of immunodeficiency.

5.2 RESULTS

5.2.1 Immunoproteasome Protein Expression and Neurocognitive Testing Scores

The relationship between the induction of immunoproteasome subunits and specific measures of HIV-associated neuropsychological impairment was determined by examining the extent of correlation between immunoproteasome protein expression levels and the normalized T-scores from individual neurocognitive tests that comprise the neurocognitive testing battery administered to HIV-positive subjects. The neurocognitive testing battery was developed by the National NeuroAIDS Tissue Consortium to perform a concise evaluation of specific domains of cognitive functioning known to be compromised in HIV-associated dementia and minor cognitive motor disorder (Morgello, Gelman et al. 2001). The Wisconsin Card Sorting Test – 64 (WCST-64) assesses abstract and executive functioning, which is primarily driven by frontal lobe circuitry. The Hopkins Verbal Learning Test – Revised (HVLT-R) and Brief Visuospatial Memory Test – Revised (BVMT-R) assesses learning and memory functioning. The Paced Auditory Serial Addition Test (PASAT) assesses working memory function through a series of mental calculations. The Wechsler Adult Intelligence Scale – III (WAIS-III) subtests Digit Symbol and Symbol Search assesses the speed of information processing, which requires the integration of visual information to perform certain tasks. The F-A-S Test measures verbal fluency. The Wide Range Assessment Test (WRAT3) is an assessment of basic reading skills, spelling, and arithmetic that determines intellectual level.

Significant correlations were found between frontal cortex immunoproteasome beta subunit LMP7 protein expression levels and select neurocognitive testing scores. Specifically, LMP7 levels were negatively correlated with the normalized test scores from the WCST-64 (**Figure 5.1**). In the assessment of WCST-64 Categories, the increase in LMP7 was significantly correlated with fewer categories completed, an indication of

poorer performance (r = -0.375, p < 0.01; **Figure 5.1A**). Increased LMP7 protein expression was also negatively correlated with the normalized scores of Total Errors (r = -0.441, p < 0.01; **Figure 5.1B**) and Perseverative Responses (r = -0.409, p < 0.01; **Figure 5.1C**), further indicating that increased LMP7 protein expression in frontal cortex is associated with declining performance on the WCST-64. Further comparisons of frontal cortex LMP7 levels with other neurocognitive testing scores revealed no other significant correlations. Additionally, no significant correlations were found when comparing LMP7 protein expression in white matter with neurocognitive testing scores. A summary of the correlation analyses comparing both cortex and white matter LMP7 protein expression and neurocognitive testing scores is provided in **Table 5.1**.

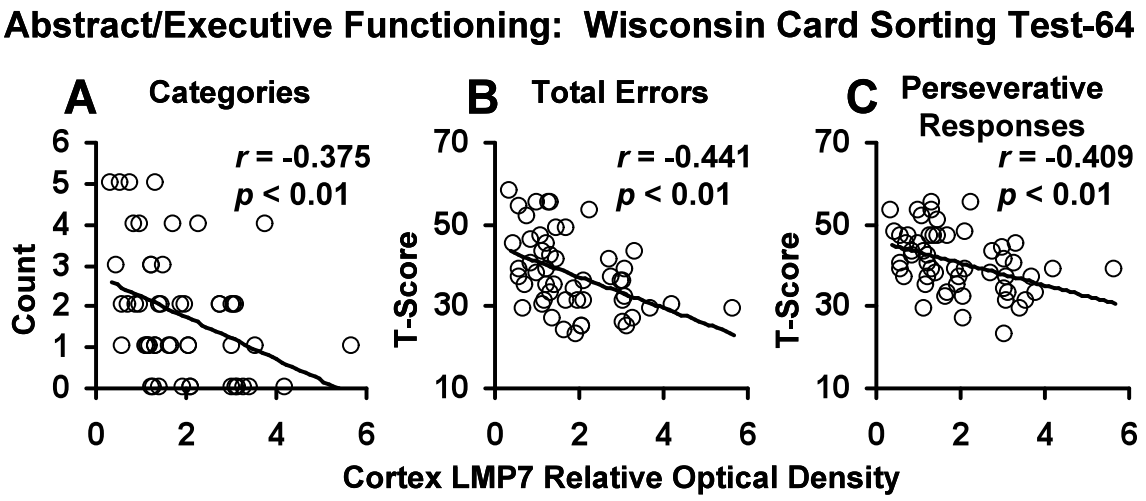

Figure 5.1 Cortex immunoproteasome beta subunit LMP7 correlates with declining performance on the Wisconsin Card Sorting Test – 64 (WCST-64). Analyses of correlations between the LMP7 protein expression level and the neurocognitive testing data assessed for each HIV-positive subject were performed. Significant negative correlations were found when cortex LMP7 levels were compared to WCST-64 testing scores of (A) Categories, (B) Total Errors, and (C) Perseverative Responses. This indicated that increased protein expression of LMP7 in the frontal cortex is associated with worsening performance in the abstract and executive functioning neurocognitive domain. Comparisons of cortex LMP7 levels with other neurocognitive tests showed no significant correlations. White matter LMP7 protein expression was not significantly correlated with neurocognitive tests.

I then explored whether the proteasome 11S subunit expression exhibited the same relationship to neurocognitive testing scores. Significant correlations were again found between frontal cortex immunoproteasome 11S subunit PA28 alpha protein expression levels and neurocognitive testing scores. Like LMP7, PA28 alpha levels also were negatively correlated with the normalized test scores from the WCST-64 scores of Categories (r = -0.407, $p < 0.01$; **Figure 5.2A**), Total Errors (r = -0.392, $p < 0.01$; **Figure 5.2B**) and Perseverative Responses (r = -0.394, $p < 0.01$; **Figure 5.2C**). Additionally, cortex PA28 alpha levels were negatively correlated with the WAIS–III subtests Digit

Symbol (r = -0.260, $p < 0.05$; **Figure 5.2D**) and Symbol Search (r = -0.267, $p < 0.05$; **Figure 5.2E**), indicating worsening performance in the speed of information processing with increases in cortex PA28 alpha. Further comparisons of PA28 alpha levels, both in cortex and white matter, with other neurocognitive testing scores did not reveal any other significant correlations. A summary of the correlation analyses comparing both cortex and white matter PA28 alpha protein expression levels and neurocognitive testing scores is provided in **Table 5.1**.

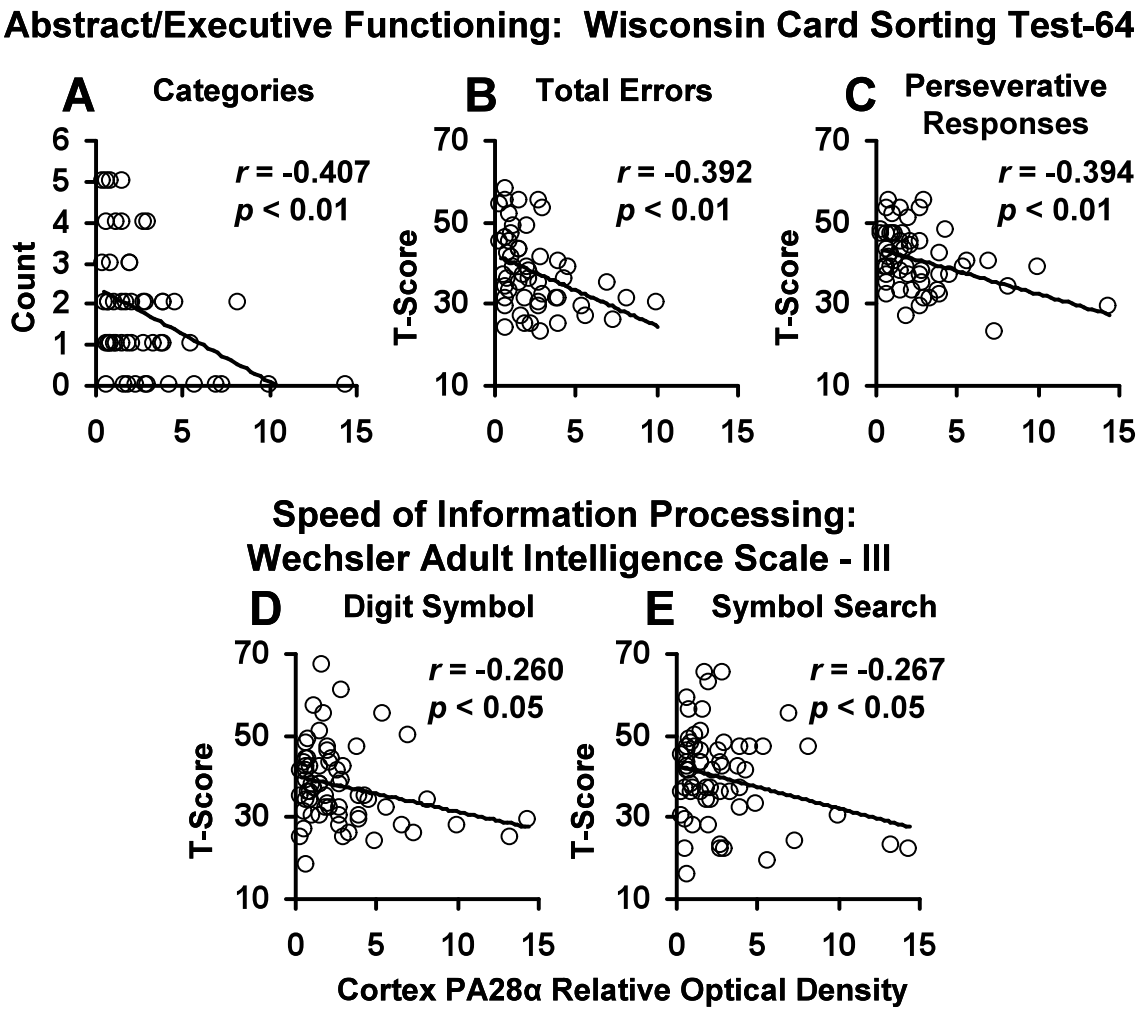

Figure 5.2 Frontal cortex immunoproteasome 11S subunit PA28 alpha protein expression levels correlated with worsening performance on the Wisconsin Card Sorting Test – 64 (WST-64) and the Wechsler Adult Intelligence Scale – III (WAIS-III). Correlations between PA28 alpha levels and neurocognitive testing scores assessed for each HIV-positive subject were analyzed. Significant negative correlations were found between frontal cortex PA28 alpha levels and the WCST-64 scores of (A) Categories (B) Total Errors, and (C) Perseverative Responses, and to the WAIS-III (D) Digit Symbol and (E) Symbol Search subtests. This suggests that increased PA28 alpha in the frontal cortex is associated with worsening performance in the abstract and executive functioning and speed of information processing domains. Comparisons of cortex PA28 alpha levels with other neurocognitive testing scores showed no significant correlations. White matter PA28 alpha expression was not significantly correlated with neurocognitive testing scores.

Table 5.1 Summary of the analyses of correlations between immunoproteasome subunit protein expression levels and neurocognitive testing scores assessed in HIV-positive subjects.

	LMP7				PA28α			
Neurocognitive	**Cortex**		**White Matter**		**Cortex**		**White Matter**	
Domain	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Abstract/Executive								
WCST Categories	**- 0.375**	**0.006** *	- 0.210	0.136	**- 0.407**	**0.003** *	0.019	0.896
WCST Total Errors	**- 0.441**	**0.001** *	- 0.159	0.260	**- 0.392**	**0.004** *	0.053	0.707
WCST Perseverative Responses	**- 0.409**	**0.002** *	- 0.101	0.462	**- 0.394**	**0.003** *	0.036	0.792
Speed of Information Processing								
Digit Symbol	- 0.058	0.650	- 0.145	0.254	**- 0.260**	**0.038** *	0.109	0.390
Symbol Search	- 0.086	0.504	- 0.185	0.149	**- 0.267**	**0.036** *	0.035	0.786
Processing Speed Index	- 0.031	0.809	- 0.134	0.294	- 0.226	0.075	0.075	0.559
Attention/Working Memory								
PASAT	- 0.046	0.780	- 0.255	0.112	- 0.095	0.561	- 0.141	0.386
Letter Number	- 0.063	0.623	- 0.194	0.125	- 0.195	0.122	- 0.070	0.583
Memory								
BVMT Free Delay Recall	- 0.079	0.548	- 0.175	0.181	- 0.228	0.080	0.069	0.601
HVLT Free Delay Recall	0.004	0.977	- 0.033	0.794	- 0.170	0.176	0.018	0.883
Learning								
BVMT Total	- 0.151	0.249	- 0.223	0.087	- 0.209	0.109	0.037	0.781
HVLT Total	0.039	0.753	- 0.080	0.522	- 0.112	0.369	- 0.026	0.839
Verbal Fluency								
FAS	- 0.209	0.085	- 0.191	0.117	- 0.197	0.104	- 0.123	0.313
Skills Assessment								
WRAT3	0.200	0.110	0.229	0.069	0.079	0.533	0.140	0.271

* Denotes statistical significance

5.2.2 Immunoproteasome Protein Expression and Virological Data

Analyses were performed to asses the extent of correlation between immunoproteasome subunit protein expression levels and the viral load measurements in brain tissue, cerebrospinal fluid, and blood, and the plasma CD4+ T-cell count. The

laboratory values of CD4+ T-cell count, blood HIV load, and cerebrospinal fluid HIV load measured by the National NeuroAIDS Tissue Consortium were obtained for each HIV-positive subject from blood and cerebrospinal fluid drawn at six-month intervals. The university hospital clinical laboratories measured CD4+ T-cell counts as an indication of the progression of HIV-infection and immunodeficiency. HIV loads were measured in blood and cerebrospinal fluid using the Roche AMPLICOR HIV-1 Monitor Test. Brain tissue viral loads were determined experimentally from RNA extracted from the fresh frozen brain tissue of each HIV-positive subject by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) using primers designed to detect HIV Gag.

The protein expression levels of immunoproteasome 20S subunit LMP7 showed strong correlations with HIV loads and CD4+ T-cell counts (**Figure 5.3**). The strongest correlations were between brain tissue viral loads and the LMP7 levels in both cortex (r = 0.403, $p < 10^{-4}$; **Figure 5.3A**) and white matter (r = 0.491, $p < 10^{-5}$; **Figure 5.3E**). HIV load in cerebrospinal fluid was also significantly correlated with LMP7 levels in cortex (r = 0.387, $p < 0.01$; **Figure 5.3B**) and white matter (r = 0.513, $p < 10^{-4}$; **Figure 5.3F**). Viral load in plasma was correlated significantly with LMP7 levels in cortex (r = 0.415, $p < 0.001$; **Figure 5.3 C**) and white matter (r = 0.325, $p < 0.01$; **Figure 5.3G**) as well. CD4+ T-cell count was negatively correlated with LMP7 levels in cortex (r = -0.333, $p < 0.01$; **Figure 5.3D**), but not white matter (r = -0.227, p = 0.052; **Figure 5.3H**). A summary of the analyses of correlation comparing both cortex and white matter LMP7 protein expression levels and virological measures is provided in **Table 5.2**.

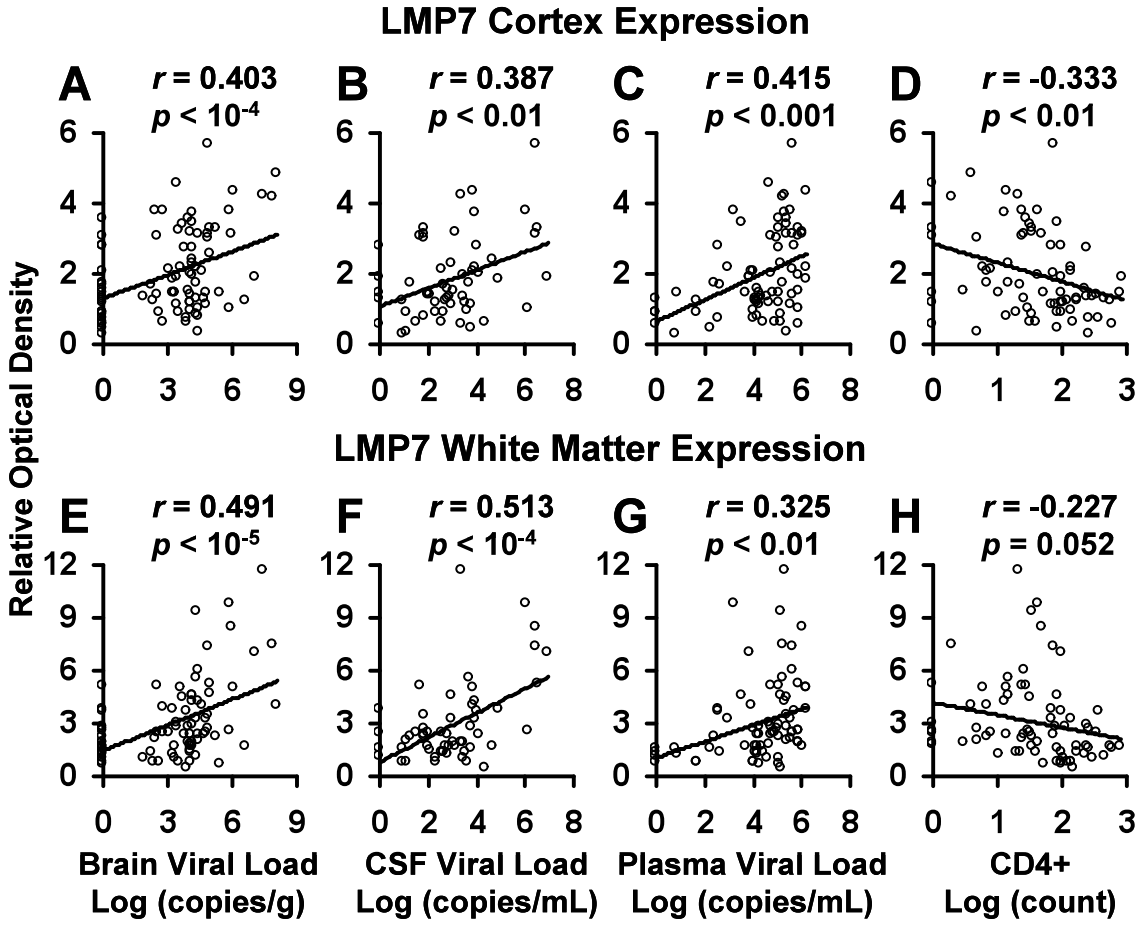

Figure 5.3 Immunoproteasome 20S subunit LMP7 protein expression levels significantly correlated with virological measurements in HIV-positive subjects. Protein expression levels of LMP7 previously measured in the cortex and white matter of HIV-positive subjects were compared to viral loads measured in (A,E) brain tissue, (B,F) cerebrospinal fluid, and (C,G) plasma, as well as to (D,H) CD4+ T-cell counts. Significant correlations were observed for both cortex (A,B,C,D) and white matter (E,F,G) LMP7 levels and these virological measurements, except for the relationship between CD4+ T-cell counts and white matter LMP7 levels (H), which did not reach the level of significance. The strongest correlations were found between LMP7 levels and brain viral load measurements.

Analysis of 11S subunit PA28 alpha protein expression levels also showed strong correlations with virological and immunological results. Cortex PA28 alpha levels were

highly correlated with the HIV loads measured in brain tissue ($r = 0.385$, $p < 0.001$; **Figure 5.4A**). Cortex PA28 alpha levels were also correlated with the viral loads measured in cerebrospinal fluid ($r = 0.342$, $p < 0.01$; **Figure 5.4B**) and plasma ($r = 0.295$, $p < 0.01$; **Figure 5.4C**), and negatively correlated with CD4+ T-cell counts ($r = -0.303$, $p < 0.01$; **Figure 5.4D**). White matter PA28 alpha protein expression levels were highly correlated with cerebrospinal fluid viral load ($r = 0.432$, $p < 0.001$; **Figure 5.4F**), and to a lesser extent, in brain tissue ($r = 0.336$, $p < 0.01$; **Figure 5.4E**) and plasma ($r = 0.227$, $p < 0.05$; **Figure 5.4G**). PA28 alpha levels in white matter were not correlated with CD4+ T-cell counts (**Figure 5.4H**). A summary of the analyses of correlations between both frontal cortex and white matter PA28 alpha protein expression levels and virological measures is provided in **Table 5.2**. Overall, these data provided robust evidence that immunoproteasome synthesis was indeed related to HIV replication to some extent, and to the severity of acquired immunodeficiency to a modest extent.

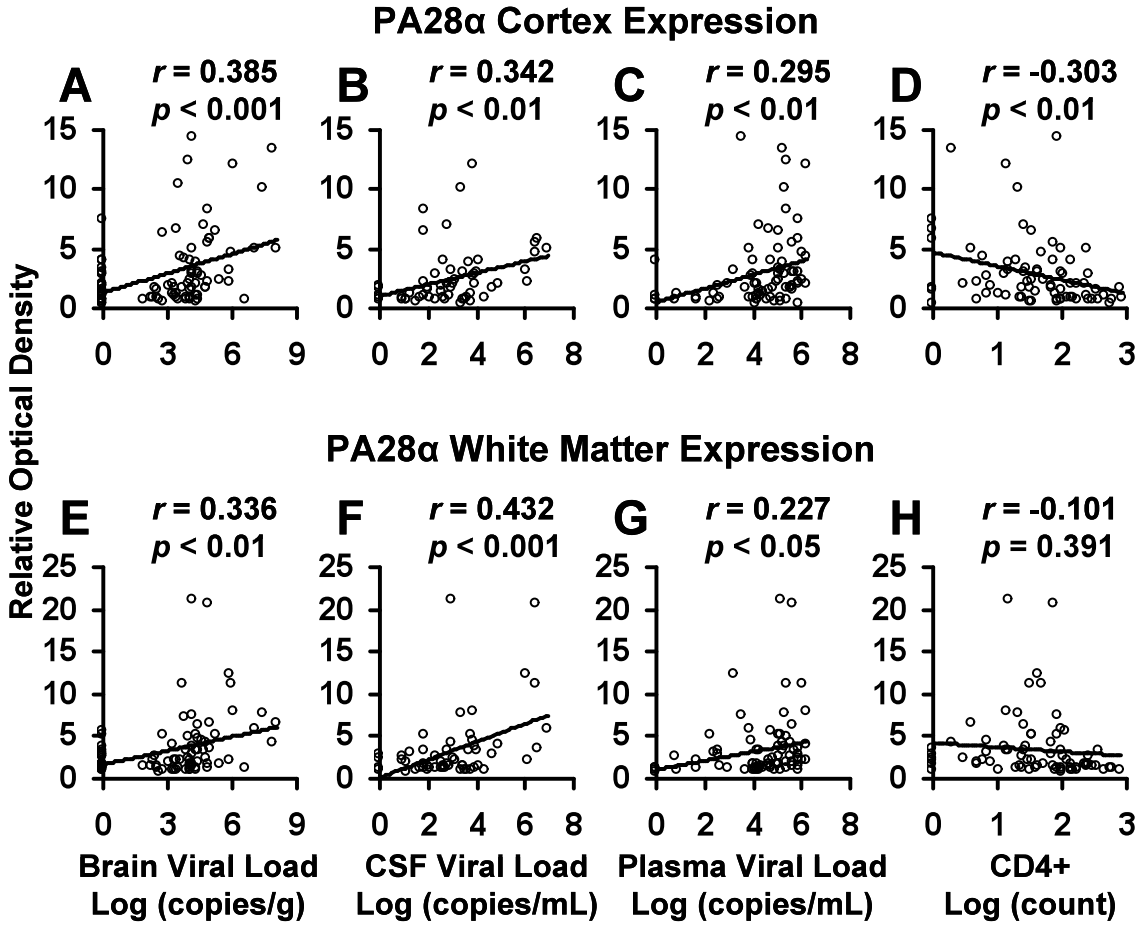

Figure 5.4 Immunoproteasome 11S regulatory complex subunit PA28 alpha protein expression levels significantly correlated with virological measurements. Protein expression levels of PA28 alpha previously measured in the frontal cortex and white matter of HIV-positive subjects were compared to HIV loads measured in (A,E) brain tissue, (B,F) cerebrospinal fluid, and (C,G) plasma, as well as to (D,H) CD4+ T-cell counts. Significant correlations were observed for both cortex (A,B,C,D) and white matter (E,F,G,) PA28 alpha expression and these virological measurements, except for the relationship between CD4+ T-cell counts and white matter PA28 alpha levels (H), which did not reach the level of significance. The strongest correlations were found between brain tissue viral load measurements and PA28 alpha expression in cortex, and CSF viral load and PA28 alpha expression in white matter.

Table 5.2 Summary of the analyses of correlations between immunoproteasome subunit protein expression levels and virological measurements in HIV-positive subjects.

Virological Measurement	LMP7					PA28α				
	Cortex			White Matter		Cortex			White Matter	
	r	*p*		*r*	*p*	*r*	*p*		*r*	*p*
Brain Viral Load	**0.403**	**1E-04**	*	**0.491**	**1E-06**	*	**0.385**	**2E-04**	*	**0.336**	**0.001**	*
CSF Viral Load	**0.387**	**0.003**	*	**0.513**	**5E-05**	*	**0.342**	**0.009**	*	**0.432**	**0.001**	*
Blood Viral Load	**0.415**	**2E-04**	*	**0.325**	**0.004**	*	**0.295**	**0.010**	*	**0.227**	**0.048**	*
CD4+ Count	- **0.333**	**0.004**	*	- 0.227	0.052		- **0.303**	**0.009**	*	- 0.101	0.391	

* Denotes statistical significance

5.3 DISCUSSION

The analyses of immunoproteasome subunit protein expression revealed significant negative correlations with measurements of neurocognitive functioning in HIV-positive subjects. When immunoproteasome levels were compared to the neurocognitive testing scores of the subjects analyzed, both LMP7 and PA28 alpha levels in frontal cortex samples were significantly correlated with declines in performance on the WCST-64. Furthermore, cortex levels of PA28 alpha were also correlated with declines in performance on WAIS-III subtests. The implication of these significant correlations is highlighted when considering the neurocognitive domains assessed during these tests. The WCST-64, which measures performance in abstract and executive functioning, and the WAIS-III, which measures speed of information processing, both assess the functioning of frontal lobe circuitry. The correlation of increased immunoproteasome subunit protein expression in frontal cortex samples with the measures of neurocognitive functioning representative of frontal lobe circuitry provide

support of an association between the induction of immunoproteasomes in the brain and HIV-associated neurocognitive decline.

Both the frontal cortex and white matter protein expression levels of LMP7 and PA28 alpha were significantly correlated with virological measurements in HIV-infected subjects, especially with the viral loads measured in brain tissue and cerebrospinal fluid. Cortex levels of LMP7 and PA28 alpha were also correlated with decreases in CD4+ T-cell counts. While the presence of HIV in the brain contributes to the development of neuropsychological impairment, studies have shown that the viral load or number of infected cells does not necessarily correlate strongly with dementia (Glass, Fedor et al. 1995; Masliah, Heaton et al. 1997). However, the correlation between brain viral loads and immunoproteasome expression indicated a strong interrelationship, with the increasing HIV burden driving the immunological induction of immunoproteasome expression.

Chapter 6: Localization of the Immunoproteasome in HIV-Infected Brains

6.1 INTRODUCTION

The preceding biochemical analysis of proteasomes in HIV-infected brains established that there was an increase in immunoproteasome subunits in frontal lobe brain tissue specimens from HIV-infected subjects that was heightened in those with neuropsychological impairment and in those with HIV encephalitis. The protein expression levels of immunoproteasome subunits were shown to be highly correlative with declining performance on neurocognitive tests measuring frontal lobe functioning as well as with increases in viral loads, particularly those measured in brain tissue and cerebrospinal fluid. These findings provided substantial insight into the proteasome composition and immunoproteasome induction in the HIV-infected brain. What remained to be clarified, however, was the determination of the types of cells induced to express immunoproteasome subunits. To that end, I approached the localization of immunoproteasome subunits in HIV-infected brains using immunohistochemistry performed on archival brain tissue sections. I initially studied the localization of immunoproteasomes using immunoperoxidase immunohistochemistry. Elaboration of those findings was performed by indirect immunofluorescence with parallel staining using antibodies directed against immunoproteasome subunits and specific cell type marker proteins. Assessment of immunofluorescence reactivity was performed using laser confocal microscopy.

6.2 RESULTS

6.2.1 Immunoperoxidase Immunohistochemical Detection of Immunoproteasome Expression

To determine which brain cell populations contained immunoproteasome subunits, preliminary analyses were performed using immunoperoxidase immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections from the prefrontal cortex of HIV-positive subjects were stained using primary antibodies directed against immunoproteasome 20S beta subunits LMP2 and LMP7 and 11S regulatory complex subunit PA28 alpha. All these immunoproteasome antigens yielded histological results that were essentially equivalent to each other. Representative immunostaining with the anti-PA28 alpha antibody in **Figure 6.1** illustrates that immunoproteasomes were present in both neurons and glial cells. Neuronal immunostaining for immunoproteasome subunits occurred in both the cell bodies of cortical gray matter (B, D) and in the axons within white matter (C). Positively stained glial cells were most apparent in white matter, especially in sections containing microglial nodules (A).

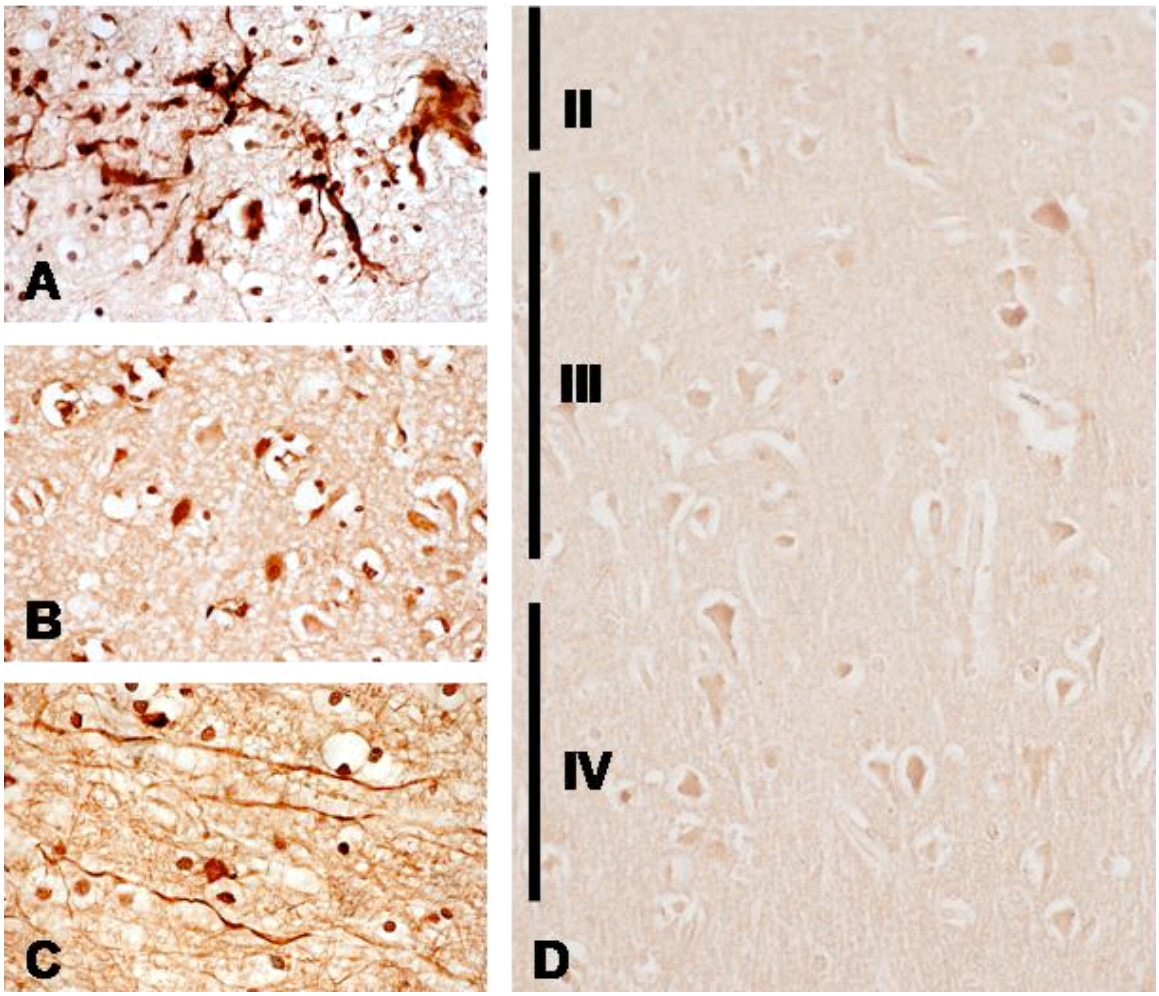

Figure 6.1 Immunoperoxidase detection of immunoproteasome 11S regulatory complex subunit PA28 alpha in neurons and glia. Immunohistochemistry was performed on formalin-fixed paraffin embedded prefrontal cortex tissue sections. Immunoreactivity for 11S subunit PA28 alpha was detected in several cell types in HIV-positive cases, including (B,D) neuronal cell bodies in cortex and (C) neuronal axons in white matter, and (A) microglial nodules in white matter. Cells known to be present in microglial nodules are positively stained, including activated microglia, macrophages, astrocytes, and oligodendrocyte nuclei. Roman numerals in (D) reflect neocortical laminae boundaries.

6.2.2 Immunoproteasome Subunits Localized to Neurons

To confirm the induction of immunoproteasomes in neurons, which would implicate direct involvement of immunoproteasomes in neuronal protein turnover and cellular functioning, simultaneous double immunofluorescence stainings were performed on tissue sections from HIV encephalitis cases using antibodies directed against immunoproteasome subunits and neuron-specific proteins. Antibodies to immunoproteasome 20S subunit LMP2 and 11S regulatory complex subunit PA28 alpha were used to elucidate the localization of two components of the inducible immunoproteasome complex. Identification of neurons and neuronal components was achieved by staining for NeuN, found in neuronal nuclei and perikarya, and neurofilament protein, found primarily in axons and dendrites. Laser confocal microscopy images obtained from double-stained sections revealed that immunoproteasome subunits were indeed localized to neurons. The staining pattern of immunoproteasome 20S subunit LMP2 (**Figure 6.2A**) revealed that immunoproteasomes were present in the perikaryal cytoplasm, with less intensity in nucleus and absence in the nucleolus. PA28 alpha staining in the perikarya (**Figure 6.2B**), in contrast to LMP2, was more evident in the nucleus compared to the cytoplasm. LMP2 and PA28 alpha colocalization with neurofilament staining in white matter showed that immunoproteasome subunits were present in axons (**Figure 6.2 A, B**). Of particular interest is the presence of PA28 alpha immunostaining in swollen axons shown in **Figure 6.2B**.

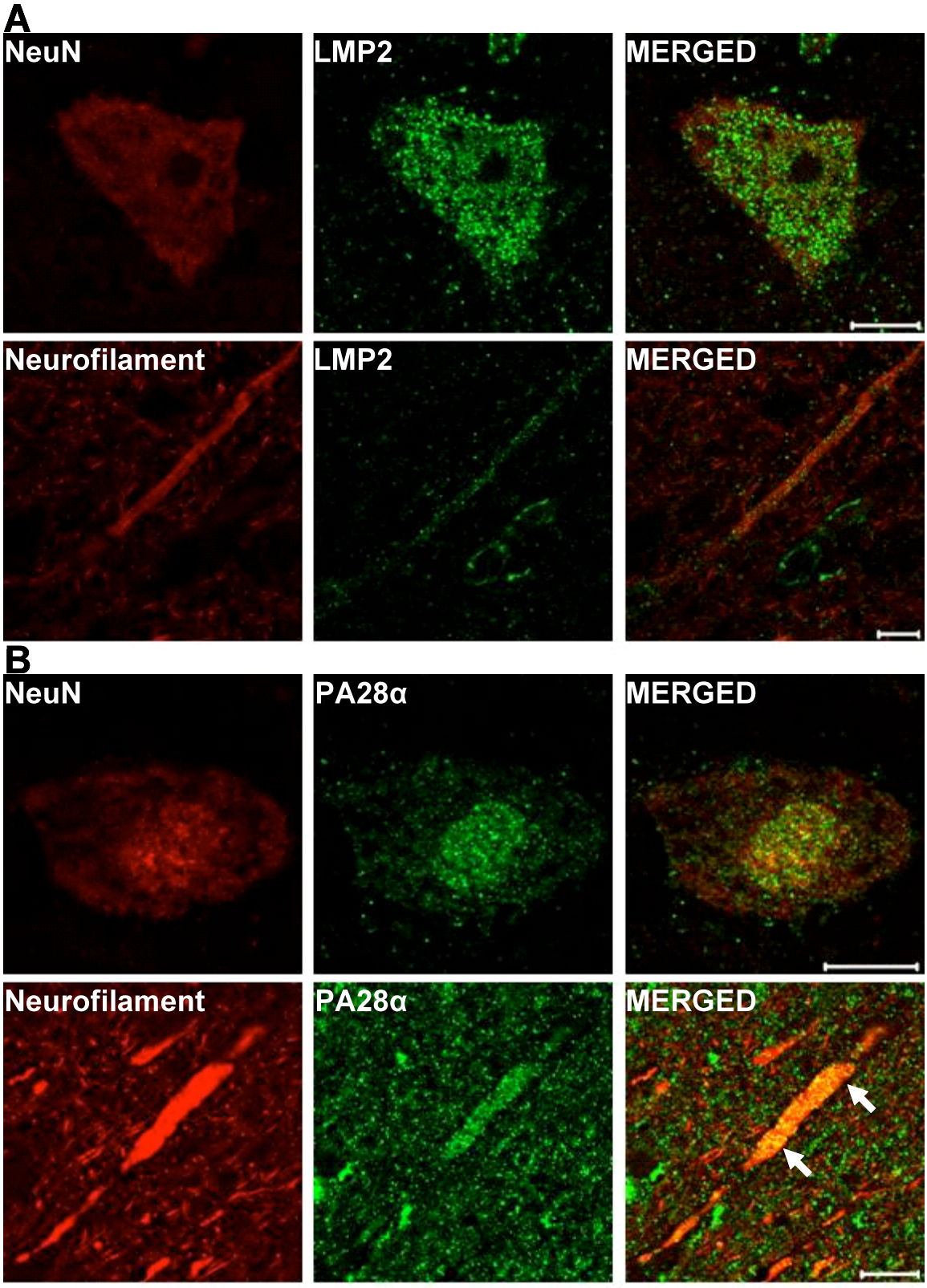
A
NeuN
LMP2
MERGED
Neurofilament
LMP2
MERGED
B
NeuN
PA28α
MERGED
Neurofilament
PA28α
MERGED

Figure 6.2 Immunoproteasome subunits localized within neuronal perikarya and distal processes. Indirect immunofluorescence staining of inducible proteasome (A) 20S subunit LMP2 and (B) 11S subunit PA28 alpha in HIV encephalitis cases localized with staining for neuronal components NeuN (Neuronal Nuclei protein, found in the nucleus and perikarya) and light-chain neurofilament (found in axons). In neuronal cell bodies, LMP2 showed more staining in the cytoplasm compared to the nucleus (A), in contrast with PA28 alpha, which showed more nuclear staining (B). PA28 alpha immunostaining in axons (B) showed a distinct presence in a swollen axon segment (arrows). Scale bar = 10 μm

6.2.3 Immunoproteasome Subunits Localized to Glial Cells

Localization of immunoproteasome subunits with regard to glial markers was assessed in HIV encephalitis cases to confirm the presence of immunoproteasomes in different glial cells. Antibodies against glial fibrillary acidic protein (GFAP), oligodendrocyte myelin glycoprotein (OMG), and CD68 were used to label astrocytes, oligodendrocytes, and macrophage/microglial cells, respectively. Double-labeling with these antibodies and the anti-LMP2 antibody revealed distinct areas of colocalization that indicated immunoproteasome synthesis in astrocytes, oligodendrocytes, and microglia/macrophages (**Figure 6.3**). Double staining for glial markers and 11S PA28 alpha similarly showed the presence of the immunoproteasome regulatory complex in all glial cell types analyzed (not shown) and were essentially equivalent to results for LMP2.

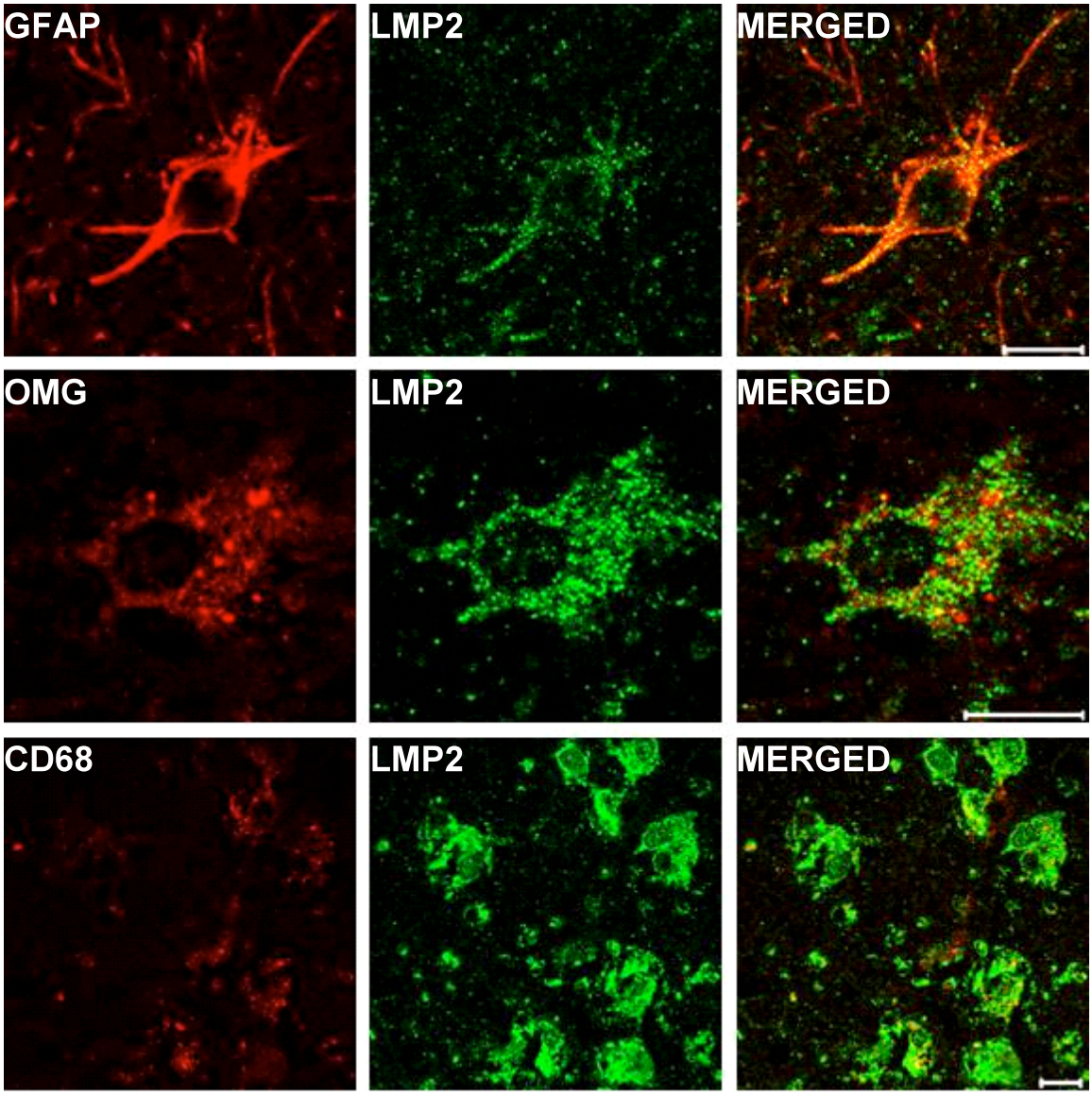

Figure 6.3 Immunoproteasomes localized within glial cells. Indirect immunofluorescence staining of inducible proteasome 20S subunit LMP2 in HIV encephalitis cases was localized with staining for astrocytes (GFAP, glial fibrillary acidic protein), oligodendrocytes (OMG, oligodendrocyte myelin glycoprotein), and microglia and macrophages (CD68). Scale bar = 10 μm

6.3 DISCUSSION

The increase in immunoproteasome subunit protein expression measured in the brain samples from HIV-infected patients prompted the determination of the cell types that undergo immunoproteasome induction. Through immunoperoxidase staining analysis of formalin-fixed paraffin-embedded frontal cortex tissue sections, I found that immunoproteasome subunits were present in neurons as well as glia in HIV-positive subjects. Immunofluorescent double staining with laser confocal microscopy image acquisition confirmed the results of immunohistology. There was colocalization between immunoproteasome subunits and cell markers for neurons (NeuN and neurofilament), astrocytes (GFAP), oligodendrocytes (OMG), and microglia/macrophages (CD68). These findings suggest that the induction of immunoproteasomes pervasively affects most cell types in the HIV infected brain.

The localization of immunoproteasome subunit PA28 alpha in a swollen axon is of particular interest. Axonal swelling indicates damage to the axon and the resulting interruption of the axonal transport system. This leads to an accumulation of transported material such as proteins, vesicles and organelles. Axonal injury associated with HIV infection of the brain is well documented, and occurs primarily in the subcortical white matter, basal ganglia, and the brainstem (An, Giometto et al. 1997; Giometto, An et al. 1997; Raja, Sherriff et al. 1997; Adle-Biassette, Chretien et al. 1999). The incorporation of immunoproteasomes in the focal areas of axon swelling in HIV-infected brains is a novel finding, and suggests possible involvement of immunoproteasomes in axonal injury.

Few studies of neurodegenerative diseases have reported comparable findings of immunoproteasome induction specifically in neurons and glia. In studies of Huntington's disease, both the mouse model and human brain tissue showed increases in

immunoproteasome subunits using immunoperoxidase immunohistochemistry particularly in cortical neurons, and also some staining of astrocytes (Diaz-Hernandez, Hernandez et al. 2003). Similar findings were reported in Alzheimer's disease, where immunoproteasome immunopositive staining was demonstrated in hippocampal and cerebellar neurons, astrocytes, and endothelial cells (Mishto, Bellavista et al. 2006). The induction of immunoproteasomes in neurons is supported by *in vitro* experiments in which murine neuroblastoma cells showed increases in inducible immunoproteasome subunits after treatment with interferon-gamma (Yang, Tugal et al. 2006). In studies using animal models for Amyotrophic Lateral Sclerosis, however, expression of immunoproteasomes in the spinal cord was limited to astrocytes and microglia (Puttaparthi and Elliott 2005; Ahtoniemi, Goldsteins et al. 2007).

The localization of immunoproteasome subunits within different cell types of the central nervous system showed that many cell types contribute to the increased expression of immunoproteasomes observed in the brain tissue homogenates of HIV-positive subjects. Since there is reactive astrocytosis and proliferation of activated microglia associated with HIV infection of the brain and HIV encephalitis, it is likely that a very substantial amount of immunoproteasome synthesis is attributable to astrocytes and microglia. However, the most functionally relevant finding with regard to HIV-associated neurocognitive impairment might be the expression of immunoproteasomes in neurons, which could detrimentally affect the handing of protein turnover necessary for proper neuronal functioning. To further explore that proposition, I next focused on the relationship between the protein expression levels of immunoproteasome subunits and synaptic proteins.

Chapter 7: Immunoproteasomes and Synaptic Protein Expression

7.1 INTRODUCTION

The results presented thus far provided increasing support for the induction of immunoproteasomes in the brains of HIV-infected subjects and its association with the development of neurocognitive deficits and neuropsychological impairment. However, the biochemical consequences of increased and prolonged immunoproteasome induction remained unexplored. Using the resources of the archival brain tissue available, I investigated the potential link between immunoproteasomes and neuropsychological impairment by attempting to identify neuronal synaptic proteins with expression levels that could be modified by immunoproteasome induction.

A preliminary analysis of increased ubiquitinated protein deposits in the brains of HIV-positive subjects revealed alterations in two neuronal proteins in AIDS (Gelman and Schuenke 2004). The presynaptic proteins synaptophysin and growth-associated protein 43 (GAP-43) were decreased in AIDS patients and correlated with an increase in a high-molecular-weight ubiquitin-protein conjugate species. To determine a possible relationship between immunoproteasome induction and altered synaptic protein composition, I pursued the expression levels of these two neuronal proteins.

To identify additional neuronal proteins with altered protein expression levels correlating with immunoproteasome induction and neuropsychological impairment, a proteomic analysis was performed. Synaptosomal fractions were isolated to enrich for proteins associated with neurons and neuronal functioning. Synaptosomes are nerve endings isolated from brain homogenates through the processes of differential centrifugation and density centrifugation using a discontinuous sucrose gradient. Synaptosomes isolated from HIV-positive and HIV-negative subjects were initially

characterized by immunoblotting to confirm the enrichment of neuronal proteins and the presence of immunoproteasomes in these isolates, as well as to investigate changes in select synaptic proteins between the two groups. Additional synaptosomes were isolated and analyzed by two-dimensional gel and mass spectroscopy to identify altered synaptic protein expression between groups with respect to neuropsychological impairment and immunoproteasome expression.

7.2 RESULTS

7.2.1 Immunoproteasomes and Synaptic Protein Expression

Analyses of synaptic proteins synaptophysin and GAP-43 required the quantitation of protein expression levels measured for each study subject. Immunoblotting and densitometry analysis of protein expression levels for synaptophysin and GAP-43 in brain cortex samples of HIV-negative and HIV-positive subjects were performed similar to the analysis of immunoproteasome subunits. The levels of the synaptic proteins were then compared to immunoproteasome subunit levels in frontal cortex homogenate samples previously measured by way of correlation analysis.

The protein expression levels of synaptophysin, but not GAP-43, were negatively correlated with immunoproteasome subunit protein expression levels (**Figure 7.1**). The correlation between the decrease in cortex synaptophysin and increase in LMP7 was highly significant (r = -0.376, $p < 10^{-5}$, **Figure 7.1A**). The correlation between synaptophysin and PA28 alpha was also significant, to a lesser extent (r = -0.223, $p <$ 0.01, **Figure 7.1B**). The analysis of GAP-43 protein expression showed no correlation with the protein levels of either LMP7 ($r = 0.112$, $p = 0.173$, **Figure 7.1C**) or PA28 alpha ($r = 0.024$, $p = 0.774$, **Figure 7.1D**).

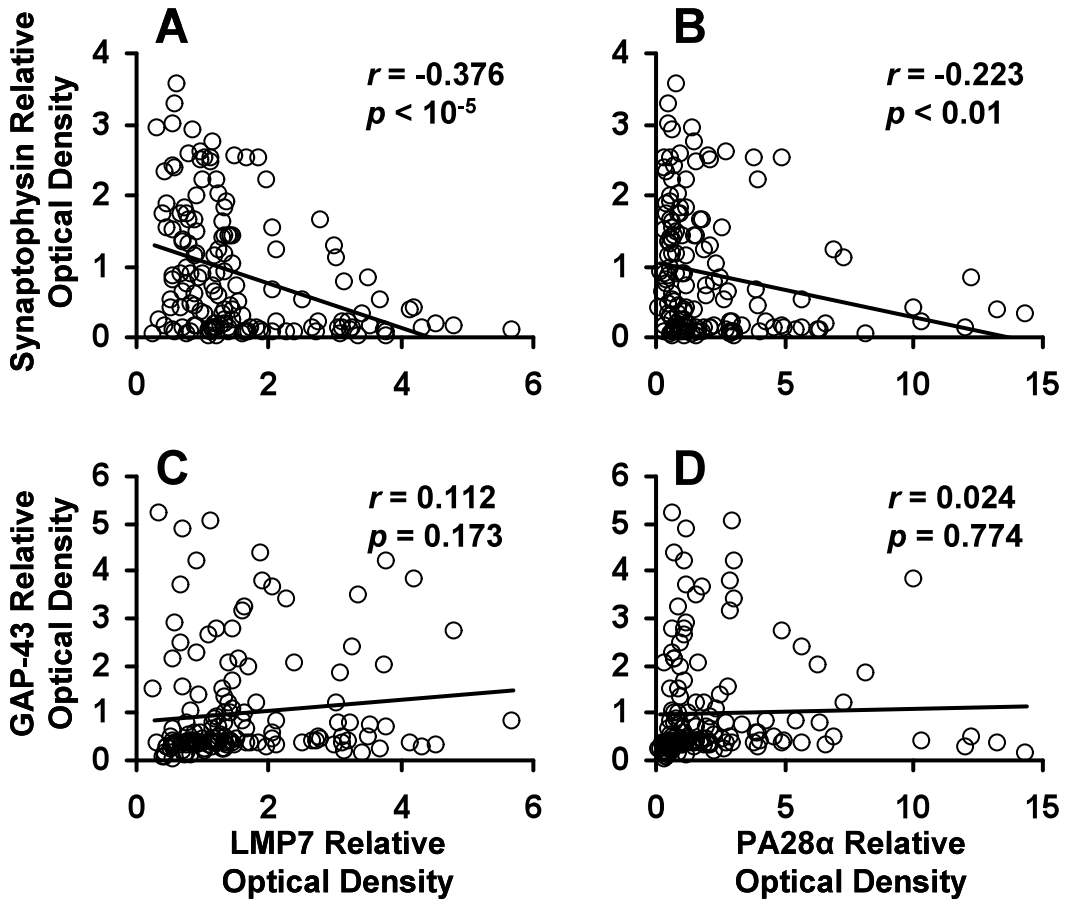

Figure 7.1 Analysis of correlation between frontal cortex protein expression levels of immunoproteasome subunits and synaptic proteins. Protein expression levels of synaptic proteins synaptophysin and GAP-43 were measured in the frontal cortex brain homogenate samples of all study subjects and compared to the frontal cortex protein expression levels of immunoproteasome subunits. Significant negative correlations were found between synaptophysin and both (A) LMP7 and (B) PA28 alpha. GAP-43 protein expression was not correlated with either (C) LMP7 or (B) PA28 alpha.

7.2.2 Characterization of Synaptosome Fraction

The search for additional protein species with altered levels in concert with immunoproteasome subunit protein expression required refinement by narrowing the pool of available proteins to those more specifically neuronal. This was approached

through the isolation of nerve endings, called synaptosomes, from the frontal cortex brain tissue homogenates. To validate this methodology, synaptosomes were initially isolated from a subset of HIV-negative and HIV-positive subjects and characterized by immunoblot analysis with comparison to the brain tissue homogenate that served as the isolation starting material.

Synaptosomes were characterized with regard to the presence of immunoproteasomes. The level of immunoproteasome subunit LMP7 was reduced in synaptosomes compared to the brain tissue homogenates (**Figure 7.2A**), but was still appreciable. Synaptosomes isolated from HIV-positive subjects exhibited an increase in LMP7 compared to the synaptosomes of HIV-negative subjects, similar to the LMP7 increase in the frontal cortex homogenate samples of HIV-positive subjects.

Homogenate and synaptosome isolations were also compared with regard to levels of both presynaptic (**Figure 7.2B**) and postsynaptic (**Figure 7.2C**) proteins. The presynaptic proteins analyzed for the characterization of synaptosomes included dynamin, secretory carrier membrane protein 1 (SCAMP1), synaptic vesicle glycoprotein 2C (SV2C), synaptophysin, and vesicular monoamine transporter 2 (VMAT2). Postsynaptic proteins analyzed included glutamate receptor 2 (GluR2), Homer1 B/C, N-methyl-D-aspartic acid receptor 2B (NMDAR2B), postsynaptic density protein 95 (PSD95), and synaptic guanosine triphosphatase (GTPase)-activating protein (SynGAP). The isolation of the synaptosome fraction was expected to result in the enrichment of both presynaptic and post-synaptic proteins compared to the frontal cortex homogenate from which it was isolated. Indeed, both the presynaptic and the postsynaptic proteins analyzed showed enrichment in the synaptosome fractions compared to homogenates, to varying degrees. However, no substantial differences in synaptic proteins could be detected in the infected brains when only a small sample of cases was used.

In addition to enriching synaptic proteins, isolation of the synaptosome fraction was expected to deplete non-neuronal proteins. Two non-neuronal proteins analyzed were cluster of differentiation 68 (CD68), specific for macrophages and microglial cells, and glial fibrillary acidic protein (GFAP), specific for astrocytes (**Figure 7.2D**). CD68 was detectable in the synaptosome fractions, but was decreased compared to homogenate. GFAP was also detectable in the synaptosome fraction, but was drastically reduced compared to homogenate. These immunoblots illustrated that the contribution of non-neuronal cells and proteins in the synaptosome fraction were diminished compared to levels in the homogenate samples, but they were not completely eliminated.

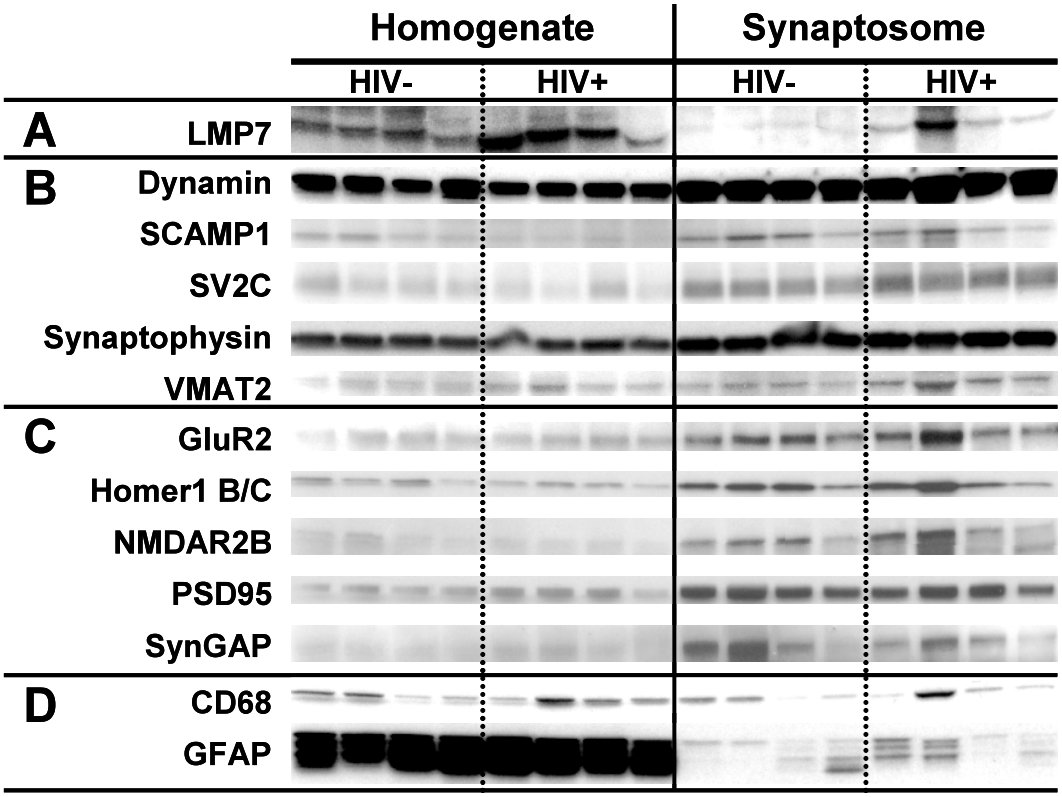

Figure 7.2 Characterization of synaptosome fractions compared to frontal cortex tissue homogenates by immunoblotting. Synaptosomes isolated from frontal cortex homogenates of HIV-positive and HIV-negative samples were analyzed for the presence of neuronal and non-neuronal proteins in comparison to the homogenate samples from which they were derived. (A) Analysis of immunoproteasome subunit LMP7 showed diminished presence in synaptosomes compared to brain homogenates. LMP7 levels in synaptosomes were greater in HIV-positive subjects compared to HIV-negative subjects. (B,C) Analysis of presynaptic (B) and postsynaptic (C) proteins showed increased levels in synaptosomes compared to homogenates. This indicated that synaptic proteins were enriched in the synaptosome isolation procedure. (D) Proteins specific for macrophages and microglia (CD68) and astrocytes (GFAP) were decreased in synaptosomes versus homogenates, which indicated that enrichment for synaptic proteins coincided with the depletion of non-neuronal proteins.

7.2.3 Synaptosome Proteomic Analysis and Western Blotting Confirmation

Proteomic analysis was performed using isolated synaptosomes to identify neuronal proteins possibly altered by HIV infection and increased neuronal immunoproteasomes. The limited yield of synaptosomes from brain homogenates during the isolation procedure and the great expense of time and effort required to procure these isolations greatly restricted the number of subjects that could be analyzed. With these limitations under consideration, the proteomic analysis was designed to compare seven synaptosome pools created from isolates of 19 subjects. Synaptosomes isolated from 12 subjects were separated into groups based on HIV status and the diagnoses of HIV-associated neuropsychological impairment and HIV encephalitis: Group A, HIV-negative subjects (n = 3); Group B, HIV-positive subjects without neuropsychological impairment (n = 3); Group C, HIV-positive subjects with neuropsychological impairment (n = 3); Group D, HIV-positive subjects with neuropsychological impairment and HIV encephalitis (n = 3). Synaptosomes from an additional seven HIV-positive subjects with neuropsychological impairment, in combination with the isolated synaptosomes from Groups C and D, were separated into the following groups: Group E, low immunoproteasome expression (n = 4); Group F, high immunoproteasome expression (n = 4); Group G, high immunoproteasome expression with HIV encephalitis (n = 4). Synaptosome pools were created with equal contribution from each subject and analyzed in triplicate by 2-dimensional gel electrophoresis. Computerized analysis of digital images of the gels were performed with the following comparisons between groups: A versus B; B versus C; B versus D; C versus D; E versus F; E versus G; and F versus G. 139 spots with at least a 2-fold difference in optical density between groups were identified. The spot list was refined to 68 spots after a manual analysis of the gel images to eliminate spots that were low in intensity or not present in at least two of the three gels.

For each remaining spot, the gel with the highest spot intensity was selected and the area corresponding to the spot was manually removed by large-bore pipette tip and analyzed by mass spectrometry for protein identification. 31 proteins were identified as listed in **Table 7.1**. A flow chart of the synaptosome proteomic analysis is shown in **Figure 7.3.**

Table 7.1 Proteins identified by synaptosome proteomic analysis.

Protein	Accession Number	Score	CI%	MW (kDa)	PI	Group Comparison
Increased with High Immunoproteasomes						
14-3-3 Epsilon	119611033	74	99	21.0	5.7	B<D
14-3-3 Zeta	49119653	379	100	30.1	4.7	B<D; C<D
α Fodrin	119608213	346	100	280.1	5.2	E<F; E<G
β Tubulin	18088719	110	100	50.1	4.8	E<G
β−Actin	15277503	245	100	40.5	5.6	B<C; B<D
Carbonic Anhydrase II	119389514	56	51	29.2	6.8	B<D
Collapsin Response Mediator Protein 2	62087970	173	100	68.6	5.9	B<D
Creatine Kinase B	49457530	365	100	42.9	5.3	B<D; C<D
Dynamin 1	123236791	58	68	96.2	6.3	B<D
Heat Shock 70kDa protein 1	147744565	150	100	70.3	5.5	B<D
Tropomyosin 3	114155146	374	100	29.1	4.8	B<D
Ubiquitin Activating Enzyme E1	35830	63	89	118.8	5.6	B<D
Ubiquitin Carboxy-Terminal Hydrolase L1	4185720	155	100	23.4	5.3	B<D
Decreased with High Immunoproteasomes						
αB Crystallin	4503057	565	100	20.1	6.8	C>D
Aldolase A	4557305	369	100	39.9	8.3	E>G
Annexin V	809185	65	94	35.8	4.9	B>D
ATP Synthase	15030240	256	100	59.9	9.1	E>G
Calmodulin	146386506	88	100	7.7	4.3	E>G
Chaperonin 10	4008131	155	100	10.6	9.4	E>F
Cytochrome C Oxidase Subunit VIb	4502985	133	100	10.4	6.5	B>D
Peroxiredoxin 2	32189392	479	100	22.0	5.7	B>D
Phosphoglycerate Kinase 1	48145549	472	100	45.0	8.3	E>G
Stathmin 1	5031851	376	100	17.3	5.8	B>D
Transgelin 3	56549135	32	0	22.6	6.8	C>D; E>G
Ubiquitin C	54300702	435	100	17.1	7.9	B>D
Increased with HIV Infection w/o NPI or HIVE						
Acyl Coenzyme A Dehydrogenase 10	119618373	66	95	54.4	9.1	A<B
Hemoglobin α2	22671717	341	100	15.3	8.7	A<B; B>C
Myelin Basic Protein	49168552	80	100	17.3	11.1	B>C
Synapsin Ib	338649	60	80	74.1	9.9	A<B
Decreased with HIV Infection w/o NPI or HIVE						
Protein Kinase C Substrate 80K-H	48255891	75	99	60.1	4.3	A>B
Increased with HIVE						
α Tubulin	109096484	61	84	46.8	5.0	F<G

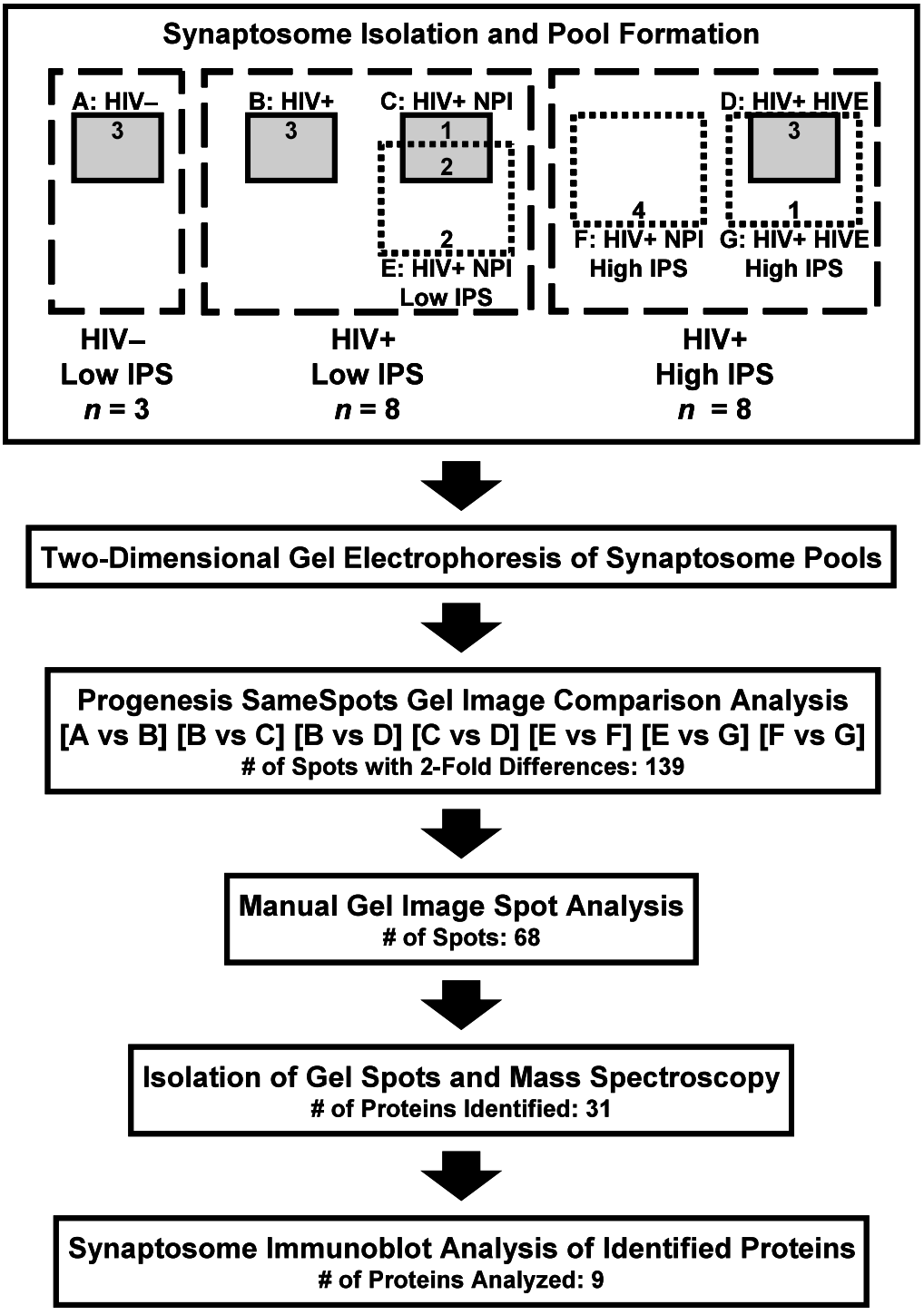
Synaptosome Isolation and Pool Formation
A: HIV–
3
B: HIV+
3
C: HIV+ NPI
1
2
2
E: HIV+ NPI
Low IPS
D: HIV+ HIVE
3
4
1
F: HIV+ NPI
High IPS
G: HIV+ HIVE
High IPS
HIV–
Low IPS
n = 3
HIV+
Low IPS
n = 8
HIV+
High IPS
n = 8
Two-Dimensional Gel Electrophoresis of Synaptosome Pools
Progenesis SameSpots Gel Image Comparison Analysis
[A vs B] [B vs C] [B vs D] [C vs D] [E vs F] [E vs G] [F vs G]
# of Spots with 2-Fold Differences: 139
Manual Gel Image Spot Analysis
# of Spots: 68
Isolation of Gel Spots and Mass Spectroscopy
# of Proteins Identified: 31
Synaptosome Immunoblot Analysis of Identified Proteins
# of Proteins Analyzed: 9

Figure 7.3 Flow chart of synaptosome proteomic analysis. Synaptosomes were isolated from a total of 19 subjects and divided into groups. Synaptosomes from 12 subjects were divided into 4 groups based on HIV status and diagnoses of HIV-associated neuropsychological impairment (NPI) and HIV encephalitis (HIVE) (3 isolations per group; shaded boxes). Additionally, 7 synaptosome isolations from HIV+ subjects with NPI were combined with isolations from C and D to form 3 groups based on frontal cortex immunoproteasome (IPS) levels (4 isolations per group; dotted boxes). Synaptosome pools representative of each group were created with equal contribution from each subject and analyzed by 2D gel electrophoresis in triplicate. The listed comparisons were performed by computer analysis of digitally averaged gel images. The spots selected by computer analysis were reviewed manually to remove erroneous spots that were low intensity and present in only one of the triplicate gels. The remaining spots were located on the gels and isolated for mass spectroscopy analysis and protein identification. Immunoblot analyses of identified proteins were performed using the individual synaptosome isolates. To simplify the immunoblot analyses, protein levels in synaptosomes were compared between three groups based on HIV status and frontal cortex IPS levels (dashed boxes).

Analysis of the proteins identified in the synaptosome proteomics study was performed by immunoblotting of the synaptosomes isolated from each of the 19 subjects. To streamline the analyses, the levels of the identified proteins measured by optical densitometry were compared between three groups based on HIV status and the immunoproteasome protein expression levels previously measured in frontal cortex homogenates: HIV-negative subjects with low immunoproteasome levels (n = 3), HIV-positive subjects with low immunoproteasome levels (n = 8), and HIV-positive subjects with high immunoproteasome levels (n = 8) (**Figure 7.3**).

Immunoproteasome subunit LMP7 protein expression levels in the synaptosomes were analyzed to confirm the characterization of these groups based on the LMP7 levels measured in the frontal cortex homogenates. Comparison between groups by ANOVA revealed significant differences in LMP7 levels ($p < 0.05$), with a 304 % increase synaptosomes from HIV-positive subjects with cortex immunoproteasome levels

compared to the HIV-negative subjects with low cortex immunoproteasome levels ($p < 0.05$; **Figure 7.4A**). Comparisons between the HIV-positive groups showed a 100 % increase in synaptosome LMP7 in the HIV-positive subjects with high cortex immunoproteasome levels compared to the HIV-positive subjects with low cortex immunoproteasome levels, but the increase did not reach the level of significance. These results showed that immunoproteasome levels measured in the isolated synaptosomes reflected those previously measured in the frontal cortex brain homogenate. Analysis comparing the LMP7 protein expression levels between frontal cortex brain homogenate and synaptosome samples indicated a significant regression ($r = 0.478$, $p < 0.05$, data not shown).

Nine proteins identified by the synaptosome proteomic analysis were analyzed by immunoblotting. Three proteins showed significant differences when protein expression levels were compared between groups (**Figure 7.4B**). Analysis of 14-3-3 zeta expression in synaptosomes showed a significant 75 % increase in the HIV-positive subjects with high cortex immunoproteasome levels compared to the HIV-positive subjects with low cortex immunoproteasome levels ($p < 0.01$). Synaptosome 14-3-3 epsilon also showed significant increase in the HIV-positive subjects with high cortex immunoproteasome levels compared to the HIV-positive subjects with low cortex immunoproteasome levels of 101 % ($p < 0.05$). The analysis of synaptosome synapsin 1 expression showed significant decreases in the HIV-positive subjects with high cortex immunoproteasome levels compared to both HIV-positive subjects with low cortex immunoproteasome levels (74 %, $p < 0.05$) and the HIV-negative subjects with low cortex immunoproteasome levels (80 %, $p < 0.05$). Six proteins analyzed by immunoblotting, including annexin V, collapsing response mediator protein 2 (CRMP2), alpha-B crystalline, dynamin 1, alpha-

Fodrin (both full-length 240kDA and cleavage product 150kDA), and stathmin, did not show significant differences between the compared groups (**Figure 7.4C**).

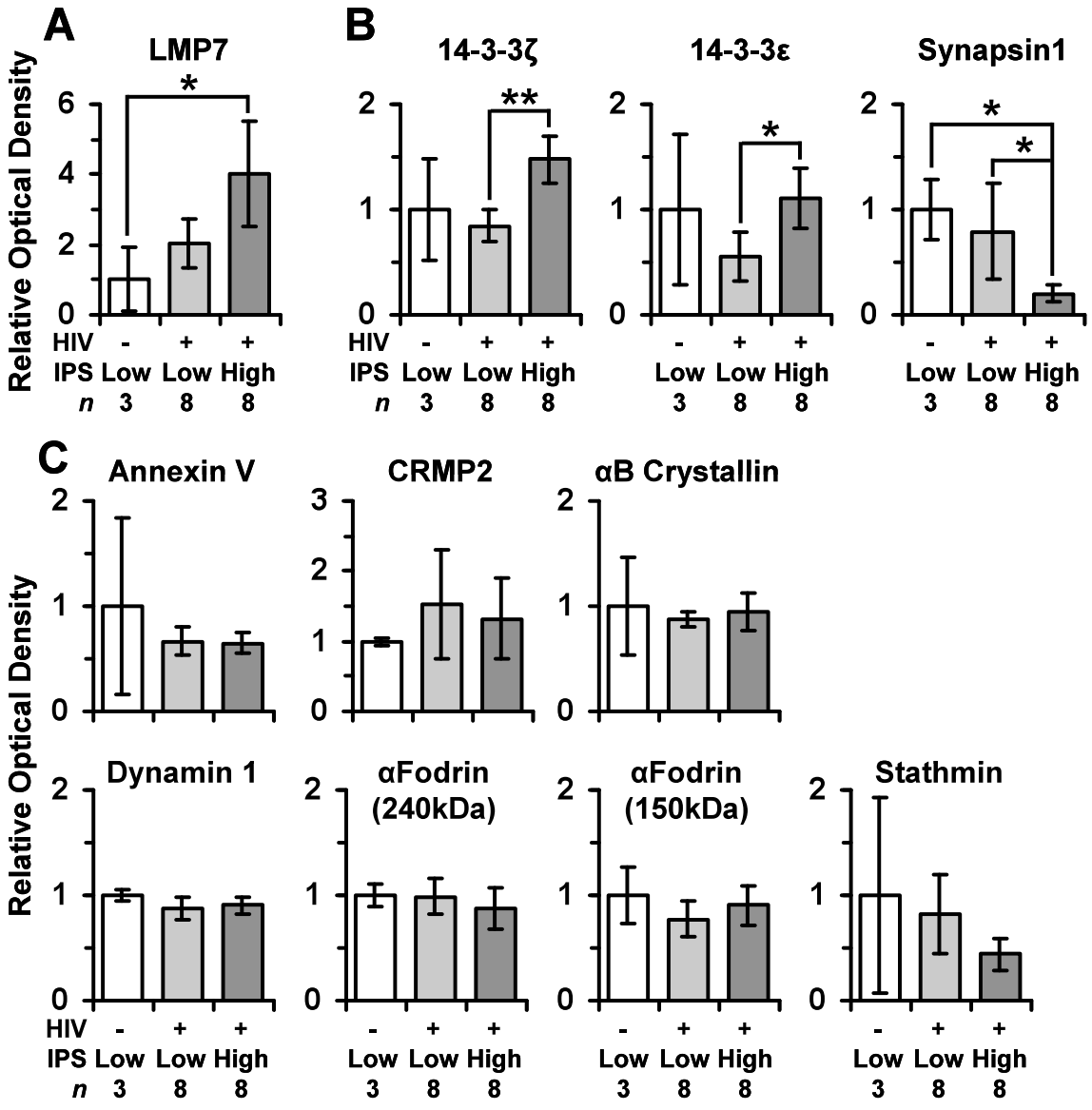

Figure 7.4 Immunoblot analyses of the proteins identified by the synaptosome proteomic study. Proteins identified by the synaptosome proteomic analysis were analyzed by immunobloting using the isolated synaptosomes from frontal cortex brain homogenate used in the proteomic analysis. Synaptosome samples were analyzed by groups based on HIV status and level of immunoproteasome protein expression previously measured in brain cortex homogenate samples. (A) Characterization of the groups by synaptosome LMP7 levels showed a 304 % increase in HIV+/High IPS group compared to the HIV-/Low IPS. Synaptosome LMP7 was also increased 100 % in HIV+/High IPS compared to HIV+/Low IPS, but was not statistically significant. These results indicate that synaptosome immunoproteasome expression levels reflect those previously measured in brain homogenate samples. (B) Three proteins identified by proteomic analysis showed significant differences between the compared groups. 14-3-3 zeta and 14-3-3 epsilon were increased in HIV+/High IPS compared to HIV+/Low IPS by 75 % and 101 %, respectively. Synapsin 1 was decreased in HIV+/High IPS compared to HIV+/Low IPS (74 %) and HIV-/Low IPS (80 %). (C) Six proteins identified by proteomic analysis showed no statistically significant differences between the compared groups. (IPS, immunoproteasome level measured in frontal cortex brain homogenates; *, $p < 0.05$; **, $p < 0.01$)

Further analyses were conducted to determine the correlations between the protein expression levels of the three newly identified proteins of interest and immunoproteasome subunit expression in synaptosomes. 14-3-3 zeta expression was positively and significantly correlated with immunoproteasome LMP7 expression in synaptosomes ($r = 0.488$, $p < 0.05$, **Figure 7.5A**). No significant correlation was detected when comparing 14-3-3 epsilon and LMP7 ($r = 0.190$, $p = 0.44$, **Figure 7.5B**). A significant negative correlation was observed with synapsin 1 and LMP7 expression in synaptosomes ($r = -0.475$, $p < 0.05$, **Figure 7.5C**). It is important to note that these newly discovered synaptosomal protein anomalies in HIV-infected brains showed stronger correlation to immunoproteasome synthesis relative to the previously studied synaptic proteins. Thus, the overall objective of finding new proteins to examine was achieved.

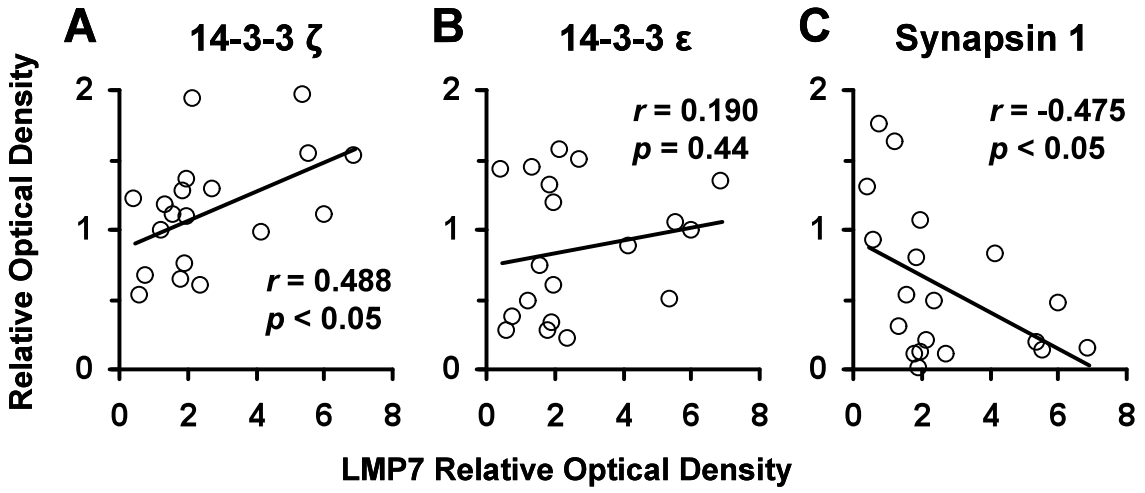

Figure 7.5 Correlations between the synaptosome protein expression levels of immunoproteasome subunit LMP7 and synaptic proteins identified by the synaptosome proteomic analyses. Proteins 14-3-3 zeta, 14-3-3 epsilon, and synapsin 1 were previously identified by synaptosome proteomic analysis to have altered synaptosomal protein expression levels associated with HIV infection and increased immunoproteasome. Synaptosomal levels of these proteins were compared to the synaptosomal level of immunoproteasome subunit LMP7. (A) Increases in 14-3-3 zeta were significantly correlated with increased LMP7. (B) No significant correlation was found between 14-3-3 epsilon and LMP7. (C) A significant negative correlation was found between synapsin 1 and LMP7.

7.2.4 Immunoproteasome and Synaptic Protein Localization

To further characterize the possible relationship between synaptosomal proteins and immunoproteasome induction, double-labeling immunofluorescence with laser confocal microscopy analysis was performed. Immunoreactivities for 14-3-3 zeta and immunoproteasome 20S subunit LMP2 were compared in prefrontal cortex tissue sections of HIV encephalitis cases. Images of neocortex revealed distinct punctate areas of colocalization within the neuronal cell bodies as well as in the surrounding neuropil, which both contain many synapses (**Figure 7.6**). The colocalization of 14-3-3 zeta and

LMP2 support the suggestion that these two proteins could interact with each other in neurons and synapses.

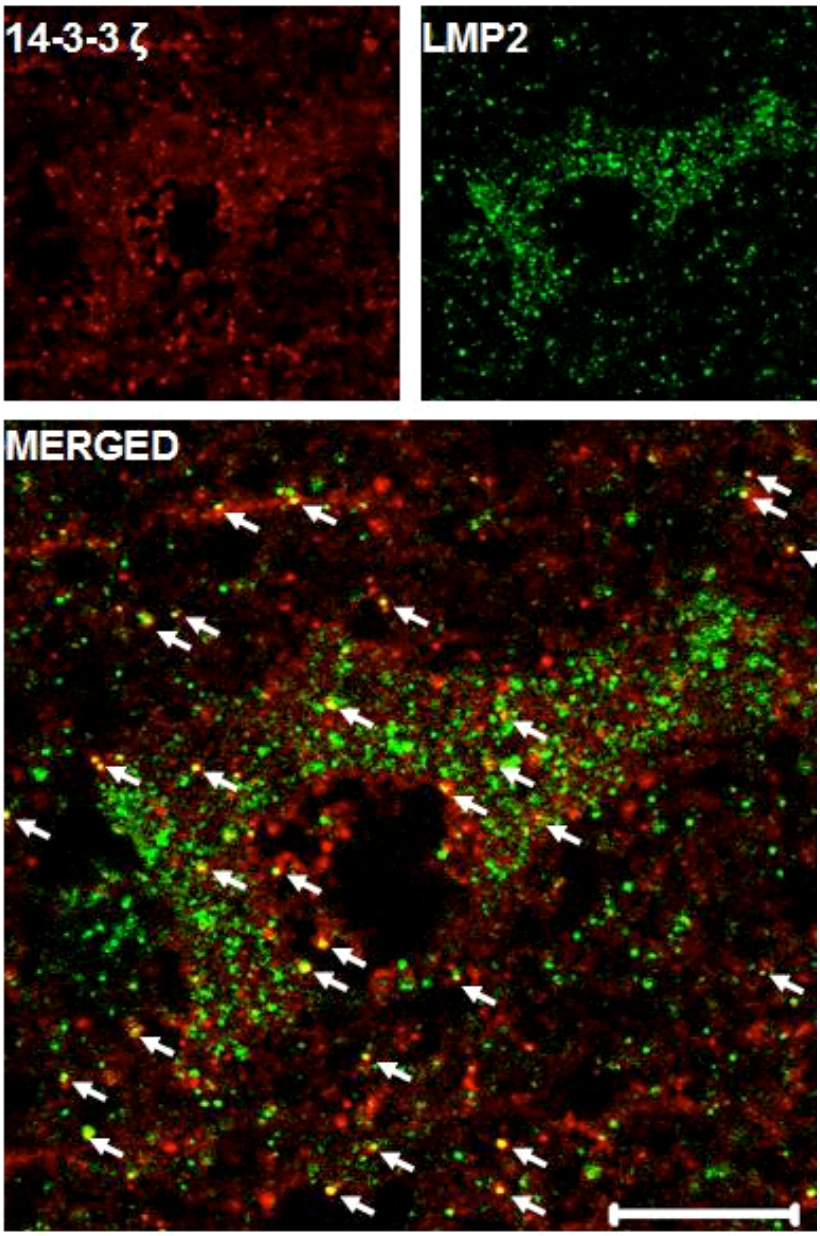

Figure 7.6 Localization of immunoproteasome subunit LMP2 with 14-3-3 zeta. Immunofluorescence and laser confocal microscopic analysis was performed to determine the spatial relationship between the immunoproteasome subunit LMP2 and 14-3-3 zeta. Results showed distinct areas of colocalization (arrows) both within a neuronal cell body and the surrounding neuropil. Scale bar = 10 μm.

Further studies were performed to analyze the localization of immunoproteasomes within synapses. Immunofluorescence and laser confocal microscopy analysis was used again to determine the relationship between immunoproteasome 20S subunit LMP2 and the presynaptic protein synaptophysin. Analysis of the neuropil in HIV encephalitis cases revealed numerous points of colocalization, which confirm that immunoproteasomes were present in or near the presynaptic terminals. The clear implication of this novel discovery is that immunoproteasomes and presynaptic proteins could physically interact with each other and modify synaptic protein turnover profoundly (**Figure 7.7**).

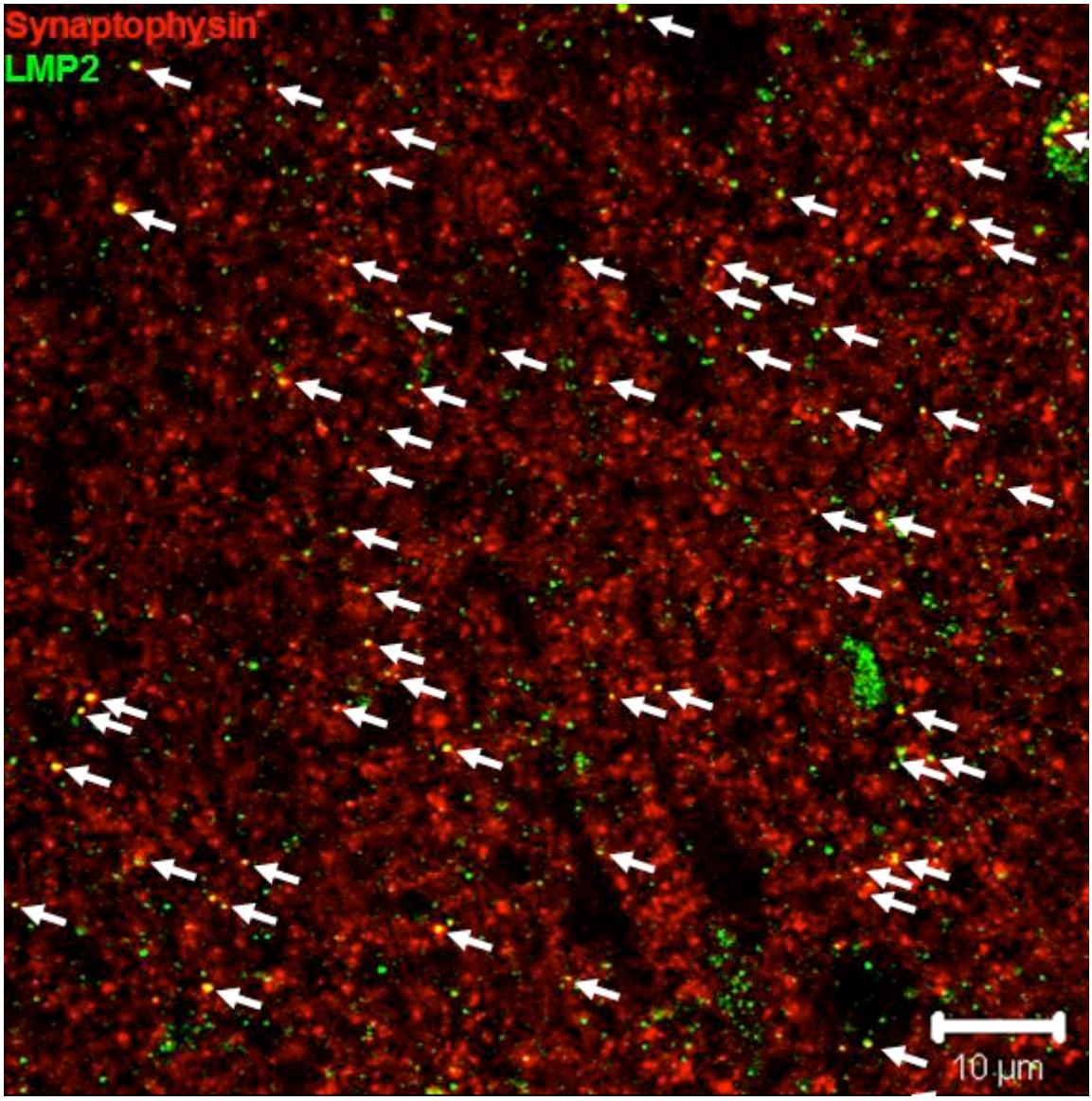

Figure 7.7 Immunoproteasomes localized to the presynaptic terminal. Localization of immunoproteasomes to the presynaptic terminal was assessed by immunofluorescence and laser confocal microscopy using antibodies against LMP2 and synaptophysin. Distinct areas of colocalization (arrows) indicate the presence of immunoproteasomes in the presynaptic terminals. In this image, about 12% of labeled presynaptic boutons also contain immunoproteasome antigenicity. Scale bar = 10 µm.

7.3 DISCUSSION

Recent reports have suggested significant importance on the role of the ubiquitin-proteasome system on synaptic development and functioning, including the modulation of presynaptic neurotransmitter release and postsynaptic molecular composition that is crucial to neural circuit plasticity (Bingol and Schuman 2005; Yi and Ehlers 2005; Patrick 2006). The current findings of an altered proteasome complex in the frontal cortex of HIV-infected subjects with the induction of immunoproteasomes have already shown strong correlations with neuropsychological impairment and neurocognitive deficits. To determine if the immunoproteasome induction could have an effect on proteasome-mediated turnover of synaptic proteins, I compared the protein levels of immunoproteasome subunits to synaptic proteins synaptophysin and GAP-43. Additionally, I conducted a proteomic analysis of isolated nerve endings to identify other synaptic proteins with altered concentrations coinciding with immunoproteasome induction.

Alterations in synaptophysin, a synaptic vesicle glycoprotein, have been previously reported to be associated with HIV-associated neuropsychological impairment. Histochemical analyses of synaptophysin have shown decreases in HIV-positive subjects that was related to the decline in cognitive performance, and suggested that neurodegeneration occurs via the loss of synaptic density and volume (Everall, Heaton et al. 1999; Moore, Masliah et al. 2006) or changes to the protein composition of the synapse. Additionally, the loss of synaptophysin measured by immunoblotting was correlated to the increased presence of high-molecular-weight ubiquitin-protein conjugates, and suggested that decreased neuronal protein turnover is possibly related to alterations in synaptic protein composition (Gelman and Schuenke 2004). I have shown here that decreased synaptophysin protein expression levels were significantly correlated

with increased immunoproteasomes, which supports the potential role of altered proteasome-mediated protein turnover in neuronal dysfunction in HIV-infected brains and specifically implicates the involvement of immunoproteasome induction.

The decrease in synaptophysin has also been reported in other neurodegenerative diseases as well. Research in schizophrenia has shown a decrease in synaptophysin protein (Eastwood and Harrison 1995) without a change in messenger RNA levels, indicating post-transcriptional abnormalities or diminished axonal projections (Glantz, Austin et al. 2000). In Alzheimer's disease, synaptophysin has been shown to be decreased in the hippocampal formation (Masliah, Mallory et al. 1994; Heinonen, Soininen et al. 1995) and frontal cortex (Reddy, Mani et al. 2005), and were also particularly absent near neurons with clusters of oligomer beta-amyloid (Ishibashi, Tomiyama et al. 2006). Besides Alzheimer's disease, decreased synaptophysin immunoreactivity has also been reported in Huntington's disease and Parkinson's disease (Zhan, Beyreuther et al. 1993). It remains unclear whether the decrease in synaptic proteins reflects the loss of the entire synapse, or instead, reflects altered composition of structurally intact synapses.

The protein expression level of GAP-43 was also compared to immunoproteasome levels to determine if a correlation was present. GAP-43, also known as neuromodulin, is a neuronal phosphoprotein associated with axonal growth and remodeling of synaptic connections that is degraded by the proteasome in both ubiquitin-dependent and ubiquitin-independent pathways (Denny 2004; De Moliner, Wolfson et al. 2005). Similar to synaptophysin, a decrease in GAP-43 was found to correlate with increased presence of high-molecular-weight ubiquitin-protein conjugates found in HIV-positive subjects (Gelman and Schuenke 2004). This suggested that GAP-43 might be correlated with immunoproteasome subunits as well, but contrary to this hypothesis, no

correlation was found. This indicates that the GAP-43 protein expression level may not be influenced by immunoproteasome induction in HIV-infected brains. It also suggests that not all synaptic proteins are influenced by immunoproteasomes. However, like many proteins, the regulation of GAP-43 is complex with various transcriptional and posttranscriptional regulating factors (De Moliner, Wolfson et al. 2005) that may mask potential effects mediated by the induction of immunoproteasomes. As well, measuring the steady state concentration of this protein in tissue does not address the question of protein turnover rate, which requires viable tissue to perform.

Deeper investigation into immunoproteasome induction in HIV-infected brains required the identification of other neuronal proteins with altered protein expression levels associated with this phenomenon. To that end I identified three synaptic proteins using proteomic analysis of isolated synaptosome fractions from brain homogenates. The synaptosome protein concentrations of 14-3-3 isoforms zeta and epsilon were significantly higher in HIV-positive subjects with high immunoproteasome levels compared to the HIV-positive subjects with low immunoproteasome levels. Presynaptic protein synapsin 1 was decreased. Additionally, significant correlations of the increase in 14-3-3 zeta and decrease in synapsin 1 were demonstrated in relation to the increase in immunoproteasome subunit LMP7 measured in synaptosomes.

Synapsins are a family of brain phosphoproteins localized primarily in presynaptic terminals, where they serve to modulate neurotransmitter release through the availability of synaptic vesicles bound to cytoskeletal proteins and subsequently released during action potential stimulation (Evergren, Benfenati et al. 2007). The consequence of decreases in synapsin is illustrated in studies of knockout mice lacking synapsin genes, which show severe synaptic depression with repetitive high stimulation suggestive of a dysregulation in the reserve pool of synaptic vesicles (Rosahl, Spillane et al. 1995; Gitler,

Takagishi et al. 2004; Sun, Bronk et al. 2006). Studies of synapsin levels suggest a possible decrease in Alzheimer's disease, although it remains uncertain whether it reflects a generalized loss of synapses versus a more specific change in this protein. Immunoblotting analysis of synaptic proteins in post-mortem tissue from Alzheimer's disease subjects showed comparable levels of synapsin I with that of controls (Sze, Bi et al. 2000). However, immunofluorescence analysis of hippocampal sections from Alzheimer's disease patients and control subjects indicated a loss of synapsin 1 in specific hippocampal regions (Qin, Hu et al. 2004). As well, cDNA microarray analysis investigating changes in gene expression associated with the early stages of Alzheimer's disease suggested a selective decrease in synapsin splice variants in the entorhinal cortex (Ho, Guo et al. 2001).

The decrease in synapsin 1 in the synaptosomal fraction with increased immunoproteasome expression that I found suggests a dysregulation of synaptic vesicles in HIV-associated dementia that is possibly related to alterations in the ubiquitin-proteasome system. Further evidence of synapsin involvement in HIV central nervous system infection can be found in the Murine Acquired Immunodeficiency Syndrome (MAIDS) model, where a proteomic analysis of hippocampal slices identified a decrease in synapsin 2 in affected animals (Takahashi, Saito et al. 2007).

The 14-3-3 protein family consists of at least seven isoforms: beta, gamma, zeta, sigma, epsilon, eta, and tau, with the phosphorylated forms of beta and zeta originally designated as alpha and delta. These proteins are involved in a diverse array of cellular processes, such as cell cycle regulation, metabolism, transcriptional control, signal transduction, and intracellular trafficking, through the modulation of protein activity, localization, and interaction with other proteins (Berg, Holzmann et al. 2003; Kjarland, Keen et al. 2006). The functions of 14-3-3 proteins in the central nervous system, where

concentrations are the highest (one percent of total soluble proteins), include the modulations of synaptic plasticity (Dai and Murakami 2003), ion channel function and localization(Zhou, Schopperle et al. 1999; Suginta, Karoulias et al. 2001; Bunney, van den Wijngaard et al. 2002; Rajan, Preisig-Muller et al. 2002), and neurotransmitter synthesis (Ichimura, Isobe et al. 1987; Kleppe, Toska et al. 2001).

14-3-3 proteins are also involved in neurodegenerative disorders. Numerous neurological disorders, including those involving inflammation, vascular disorders, dementias, and malignancies, are associated with the detection of 14-3-3 proteins in the cerebrospinal fluid of afflicted patients, suggesting a possible leakage of neuronal proteins into the cerebrospinal fluid as a result of tissue destruction (Berg, Holzmann et al. 2003). In Creutzfeldt-Jakob disease, the levels of 14-3-3 proteins in the cerebrospinal fluid are particularly elevated (Kenney, Brechtel et al. 2000), and have shown high sensitivity and specificity in diagnosing suspected patients (Lemstra, van Meegen et al. 2000). As well, 14-3-3 zeta was localized to amyloid plaques of Creutzfeldt-Jakob disease, suggesting a possible role in deposit formation (Richard, Biacabe et al. 2003). In Alzheimer's disease, 14-3-3 zeta is involved in tau phosphorylation (Hashiguchi, Sobue et al. 2000) and has been localized to neurofibrillary tangles consisting of hyperphosphorylated tau (Umahara, Uchihara et al. 2004), supporting its involvement in this pathological process. 14-3-3 proteins are also found in the Lewy bodies of Parkinson's disease and diffuse Lewy body disease (Kawamoto, Akiguchi et al. 2002; Berg, Riess et al. 2003).

Previous reports have linked 14-3-3 proteins with HIV-infection of the brain. An analysis of 14-3-3 proteins in the cerebrospinal fluid of 17 HIV-positive subjects, six of whom had neuropsychological impairment, found detectable levels of 14-3-3 gamma in only one subject with neuropsychological impairment, and two without impairment,

(Miller, Green et al. 2000). However, a following study detected significant elevations of 14-3-3 proteins epsilon, gamma, and zeta (but not beta, eta, or tau) in the cerebrospinal fluid of AIDS patient with dementia or cytomegalovirus encephalitis, but not in AIDS patients without neurological symptoms or in those who were HIV-negative (Wakabayashi, Yano et al. 2001). Similar findings were reported in the simian immunodeficiency virus (SIV)/macaque model of HIV central nervous system disease, where 14-3-3 proteins in the cerebrospinal fluid were elevated in the SIV-infected animals with highest viral loads in cerebrospinal fluid and brain tissue (Helke, Queen et al. 2005). Most recently, a proteomic analysis of hippocampus tissue from mice of the MAIDS model of HIV central nervous system disease identified 14-3-3 zeta as having decreased expression compared to non-infected animals (Takahashi, Saito et al. 2007). The identification of the same protein species in the hippocampus of MAIDS mice and in synaptosomes of frontal cortex samples of human HIV-infected subjects, but with conflicting change in protein expression, could be due to the various differences between an animal model and human disease, but could also represent the diverse roles of 14-3-3 zeta in different brain regions and subcellular fractions.

The analysis of synaptic proteins in HIV-infected brain tissue has revealed significant associations between the increase in immunoproteasome subunits and the protein expression levels of synaptophysin, synapsin 1, and 14-3-3 zeta. The immunofluorescence analysis showing colocalization between immunoproteasomes and both synaptophysin and 14-3-3 zeta indicates the presence of immunoproteasomes in the synapses and possible influences on proteins in the synaptic compartment. These results support a possible connection between alterations in proteasome-mediated protein turnover by immunoproteasome induction and modified synaptic protein composition. The biochemical interplay between immunoproteasomes and synaptic proteins has not

been investigated, and warrants further study to determine the role of immunoproteasomes in altered synaptic protein concentrations found in HIV-associated neuropsychological impairment.

Chapter 8: Summary and Conclusion

8.1 SUMMARY

The neuroscientific results I have obtained show that the ubiquitin-proteasome system is altered by induction of immunoproteasomes in the brains of HIV-infected subjects and is associated with neuropsychological impairment, neuropathology, and changes in neuronal proteins. Along with increasing evidence for the role of the ubiquitin-proteasome system in neurodegenerative diseases, the reports of increased ubiquitin-protein deposits and high-molecular-weight ubiquitin-protein conjugates in the brains of HIV/AIDS patients prompted the investigation into changes to the proteasome complex in HIV-infected brains. My initial analysis of proteasome enzymatic activity revealed increases in chymotrypsin-like activity in HIV-positive subjects and alterations in the proteasome proteinase profile suggestive of proteasome 20S beta subunit modification attributable to the induction of the immunoproteasome. Proteasome subunit immunoblot screening and semiquantitative analysis indeed revealed increased protein expression of inducible immunoproteasome subunits in HIV subjects that correlated with the extent of proteinase profile alterations. I also demonstrated significant increases in both the inducible 20S beta subunit LMP7 and the inducible 11S regulatory complex subunit PA28 alpha in HIV-positive subjects, particularly in the frontal cortex of those with neuropsychological impairment and in those with HIV encephalitis. Though the majority of constitutive proteasome subunits showed no alterations with HIV infection, significant decreases in 19S regulatory complex subunit Rpn2 indicated that constitutive proteasomes were affected as well.

Further analysis revealed significant correlations between increases in immunoproteasome subunits and declines in the performance of HIV-positive subjects on

neurocognitive tests of frontal lobe functioning, suggesting that immunoproteasome induction was associated with the underlying neuronal dysfunction. Increased immunoproteasome protein expression also coincided with increased HIV loads measured in brain tissue, cerebrospinal fluid, and plasma, and decreases in CD4+ T-cell counts, which implicated the importance of HIV replication, immunodeficiency, and disease progression with immunoproteasome induction.

I used immunohistochemical and immunofluoresence analysis to determine the populations of cells with immunoproteasomes, and showed immunoproteasome subunits in several brain cells, including neuronal cell bodies and cell processes. This supported the suggestion that there is direct involvement of immunoproteasomes in neuronal dysfunction. As well, I showed immunoproteasome subunits in oligodendrocytes, astrocytes and microglial cells. Since the latter two cell types demonstrate hypertrophy or hyperplasia during the progression of HIV infection of the brain, they most likely account for a substantial proportion of the increase in immunoproteasomes previously measured. However, the neuronal immunoproteasomes probably have the most functional significance with regard to neuronal dysfunction and HIV-associated neuropsychological impairment.

The analysis of immunoproteasome subunit protein expression levels compared to synaptic proteins showed significant negative correlations with the level of synaptic protein synaptophysin, suggesting a possible link between immunoproteasome induction and the dysfunction and dysregulation of protein composition localized to neuronal synapses. However, the lack of correlation between immunoproteasomes and GAP-43 indicates that not all synaptic proteins are modified in a similar manner and that a generalized dropout of presynaptic boutons may not occur. The subsequent investigation focused on the analysis of isolated nerve endings, or synaptosomes, from frontal cortex

brain tissue. In line with the previous data, immunoproteasome subunit LMP7 was increased in synaptosomes of HIV-positive subjects compared to HIV-negative subjects. Proteins 14-3-3 zeta and synapsin 1 were identified by the proteomic analysis of synaptosomes as having altered protein expression levels associated with HIV infection and increases in immunoproteasomes. Subsequent analyses showed that the protein expression levels of these proteins correlated with the levels of synaptosomal immunoproteasomes. Double-labeling immunofluorescence analysis showed that immunoproteasomes and 14-3-3 zeta were colocalized in neuronal cell bodies and the surrounding neuropil, suggestive of possible interactions within these two neuronal compartments. Additional double-labeling showed the colocalization of immunoproteasomes and synaptophysin, which highlighted the presence of immunoproteasomes in the presynaptic terminal and suggested possible interactions with presynaptic proteins.

8.2 CONCLUSION

The evidence showed that the increase in immunoproteasomes in HIV-infected brains is (A) associated with neuropsychological impairment, (B) correlated with declines in neurocogntive performances, (C) present in neurons, and (D) associated with alterations in synaptic protein composition. With these findings, I hypothesize a novel concept that the continual induction of immunoproteasomes, due to the persistent inflammatory response in HIV-infected brains, alters the ubiquitin-proteasome system and diminishes the capacity to degrade neuronal proteins. The dysregulation of neuronal protein turnover would ultimately lead to the neuronal dysfunction underlying HIV-associated neuropsychological impairment (**Figure 8.1**).

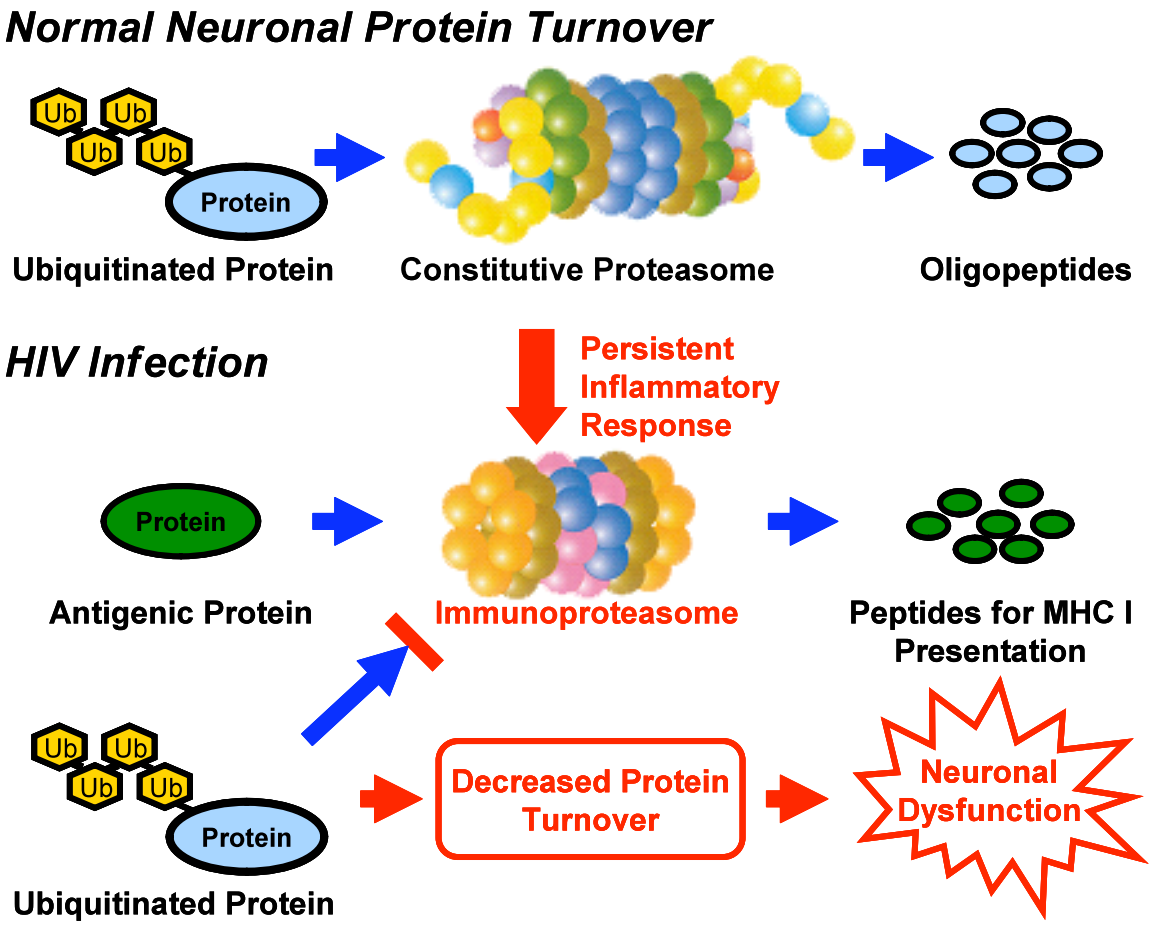

Figure 8.1 Hypothesis of persistent "hijacking" of brain proteasomes that leads to neuronal dysfunction. Under normal conditions, the constitutive proteasome complex is responsible for the majority of cellular protein turnover mediated by the ubiquitin proteasome system. Inflammatory cytokines such as interferon-gamma induce immunoproteasomes and divert the substrate repertoire towards processing of peptides for antigen presentation (MHC Class I). Borrowing the proteasome apparatus for heightened antigen presentation is designed to be temporary and persists only until the pathogen is eradicated. Infection of the brain with HIV-1 provokes a persistent inflammatory response that does not eradicate the pathogen. The resulting persistent diversion from routine protein turnover could upset the precise regulation of neuronal protein content, leading to neuronal dysfunction and ultimately dementia.

The induction of immunoproteasomes to replace constitutive proteasomes serves to optimize production of appropriate antigenic peptides for MHC class I antigen presentation. It is a tightly regulated and transient response, with a rapid remission of immunoproteasomes once the induction ceases to allow for the repopulation of constitutive proteasomes (Heink, Ludwig et al. 2005). The inducibility by inflammatory cytokines suggests that immunoproteasomes are expressed only when needed because they lack the full functionality of constitutive proteasomes (Groettrup, Khan et al. 2001; Strehl, Seifert et al. 2005).

HIV infection of the brain, however, provokes a persistent inflammatory response that prolongs the heightened expression of immunoproteasomes indefinitely since the pathogen is never eradicated. The consequence of persistent immunoproteasome induction is a diminished capacity for protein degradation. Though the immunoproteasome functions to generate antigenic peptides, its ability to perform routine and ubiquitin-targeted protein turnover is brought into question since the immunoproteasome 11S regulatory complex fails to degrade larger intact or ubiquitinated proteins usually mediated by the constitutive 26S proteasome complex (Dubiel, Pratt et al. 1992; Ma, Slaughter et al. 1992). Immunoproteasome induction also coincides with the destabilization of the constitutive 26S proteasome complex (Bose, Brooks et al. 2001; Bose, Stratford et al. 2004), which further reduces the capacity for ubiquitin-dependent proteolysis. Therefore, the induction of immunoproteasomes could be responsible for the increase in ubiquitinated protein deposits reported in AIDS cases (Gelman and Schuenke 2004). Additionally, the ubiquitin proteasome system plays a critical role in the precise regulation of proteins involved in proper neuronal functioning, including neuronal development, synaptic plasticity and remodeling, and long term potentiation and depression (Johnston and Madura 2004). The impairment of proteasome-mediated

protein turnover by long-term immunoproteasome induction would have profound effects on neuronal functioning, resulting in neuronal dysfunction and neurodegeneration. This proposed pathophysiologial role of immunoproteasomes presents a novel mechanistic concept for the neurodegeneration associated with HIV infection that warrants further investigation for the advancement of HIV dementia research.

The impact of immunoproteasome induction on neuronal ubiquitin-mediated protein turnover has additional implications with regard to other neurodegenerative diseases. The increased survival rate of HIV-positive people since the introduction of highly active antiretroviral therapy and seroconversion in the aging population may increase the susceptibility to age-associated neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. The effects of neuronal immunoproteasome induction in HIV-infected brains may synergize with the impairment of the ubiquitin-proteasome system associated with other neurodegenerative diseases to possibly reduce the age of onset or increase the severity of neurodegeneration and neuropsychological impairment.

Neuroinflammation often accompanies neurodegenerative diseases and can induce immunoproteasomes independent of infections by HIV or other pathogens. Indeed, increases in immunoproteasomes were described in studies of Huntington's disease (Diaz-Hernandez, Hernandez et al. 2003) and Alzheimer's disease (Mishto, Bellavista et al. 2006). In these studies, immunoproteasome induction was suggested to be secondary to neuroinflammation or a compensatory reaction to an impaired ubiquitin-proteasome system, though the possibility of a pathogenic role was not dismissed. The impairment of protein turnover by immunoproteasome induction is a possible mechanism for neuronal dysfunction in the presence of neuroinflammation that should be explored in

the different types of neurodegenerative diseases and compared to the findings in HIV infection of the brain.

To further explore the impact of immunoproteasome induction on neurons in HIV infection and in other neurodegenerative diseases, assessments of the neuronal activities that show involvement of the ubiquitin-proteasome system should be performed in the presence of neuroinflammation and immunoproteasome induction. One such process is the modulation of the postsynaptic density composition with changes in activity levels. Ehlers (2003) showed that in cultured rat hippocampal neurons, increased neuronal excitation after bicuculline administration and decreased excitation with tetrodotoxin produce reciprocal changes in the postsynaptic density protein expression pattern. Pulse-chase analysis revealed that the turnover of total protein and select activity-related postsynaptic proteins both run in parallel with the level of neuronal activity. Ubiquitination and the presence of ubiquitin-protein conjugates were also in parallel with changes in neuronal activity. Treatment of neurons with proteasome inhibitors inhibited these change in the postsynaptic density protein expression pattern and resembled the effects of decreased activity in untreated neurons. Additionally, the ubiquitin-proteasome system has recently been implicated in the induction of long-term potentiation (Fonseca, Vabulas et al. 2006; Karpova, Mikhaylova et al. 2006; Dong, Upadhya et al. 2008). In these reports, the pharmacological inhibition of proteasome activity resulted in the impairment of late-phase long-term potentiation measured by the field excitatory postsynaptic potential in rat hippocampal slices. Increases in interferon-gamma, which induces immunoproteasome expression, has also been associated with the impairment of long-term potentiation in rat hippocampal slices (Griffin, Nally et al. 2006; Maher, Clarke et al. 2006), though the involvement of proteasomes or immunoproteasomes was not addressed in these studies. Determining the effects of immunoproteasome induction

in these experimental models would provide the evidence for its involvement in neuronal dysfunction.

Further studies should also be directed to analyzing the relative abundance of immunoproteasomes in comparison to the constitutive proteasomes. Though the presented data depicts increased immunoproteasome subunit protein expression levels with regard to HIV infection, neuropsychological impairment and neuropathology, the extent that immunoproteasomes replace constitutive proteasomes in the brain is still unknown. One possible method that can be used to shed light on this issue is Blue-Native PAGE, which has been used in conjunction with SDS-PAGE and mass spectrometry to identify proteasome complex populations (Shibatani, Carlson et al. 2006). As described by the authors, the Blue-Native-PAGE allows for the analysis of intact multimeric protein complexes, the components of which can be separated by SDS-PAGE in the second dimension. Applying this method to HIV-infected brain specimens and isolates of glial cells, neurons, and synaptosomes can identify and compare the levels of 11S-capped 20S immunoproteasomes with 19S-capped 20S (26S) constitutive proteasomes and uncapped 20S proteasome complexes. This would provide further insight into the level of immunoproteasome induction and constitutive proteasome replacement in the different stages of HIV infection of the brain and associated neuropsychological impairment.

Finally, though the present study serves to characterize immunoproteasome induction in the frontal cortex of HIV-infected subjects, other brain regions warrant specific attention. For example, the basal ganglia have often been shown to be involved in HIV infection of the brain. Compared to other regions of the brain, the caudate nucleus has been shown to have he highest concentrations of HIV RNA (Kumar, Borodowsky et al. 2007). Other studies have shown decreases in the size and blood flow

of the caudate nucleus associated with HIV-associated dementia and neurocognitive impairment (Dal Pan, McArthur et al. 1992; Aylward, Henderer et al. 1993; Ances, Roc et al. 2006). Additionally, analysis of the striatum showed changes in proteins associated with the dopaminergic system resembling an increase in dopaminergic tone (Gelman, Spencer et al. 2006). The substantia nigra has also shown changes associated with HIV infection, including decreases in numerical density and size of total neurons and pigmented neurons (Itoh, Mehraein et al. 2000), and decreases in tyrosine hydroxylase (Silvers, Aksenov et al. 2006). Determining the extent of immunoproteasome induction in these regions may provide further insight into the neuronal dysfunction specific to these regions.

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