SUBSTANCE P AND NEUROKININ-1 EXPRESSION IN THREE BRAIN REGIONS OF HIV-INFECTED INDIVIDUALS FROM THE NATIONAL NEUROAIDS TISSUE CONSORTIUM COHORT:

Findings and Implications of Drug Use and Neuropathology In The Management Of NeuroAIDS

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ABSTRACT

INTRODUCTION: HIV- associated neurocognitive disorder (HAND) and pathology are common manifestations of HIV-infection, and often persist in spite of controlled peripheral viremia. Severity of HAND can range from loss of concentration and psychological changes to frank dementia. Inflammatory host-immune responses and chemotaxis of immune cells into the CNS are thought to be integral to development of NeuroAIDS and HAND.

OBJECTIVES: This study's primary aim was to determine if significant differences existed between Substance P and NK1R expression in brain tissue samples of HIV-infected individuals with neurocognitive disorder or pathology. The secondary aim was to determine whether expression of HIV viral entry receptors CCR5 and CXCR4 correlate with expression of Substance P or NK1R. The tertiary aim of this study was to determine if age at death, CNS penetration-effectiveness of antiretroviral therapy, diagnosis before HAART, average plasma CD4, or abnormal alcohol or drug use increased prevalence of neurocognitive disease.

STUDY DESIGN: Cross-sectional study of HIV-infected individuals (n=60) from the larger National NeuroAIDS Tissue Consortium Cohort. Pre-death demographic data, neurocognitive assessment, alcohol and drug use, ART regimens, date of diagnosis and death, and plasma CD4 levels, as well as pathology findings at autopsy and brain tissue samples were provided by the NNTC; expression levels of Substance P, NK1R, CCR5, and CXCR4 from brain samples were provided by Dr. Steven Douglas of The Children's Hospital of Philadelphia. All data was de-identified.

RESULTS: In this sample of HIV-infected individuals, Substance P expression was significantly less in the cingulate cortex of individuals with (p=0.003). Within-subject expression patterns of CCR5 and truncated-NK1R in the cingulate cortex and cerebellum were both significantly altered by neuropathology and cannabis use; CCR5 expression was also significantly affected by opiate use. CCR5 and CXCR4 expression correlated strongly with truncated-NK1R expression. No between-subject factors significantly altered prevalence of neurocognitive impairment in this HIV-infected population.

CONCLUSIONS: The study found significant changes in Substance P, NK1R, and CCR5 expression associated with neuropathology. Furthermore, in heterogeneous populations, expression patterns may be more important than overall level of expression in identifying risk factors for NeuroAIDS and other chronic diseases.

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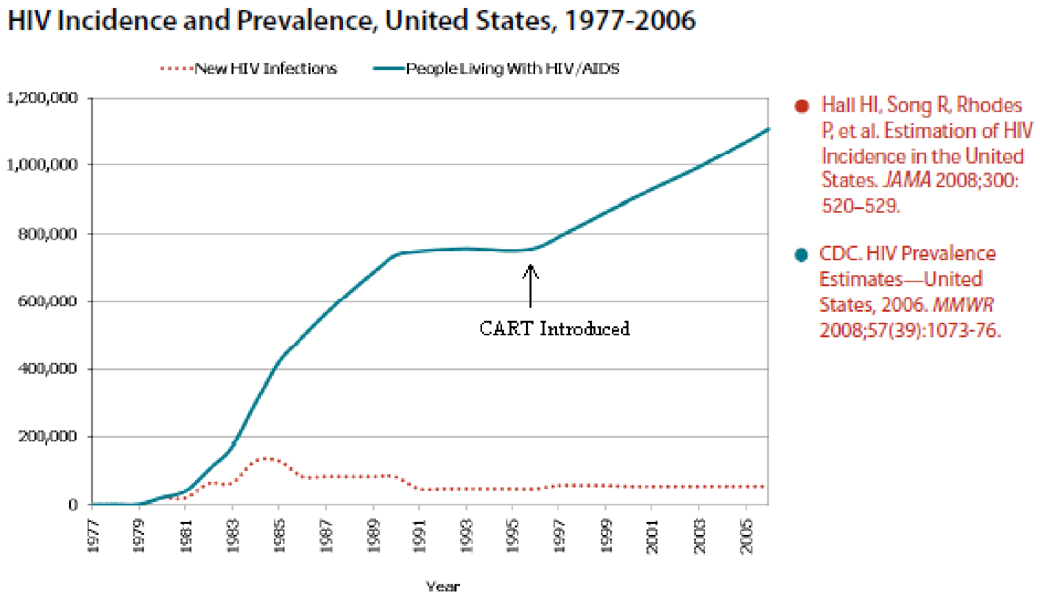
CHAPTER 1. INTRODUCTION

In 1981, five previously healthy, homosexual men reported to a clinic in Los Angeles with rare and fatal cases of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma (MMWR, 1981). In the year that followed, hundreds of homosexual men- and later, needle drug-users- presented at clinics across the country with rare illnesses associated with an underlying condition of severe immune suppression of unknown etiology. In 1982, the name acquired immune deficiency syndrome (AIDS) was given to this condition, and in 1983, human immunodeficiency virus (HIV) was identified as the causative agent (MMWR, 2011).

In the thirty years since the beginning of the AIDS epidemic, more than 600,000 individuals have died in the United States alone (HIV Surveillance Report, 2008, CDC). Worldwide, over 60 million people have been diagnosed with HIV since the beginning of the epidemic, and almost 30 million have died of AIDS. In 2009, an estimated 33.3 million people were living with HIV globally, with 2.6 million new infections and an estimated 1.8 million deaths per year (AIDS Epidemic Update, UNAIDS, 12/2009). Over 1.2 million people are living with HIV in the United States, with over 17,000 deaths per year (HIV Surveillance Report, 2008, CDC).

The introduction of combination, highly-active antiretroviral therapy (HAART) in 1996 significantly decreased AIDS-related mortality; in the years that followed, increased life expectancy led to a dramatic rise in the prevalence of HIV and AIDS following diagnosis (Figure 1). In the pre-HAART era, annual AIDS-related mortality grew quickly and steadily from 451 in 1981 to 50,628 in 1995; following the introduction of HAART, AIDS-related mortality plummeted 63% from 18, 851 in 1998, and has remained steady since. Between 1996 and 2008, the number of people >13 years of age living with Stage C AIDS has steadily risen from 219,318 to 479,161 (MMWR, 2011)

Figure 1. HIV Incidence and Prevalence in the United States from 1977 to 2006, (CDC Factsheet, June 2010)



HIV Incidence and Prevalence, United States, 1977-2006

● Hall HI, Song R, Rhodes P, et al. Estimation of HIV Incidence in the United States. *JAMA* 2008;300: 520–529.

● CDC. HIV Prevalence Estimates—United States, 2006. *MMWR* 2008;57(39):1073-76.

Despite the introduction of combination HAART, HIV-associated neurocognitive disorder (HAND) and HIV-neuropathology (e.g. HIV-encephalopathy, gliosis) persist as common manifestations of HIV-infection. Features of AIDS-related neurological disease and dementia were described in one-third of AIDS patients as early as 1984, and HIV-associated dementia and HIV-encephalopathy were added as AIDS indicator illnesses in 1987 (Wolcott et al, 1985 and MMWR, 1987). Characterized by behavioral changes and declines in cognition and motor skills, HAND often impairs daily activities and functioning, and can progress to frank-dementia in later stages. Extensive neuropathology at autopsy is characteristic of individuals with AIDS dementia, but neuropathology also occurs at high rates in individuals without diagnosed neurocognitive impairment (Robertson et al, 1998). HIV-associated neurocognitive disease was formally defined with diagnostic criteria in 1991 by the American Academy of Neurology (Janssen et al, 1991); in 2007, diagnostic updates were made by the HIV- Neurobehavioral Research Center and universally adapted (Antinori et al, 2007).

The first formal definition for HAND adopted by the American Academy of Neurology in 1991 defined two levels of clinical manifestation: minor cognitive motor disorder (MCMD) and HIV-associated dementia (HAD) (Janssen et al, 1991). MCMD is characterized by impaired behavioral or cognitive function in two areas (e.g. attention or memory, mild personality changes, and mental slowing) with mild impairment in work and activities of daily living; the more severe HAD was further characterized by functional impairments resulting from cognitive abnormalities in two or more domains with significant impairment in work or activities of daily living, and additional abnormalities in either motor function or neuropsychiatric function (e.g., behavioral changes, apathy).

Updated diagnostic criteria introduced in 2007 by the HIV Neurobehavioral Research Center, and subsequently employed by the World Health Organization, identified three diagnostic levels for HAND: asymptomatic neurocognitive impairment (ANI), minor neurocognitive disorder (MND), and HIV-associated dementia (HAD) (Antinori et al, 2007). According to this criteria, ANI is defined by "performance at least 1 SD below the mean of demographically adjusted normative scores in at least two cognitive areas (attention-information processing, language, abstraction-executive, complex perceptual motor skills, memory, including learning and recall, simple motor skills *or* sensory perceptual abilities)", which does not interfere with daily activities and cannot be attributable to opportunistic infection or other co-morbidities. Like ANI, minor neurocognitive disorder (MND) is also defined by performance at least one standard deviation below the adjusted mean in two cognitive areas, but is further characterized by impairments in daily living and work. The final, and most severe category, HIV-associated dementia, is defined by severe impairment in daily functioning and work, and performance at least two standard deviations below the mean in two or more of 5 cognitive areas. (Antinori et al, 2007).

Introduction of HAART in 1996 controlled HIV viremia, facilitated immune reconstitution, and improved medical outcomes in HIV-infected individuals. Similarly, HAART therapy was associated with a decrease in prevalence of the most severe forms of AIDs dementia, from 16% in 1993 (McArthur et al, 1993) to less than 5% in 2010 (Heaton et al, 2010). Nevertheless, the overall prevalence of HIV-associated neurocognitive disease (including ANI, MND, and HAD) persists in an estimated 36-45% of individuals with HIV, and pathologies at death remain high. Further, a study of pre-HAART (1988-1995) and post-HAART (2000-2007) HIV-infected individuals using diagnostic criteria outlined by the HNRC found a significant increase in the rate of neurocognitive impairment (36% versus 25%) in CDC Stage A HIV infection in individuals in the post-HAART era (Heaton et al, 2011).

Before the introduction of HAART, HAND was strongly associated with advanced stages of HIV-disease, low CD4+ T cell counts ($<0.2\text{mm}^3$), and increased CSF viral load (Robertson et al, 1998). Following HAART, prevalence of HAND continues to increase with progressed stages of disease (36% in CDC Stage A, 40% in Stage B, and 45% in Stage C), but incidence in Stage C has decreased since the pre-HAART era (Heaton et al, 2011). Studies by Robertson et al (2007) and Tozzi et al (2007), respectively, have found that overall prevalence of HAND within populations is not dramatically altered following introduction of HAART, and that neurocognitive impairment persists in most individuals even at 5 years following initiation of therapy. In contrast to the pre-HAART era, there are no longer significant differences in CD4+ T cell counts between those with and without neurocognitive impairment. Letendre et al. (2008) proposed a model by which NeuroAIDS progression is determined by effectiveness of antiretrovirals to penetrate through the blood-brain barrier into the CNS (CNS penetration score); this study proposed that the greater the CNS penetration score of the drug regimen, the better it is able to penetrate into the CNS, control CSF viral load, and to prevent progression of NeuroAIDS. However, after introduction of HAART, CSF viral load is not significantly different between individuals with and without neurocognitive impairment (Heaton et al, 2011). Taken together, these findings indicate that neurocognitive impairment is not the direct result of systematic immune failure and increased viremia in the CNS, but more likely the result of complex molecular mechanisms that have yet to be elucidated.

Several explanations have been proposed to understand the persistence of HIV-associated neurocognitive disorder and neuropathology despite immune reconstitution and low viremia following antiretroviral therapy. One possible explanation is that the molecular mechanisms of HIV infection, replication, and disease may be different in the central nervous system than the periphery. The CNS is separated from the periphery by the blood brain barrier (through which antiretroviral drugs may not be able to pass), and as a result is composed of a unique population of cells not seen elsewhere. Soon after HIV infection, HIV transgresses the blood brain barrier, and infection persists and propagates in microglia and astrocytes (Banks W., 2005). Infection of these cell lines induces expression of chemokines MCP-1 and fractalkine, which promotes adhesion of peripheral, circulating monocytes to the endothelial cells of the brain barrier, and subsequent transmigration into the CNS (Gras et al., 2010) Viral entry into cells is achieved by binding of HIV envelope protein gp120 binding to CD4, CCR5, or CXCR4; viral tropism is characterized by mutations in gp120 that increase the virus' affinity for one cell receptor over another (Ghorpade, 2005). HIV infection in the CNS is predominantly of the macrophage tropic CCR5-binding R5 HIV-viral tropism that infects microglia, while in the periphery both X4 and R5 viral tropisms are observed and predominately infect CD4+ T cells (Levy, 2007). Expression of host and viral proteins may be unique and distinct in the CNS, and may yield traditional antiretroviral therapies ineffective even if they are able to penetrate into the CNS. Alternatively, antiretroviral drugs may themselves elicit an immune response resulting in neurotoxic consequences. Consistent with either proposition, neurocognitive impairment and pathology are associated with increased inflammation. Cytokines, chemokines, and other signaling molecules of the immune and neuroendocrine systems are believed to play a significant role in neurological disease progression, and- if the pathways can be mapped- provide important points of intervention and prevention in neurocognitive impairment of HIV-infected individuals.

Substance P and the Pro-inflammatory Response

Substance P (SP) is an eleven amino-acid tachykinin present in the central and peripheral nervous and immune systems (Chang, 1971 and Satake, 2006); it is known to play a role in complex neural signaling associated with depression and stress response.

The tachykinin peptide acts by binding to its cognate receptor, neurokinin-1 (NK1R), which can be expressed on the surface of cells in a truncated or full-length form; binding of substance P to the different isoforms results in two distinct signaling pathways, which may be important to progression of HIV infection in the CNS (Lai JP et al., 2008). In vitro studies have shown that binding of SP to truncated NK1R induces expression of immune receptor CCR5 and increases infectivity of HIV type R5 into monocyte-derived macrophages (Lai JP et al, 2001). Binding of SP and full-length NK1R activates the NF-kB signaling pathway, resulting in increased production of pro-inflammatory cytokines consistent with neuropathy, including interleukin-6 (IL-6), fractalkine (CX3CL1), tumor necrosis factor-alpha (TNFa), and interferon gamma (IFNg) (Douglas and Leeman, 2011). Substance P expression is increased significantly in brain lesions from SIV-infected macaques with encephalopathy, indicating that it may play a role in advanced stages of HIV-infection of the CNS (Vinet-Oliphant et al., 2010). As it elicits a strong pro-inflammatory response and has been demonstrated to increase HIV infectivity, it is thought that substance P and NK1R may provide an important link between HIV infection, HIV-associated neurocognitive disorder, and HIV-encephalopathy. Further, the FDA-approved SP-antagonist, aprepitant, may offer therapeutic or preventative treatment for HIV-infected individuals at risk of developing HIV-associated neurocognitive disorder.

This study is intended to examine the relationship between Substance P, NK1R (truncated and full-length isoforms), the chemokine receptors CXCR4 and CCR5, lifestyle risk-factors, and neurocognitive and neuropathological changes in HIV-infected individuals. It is hypothesized that SP and NK1R (both full-length and truncated) expression will be different in HIV+ individuals with and without HIV-associated neuropathology. Because between-subject variation expression levels can be quite high, a within-subject assessment of substance P and NK1R expression in the cerebellum and cingulate cortex may provide further insight into their role in NeuroAIDS. It is hypothesized that differences of within-subject expression of substance P, full-length NK1R, or truncated NK1R between the cerebellum (a region responsible for motor coordination) and the cingulate cortex (a region responsible for higher cognition and memory) may be different by neuropathology status or presence of neurocognitive disorder.

Binding of substance P to NK1R alters expression of various genes, including upregulation of CCR5, which results in increased HIV-infectivity. In this study, it is hypothesized that CCR5 expression will be positively correlated with full-length and truncated NK1R expression. It is also hypothesized that truncated or full-length NK1R expression will correlate with CXCR4 expression, although the direction is not known.

Finally, this study will determine if prevalence of symptomatic neurocognitive impairment (MCMD or HAD) is significantly affected by host risk factors including diagnosis before HAART, CNS penetration of anti-retroviral regimen, drug and alcohol use, increased age, and substance P expression in the cerebellum and cingulate cortex. Previous studies have suggested protective effects of cannabinoid use and detrimental effects of opiate, cocaine, methamphetamine, and alcohol abuse in the development of neurocognitive impairment in individuals, regardless of HIV status. Other studies have suggested that anti-retroviral regimens with greater ability to penetrate into the CNS may have protective effects against development of neurocognitive disorder. Substance P has been linked to changes mental state from experiences of pain and nausea to depression, and thus may play an integral role in development of cognitive impairment. It is hypothesized that increased CPE score and cannabinoid use will be associated with decreased prevalence of neurocognitive impairment, while increased age, increased expression of substance P, use of opiates, cocaine, and methamphetamine, and alcohol abuse will be associated with increased prevalence of impairment. In addition to main effects, interactions between opiate, cocaine, and methamphetamine use and substance P will be tested, and it is hypothesized that this interaction will increase prevalence of neurocognitive disorder.

CHAPTER 2. BACKGROUND

HIV-associated neurocognitive disorder (HAND) is a common neurological manifestation of HIV-infection, both before and after the introduction of combination highly-active antiretroviral therapy in 1996. HAND is more frequently associated with advanced stages of disease, but is still observed in individuals with Stage A HIV-infection, characterized by undetectable or low plasma viremia and high CD4+ counts. (Heaton et al, 2011).

HIV infection and direct neurotoxicity of HIV proteins are known to elicit strong pro-inflammatory immune responses and to alter signaling pathways in the immune and resident cells of the CNS. These alterations correlate strongly with the presence of HIV-associated neurocognitive disease, but the pathways by which these processes impact development and severity of neurocognitive impairment and pathology have yet to be resolved. The purpose of this study is to increase knowledge of the neuroendocrine signaling molecule Substance P and its cognate receptor neurokinin-1 (NK1R), chemokine receptors CCR5 and CXCR4, and the role of illicit drugs, substance abuse, and antiretroviral therapy in the context of HIV-associated neurocognitive disorders and pathology.

Specific Aim 1: To determine if expression of substance P, full-length NK1R and truncated NK1R in three regions of the brain (cerebellum, cingulate cortex, and frontal cortex) are significantly different in HIV-infected individuals by neuropathology or neurocognitive impairment status.

Specific Aim 2: To determine if expression levels of NK1R correlate with expression levels of chemokine receptors CCR5 and CXCR4 within each of three brain regions (cerebellum, cingulate cortex, and frontal cortex) in HIV-infected individuals.

Specific Aim 3: To determine if prevalence of symptomatic neurocognitive impairment (MCMD or HAD) is significantly affected by diagnosis before HAART, CNS penetration of anti-retroviral regimen, drug and alcohol use, increased age, and substance P expression in the cerebellum and cingulate cortex.

In the pages that follow, a brief history of HIV-associated neurocognitive disease and HIV neuropathology with diagnostic criteria will be provided. An overview of the brain regions to be considered in this study will be discussed. Neuroinflammation, and the molecular mechanisms underlying HIV-infection in the CNS will be explained; cognitive impairment and pathology will be discussed, with special consideration given to the role of substance P and NK1R. In addition, the molecular mechanisms by which drug or alcohol use may enhance or protect against the neurotoxic events that occur in HIV-associated neurocognitive disorders and neuropathology will be addressed. Finally, a potential point of intervention- aprepitant- will be offered. Aprepitant is a substance P antagonist, which blocks SP-mediated up-regulation of CCR5 and release of pro-inflammatory cytokines, and has been shown to decrease HIV-infectivity in vitro.

History of HIV-associated Neurocognitive Disorders: Pre- and Post- HAART

HIV-associated neurocognitive disorder was initially defined as two stages - minor cognitive motor disorder (MCMD) and the more severe HIV-associated dementia (HAD) - by the American Academy of Neurology (AAN) in 1991 (Janssen et al., 1991, Table 1). MCMD was characterized by impairment in two functions (e.g., mental slowing, decreased memory or attention, slowed or impaired movement or coordination, or behavioral changes) that led to mild disruptions in daily activities or work, and could not be attributed to other morbidities. The more severe diagnosis of HAD was defined by at least two non-motor cognitive impairments that affect work or daily activities, and the presence of abnormalities in motor or behavioral function (Antinori et al, 2007)

In 2007, Antinori et al. introduced a revision to the definitions of HIV-associated neurocognitive disorder that has been accepted by the World Health Organization. Developed by the HIV Neurobehavioral Research Center (UCSD), the new criteria defined three categories of HAND: asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated Dementia (HAD) (Table 1). This new criteria emphasizes cognitive performance, while the previously accepted AAN criterion focused more readily on motor function, and behavioral changes. Both ANI and MND are characterized by impairment (as determined by performance of at least 1.0 standard deviation below the age- and education-adjusted mean) in two of the following domains: language and verbal skills, abstraction and executive skills, attention and working memory, learning and recall, motor skills, sensory and perception, and information processing. While ANI does not interfere with daily activities or work, impairment in MND is accompanied by reductions in mental acuity or inefficiencies in work, homemaking, or social functioning (Antinori et al, 2007). Upon publication of the new criterion, Antinori et al. reported 79% agreement between the new diagnostic criterion and the former AAN criterion, but the HNRC displayed greater predictive power at predicting HIV encephalopathy at death (Antinori et al, 2007).

HAND is often accompanied or preceded by a multitude of pathological changes within the brains of HIV-infected individuals that confirm the importance of inflammation and subsequent alterations in host cell-signaling, but again none of these are sufficient as an absolute diagnostic tool. Computerized tomography (CT) scans and magnetic resonance imaging (MRI) have commonly shown ventricular enlargement and cerebral atrophy in patients with HAND, and the severity of these findings generally correlate with neuropsychological performance. Still, atrophy is not limited to those with neurocognitive impairments and is often observed in infected individuals without clinical symptoms (Navia and Price, 2005) Protein density MRI often reveals abnormalities in the white matter of HIV-infected individuals, indicative of HIV- encephalopathy. Functional imaging reveals metabolic dysfunction at the regional and cellular level accompanying HIV infection. More specifically, hyper metabolism is observed in the basal ganglia and thalamus, while hypo metabolism is found in the cortex (Rottenberg et al, 1987). Increased choline and myoinositol, a marker of glial activation, levels are observed in HIV-infected individuals, even in asymptomatic patients. Decreases in N-acetyl aspartate, a marker of mature neuron function, particularly in the frontal cortex, have been found in individuals with advanced HIV-dementia (Navia and Price, 2005).

Table 1. Diagnostic Criteria for HIV-associated Neurocognitive Disorder

American Academy of Neurology	Minor Cognitive Motor Disorder (MCMD)	-Impaired in two or more areas of cognitive, motor or behavioral function (e.g. abnormal memory or concentration, slowed movement, incoordination, or increased irritability) -Mild impairment in work or activities of daily living -Cannot be attributed to opportunistic infections or co-morbidities
	HIV-Associated Dementia (HAD)	-Presence of abnormalities in two or more cognitive areas -AND- -Presence of an abnormality in motor and/or psychosocial/ neuropsychotic (e.g. emotional control, apathy, depression) function -Impairment in work or activities of daily living -Cannot be attributed to opportunistic infections or co-morbidities
HIV Neurobehavioral Research Center	Asymptomatic Neurocognitive Impairment (ANI)	-Performance greater than 1 Standard Deviation below the demographically adjusted mean scores on 2 of at 5 or more measured cognitive assessments -Cognitive measures include: attention-information processing, language, abstraction-executive, complex perceptual motor skills, memory, simple motor skills, sensory perception -Impairment does not interfere with work or daily activities -Impairment cannot be attributable to opportunistic infection or other co-morbidities
	Minor Neurocognitive Disorder	-Meets diagnostic criteria for ANI -AND- -Impairment interferes with activities of daily living or work function
	HIV-Associated Dementia	-Performance greater than 2 Standard Deviations below the demographically adjusted mean scores on 2 of at 5 or more measured cognitive assessments -Cognitive measures include: attention-information processing, language, abstraction-executive, complex perceptual motor skills, memory, simple motor skills, sensory perception -Severe impairment in activities of daily living and work function

HIV and Neuropathology

Neuropathology was observed in brain autopsies of HIV-infected individuals early in the AIDS epidemic. As early as 1983, it was established that neuropathological changes in individuals with AIDS were not simply the result of opportunistic infections resulting from immune suppression (Bell and Gray, 2005). Autopsy studies conducted between 1986 and 1993 revealed neuropathological changes in 55-95% of individuals with HIV-1 (Budka, 2005). Of these, an estimated 25-65% of neuropathological changes resulted from opportunistic infection of the central nervous system resulting from immune suppression. Still, an estimated 11-40% of neuropathologies in HIV-1 infected individuals arise from direct infection of CNS cells (Budka, 2005).

Introduction of combination antiretroviral therapy in 1996 did not lessen the rate of HIV-associated neuropathology. An examination of autopsy findings from 394 HIV-infected individuals that died between 1979 and 2000 in New York City revealed that while individuals who died after the introduction of HAART had decreased prevalence of many viral and fungal illnesses, there was an increase in individuals presenting with CNS findings (Morgello et al., 2002). Prevalence of HIV encephalopathy, aseptic leptomeningitis, progressive multifocal leukoencephalopathy, and CNS lymphoma at autopsy increased from 58% in those that died before 1996 to 80% in those that died between 1996 and 2000 (Morgello, 2002).

HIV-associated neuropathologies fall into two major diagnoses: HIV-encephalopathy or HIV-leukoencephalopathy, and are characterized by lesions containing multinucleated giant cells (MNGCs) at autopsy (Table 2). CNS infection by HIV-1 is most prominent in the macrophage-derived microglia, which express both CD4 and

CCR5 (Clapham and McKnight, 2001), but has also been commonly observed in astrocytes (Brack-Werner, 1999). Viral infection of oligodendrocytes, endothelial cells, and neurons have been observed using in situ hybridization in post-mortem analysis of brains from patients with HIV-associated Dementia (3295, 4422, Levy), but has not been widely reported.

HIV- encephalopathy (HIV-E) is characterized by multiple and widely-scattered foci, increased inflammatory responses, and the presence of large microgranulomas composed of elongated microglia cells, macrophages and MNGCs, and occasionally lymphocytes (Budka, 2005). In the early 1990s, HIV-E foci were predominantly seen in the basal ganglia (Kure et al, 1991), but were also observed in the white matter of other regions, including the corpus callosum, anterior commisurie, and brainstem (Budka, 2005). Following the introduction of HAART, a study by Anthony et al. (2005) reported significantly higher numbers of microglia and macrophages in the basal ganglia of HAART- treated patients. Interestingly, HIV-E lesions became more prevalent in the hippocampus and entorhinal and temporal cortexes of autopsied brains after the introduction of HAART (Gras, 2010).

HIV leukoencephalopathy (HIV-L) affects the cerebral and cerebellar white matter, and is characterized by myelin loss, reactive astrogliosis, and the presence of microglia and less commonly MNGCs. Unlike HIV-encephalopathy, the pattern of damage is diffuse and little to no inflammation is present. Immunostaining for HIV-1 p24 antigen confirms suspected HIV-L in cases without MNGCs (Neuenburg, 2009).

In addition to HIV-encephalopathy, leukoencephalopathy, and various damage from opportunistic infections, HIV-1 infection is associated with increased amyloid-beta deposition in the brain (Green et al., 2005), a commonly-presenting condition in various forms of other dementias, including Alzheimer's. Interestingly, a study by Green et al (2005) found elevated levels of beta-amyloid precursor protein in the brain of HAART-era brain tissues compared to age and gender matched historical controls, indicating that patients on combined antiretroviral therapies may be at increased risk of cognitive impairments due to beta-amyloid deposition.

Table 2. Diagnostic Criteria for HIV-associated Neuropathology

HIV Encephalopathy
-Presence of large Multi-nucleated giant cells (MNGCs) -Multiple, widely scattered foci -Increased inflammation -Presence of large microgranulomas composed of elongated microglia, macrophages and MNGCs - Foci are prominent in the basal ganglia, hippocampus, and cerebral cortex
HIV Leukoencephalopathy
-Diffuse and disseminated damage -Myelin Loss -Reactive Astrogliosis -Presence of microglia and sometimes MNGCs (in absence of MNGCs, immunostaining for HIV p24 antigen required to confirm suspected cases) -Minimal inflammation

Functional importance of brain regions

In this study, expression of signaling molecules and chemokine receptors will be examined in three areas of the brain: the cingulate cortex, cerebellum, and frontal cortex. Each of these three regions has unique functions, and inflammation and damage in each may result in different outcomes.

The cingulate cortex encompasses the cingulate gyrus and the cingulate sulcus. It sits in the limbic lobe above the corpus callosum and hippocampus, and is considered an integral part of the limbic system. It receives input from the neocortex and thalamus, and projects to the entorhinal cortex. As a part of the limbic system, the cingulate cortex is expected to play a key role in learning and memory, emotion formation and processing, motivation, and executive function.

The cerebellum is located just below the cerebrum, and attached via the pons to the brain stem. It plays an integral role in receiving input from periphery nerves, and coordinating fine motor function. Damage to the cerebellum leads to problems with fine motor control, walking, slurred speech, and vertigo; more recently fMRI analyses have shown activation within the cerebellum in response to language and attention, indicating that the cerebellum may play a larger role in cognition than originally thought.

The frontal cortex is located in the anterior position of the cerebrum and encompasses 1/3 of the total human brain mass. The frontal cortex contains most of the dopamine-sensitive neurons in the cerebrum, so it plays it an integral role in attention, drive, and short-term memory. Damage to the frontal lobe is common after stroke, and can lead to changes in personality and behavior, difficulty with problem solving and organizing tasks, and even paralysis.

Since the introduction of HAART, lesions in the limbic lobe, including the hippocampus and entorhinal cortex, have become more prevalent. At the same time, changes in the epidemiology of HAND have changed, such that frank dementia and motor impairment have become less common and behavioral changes, and alterations in concentration and memory have become more common. Taken together, this suggests that localization of lesions in patients with HIV-encephalopathy may play a role in the determination of symptomatic neurocognitive outcomes.

Inflammatory response, Substance P, NK1R, and NeuroAIDS

HIV infection is characterized by a strong and prolonged inflammatory immune response, and many have suggested that augmenting this inflammatory response could prevent the immune exhaustion that precedes AIDS (Lori F., 2008). HIV-associated neurocognitive impairment and pathology are characterized by production of pro-inflammatory cytokines in the CSF, increased iNOS activation and increased cellular oxidative stress, decreased beta-amyloid degradation resulting in deposition, and dysregulation of glutamate homeostasis resulting in neuronal excitement and apoptosis, and ultimately tissue damage.

Severity of neurocognitive impairment correlates significantly with low nadir CD4+ counts, and increased levels of beta-2-microglobulin, monocyte-chemoattractant protein-1, and neopterin, a marker of macrophage activation, in the CSF (Ghorpade, 2005), confirming that inflammation in the CNS plays a pivotal role in the development of HIV-associated neurocognitive disorder. Consistent with a trend towards inflammation, elevated substance P is found in the plasma of HIV-infected individuals in comparison to uninfected controls (Douglas et al, 2008).

Substance P in an undecapeptide tachykinin encoded for by the TAC1 gene and expressed by cells of the immune system from bone marrow stem cells to macrophages, dendritic cells, and lymphocytes (Douglas and Leeman, 2010). Binding of SP to its full-length cognate receptor, NK1R, elicits a strong pro-inflammatory response, leading to production of pro-inflammatory cytokines IL-1, IL-6, IL-8, IL-12, and TNFalpha (Lai et al, 2001 and 2008), consistent with propagation of neuroinflammation. Substance P can also bind to a truncated form of NK1R, which results in chemotaxis of monocytes, presumably enhancing transmigration across the blood-brain barrier (Chernova et al, 2009). Binding of substance P to NK1R has been shown to increase infectivity of HIV in vitro by upregulating expression of chemokine (and HIV entry co-receptor) CCR5 in monocyte-derived macrophages (Lai et al. 2001).

Substance P's cognate receptor, neurokinin-1 (NK1R), is a G-protein coupled receptor (GPCR) characterized by seven transmembrane domains, an extracellular amino-terminus, and intracellular carboxy-terminus. NK1R is a Class-A (Rhodopsin-like), pertussis-toxin insensitive GPCR (Khawaja and Rogers, 1996) expressed on immune cells, cells of the central nervous system, and within cells of the periphery. NK1R has two common isoforms- a full-length isoform (407 amino acids) and a shorter isoform (311 amino acids) resulting from alternative splicing that creates a c-terminal truncation (Fong et al, 1992).

In human peripheral blood monocytes, NK1R is expressed only in the truncated form (Chernova et al., 2009). Upon activation, monocytes induce expression of the full-length form of NK1R. Treatment of THP-1 cells with PMA (Phorbol 12-myristate 13-acetate) leads to differentiation into activated macrophages; this differentiation is characterized by an increase in full-length NK1R expression (>10-fold increase by 12 days following treatment), but no change in truncated NK1R mRNA levels (Lai et al, 2006). Immunoflourescence staining indicated that the undifferentiated THP-1 cells expressed only the truncated NK1R protein, while the differentiated cells expressed both isoforms. Binding of SP to the two isoforms initiates different signaling pathways that may play a role in HIV infection in the central nervous system; these are discussed in detail below, and outlined in Table 3.

A study by Caberlotto et al. (2003) examined localization of NK1R expression in human brains utilizing in situ hybridization and RT-PCR techniques, and found that expression was highest in the striatum, moderate in the hippocampus, amygdala and cerebral cortex, and low in the cerebellum and thalamus, consistent with localization of radio-labeled substance P localization (r=0.96, p=0.002) (Caberlotto et al, 2003). With the exception of the cerebellum, substantia nigra, and thalamus, the NK1 long isoform was preferentially expressed over the short isoform in the central nervous system. Overall NK1R expression was similar in the frontal and cingulate cortex, but decreased in the cerebellum. Using in situ hybridization histochemistry, Whitty et al (1997) found NK1R mRNA localized to the midbrain of four human brain samples, exclusively in melanin-containing dopaminergic neurons, indicating that NK1R signaling may play a role in dopamine signaling. Within the CNS, SP levels are highest in the striatal regions, substantia nigra, and hypothalamus (Kanazawa and Jessell, 1976 and Cooper et al, 1981).

In vivo studies have found a relationship between HIV infection and increased substance P/ NK1R expression. Both substance P and NK1R expression are altered in SIV-infected rhesus macaques (Vinet-Oliphant, 2010), and in limited studies of human patients with HIV. Plasma levels of substance P are elevated in individuals with AIDS (Douglas et al, 2001 and 2008). In a study of human brain tissue from HIV-infected individuals and uninfected controls, full-length NK1R expression was decreased in the cingulate cortex of HIV-infected individuals, while no significant difference in expression was observed in the cerebellum (Douglas et al, 2008).

SIV-infected rhesus macaques with SIV-encephalopathy showed increased substance P levels in the white matter, particularly within and around SIV-encephalopathy lesions (Vinet-Oliphant et al, 2010). SP expression in uninfected and SIV-infected macaques without encephalopathy was limited to astrocytes and neurons within the grey matter; full-length NK1R expression was not seen in immune cells of normal brains.

Table 3. Substance P Signaling: Roles for the Two Neurokinin-1 Receptor Isoforms

Full Length NK1R	Truncated NK1R
Induces SP-dependent intracellular calcium release	Does not induce SP-dependent intracellular calcium release; enhances (2-fold) CCL5- dependent intracellular calcium release
Quickly activates ERK1/2 (peaks at ~1 min)	Slowly activates ERK1/2 (peaks at 20-30 minutes). Enhances CCL5-mediated activation of ERK1/2.
Phosphorylation PKCδ at threonine-505	No activation of PKC
Increased expression of IL-8 (8-10 fold over control)	Reduced expression of IL-8 over control
Activation of NFkB	Does not activate NFkB transcription
	Phosphorylation of CCR5

HIV infection in the CNS and Viral Tropism

HIV-1 initially enters the central nervous system during acute infection. Two pathways of HIV neuro-invasion have been suggested: HIV-1 may directly bypass the blood brain barrier during acute infection, infect microglia, and persist at low levels for several years, or break-down of the blood brain barrier during later stages of infection may lead to increased transmigration and chemotaxis of infected monocytes, macrophages, and in some cases leukocytes into the CNS (Gras, 2010).

Diapedesis is the process by which circulating immune cells adhere to brain endothelial cells and cross through the blood brain barrier (BBB) (Banks, 2005). BBB premeabilization is tightly regulated through tight junction protein expression; HIV viral proteins are specifically capable of altering expression and activation of regulatory proteins that maintain BBB integrity (Banks, 2005). HIV infection activates focal adhesion kinase (FAK). Envelope protein gp120 degrades tight junction proteins, specifically, ZO-1 and ZO-2, in HBMECs, and Tat reduces expression of occludin, ZO-1, and ZO-2 in a caveolin-1, Ras pathway dependent manner (Nakamuta et al., 2008).

In addition to disrupting tight junction integrity, HIV infection produces a pro-inflammatory response that stimulates the release of chemokines, such as monocyte-chemoattractant protein 1 (MCP-1) and fractalkine, that promote adhesion of circulating monocytes to endothelial cells and transmigration into the CNS (Gras, 2010). Transmigration of monocytes is believed to be extremely important in the development of neurocognitive impairment, as both increased numbers of microglia and monocytes in the CSF and increased expression of MCP-1 in plasma and CSF are independent predictors of neurocognitive impairment. A study by Sevigny et al. (2007) found that MCP-1 expression in plasma and CSF independently predicted progression of HIV-associated dementia.

It is believed that Substance P may play a role in chemotaxis, and thus may facilitate transmigration of monocytes into the CNS, similar to MCP-1. In studies with human peripheral blood monocytes, substance P interaction with the truncated NK1R isoform significantly enhanced ERK1/2 dependent, CCL5-mediated chemotaxis (Chernova et al, 2009). Substance P has chemotactic qualities indistinguishable from CCL5 and pre-treatment of monocytes with SP increases chemotaxis in response to substance P, and to a smaller degree, CCL5 (Vinet-Oliphant, 2010)

Once inside the CNS, the primary reservoir for HIV infection is microglia (Williams et al., 2001), which express both CD4 and CCR5 chemokine receptors on their surface. Upon activation, infected microglia upregulate expression and cell-surface presentation of CD14, CD16, CD68, and MHCII (Anthony et al, 2005); microglial activation is further characterized by the release of TNFalpha, IL-1, oxidative radicals, and nitric oxide. On average, CD14+/ CD16+ monocytes compose 6% of the total monocyte population in healthy individuals, 16% of the monocyte population in those with late stage AIDs, and 37% of the monocyte population in individuals with HIV-associated dementia (Pulliam et al, 1997). This monocyte population is more phagocytic than CD16- monocytes, and produce greater levels of TNFalpha, MHCII and IL-1. (Pulliam, 1997). Astrocytes express receptors CCR5 and CXCR4, but not CD4 (Brack-Werner, 1999), so HIV entry is possible, but occurs in a less-efficient, CD4-independent manner.

Infection of the CNS is characterized by a greater presence of R5, macrophage-tropic viral isolates. Substance P treatment increases NK1R and CCR5 expression in monocytes, thus increasing infectivity of R5 tropic viruses in these cells. Interestingly, viral isolates from brain tissues of HIV-infected individuals demonstrated that those with HIV-associated dementia and HIV-encephalopathy had a greater heterogeneity in tropism (R5, X4, and X4R5 strains that are able to infect without CD4) than those without pathology (Levy, 2007). Initial viral infection and viral isolates from individuals with non-progressive HIV-1 infection of more than 14 years are predominantly of the R5 tropism, while X4 isolates are associated with faster progression to AIDS and greater T cell decline (Pantaleo, 1997). The syncytium-forming X4 isolates are responsible for formation of multinucleated cells indicative of HIV-associated neuropathologies (Devadas, Lal, and Dhawan, 2005). In SIV-infected rhesus macaques with encephalopathy, substance P immunoreactivity is predominately associated with MNGCs and perivascular macrophages (Vinet-Oliphant, 2010), indicating that substance P may modulate expression of CXCR4 as well. Similar to substance P, NK1R expression in SIV-E brains was highest within SIV-E lesions, and associated with MNGCs (Vinet-Oliphant, 2010).

Molecular Mechanisms of Neurodegeneration

Neurocognitive impairment and pathology from HIV infection is achieved by disruption of cell-signaling cascades and dysregulation of homeostasis in response to pro-inflammatory signals. NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) expression, glutamate regulation and NMDA receptor binding, iNOS production and oxidative stress, beta-amyloid deposition, and dopaminergic signaling are believed to play key roles in the development of neurocognitive impairment and tissue damage associated with neuropathology in HIV infection and other neurodegenerative diseases. Many host factors, as well as viral proteins, play a role in modulation of these homeostatic elements. The neurotoxic effects of pathway dysregulation are discussed in detail below, with an emphasis on the contribution of substance P- NK1R signaling. Alcohol and substance abuse will also be discussed later, as both drug and alcohol use can potentiate the neurotoxic effects of viral proteins and inflammation.

NFkB and intracellular Signaling Cascades

NFkB is a cellular transcription factor responsible for cellular responses to various forms of stress, including oxidative stress and viral and bacterial antigens. NFkB expression is tightly regulated and changes in expression are linked to changes in neuronal plasticity, memory, learning and depression (Kaltschmidt and Kaltschmidt, 2009). NFkB not only regulates host gene transcription, but also binds to HIV long terminal repeats and modulates viral gene expression.

HIV Tat, or trans-activator of transcription, is a regulatory protein that binds to sequences of the HIV-1 long terminal repeats (LTRs) and enhances viral transcription and RNA processing in an NFkB-dependent manner (DiStefano et al, 2005). Tat also binds to host DNA sequences, and is known to upregulate expression of pro-inflammatory cytokines and adhesion molecules in monocytes; it is released from infected cells and can be taken up distally by other cells, thus affecting NFkB signaling in distal locations (DiStefano et al., 2005).

Binding of Substance P to full-length NK1R specifically activates the NF-kappa-B signaling cascade and leads to increases in intracellular calcium and production of pro-inflammatory cytokines IL-1, IL-6, IL-8, IL-12, and TNFalpha (Lai et al, 2001, and Lai et al, 2008). In HEK293 cells expressing only the truncated NK1R, binding of substance P decreased expression of IL-8 mRNA and failed to activate NFkB signaling or intracellular calcium release, indicating that the carboxy-terminus is required for these signaling cascades (Lai et al, 2008). Binding of SP to the truncated isoform may play an inhibitory role in NFkB activation by depleting the extracellular environment of free substance P. SP-binding to the full-length isoform quickly and potently activates ERK1/2 (within one minute), while binding to truncated NK1R results in a delayed activation (peaking at 20-30 minutes). Indeed, binding of SP to truncated NK1R only results in phosphorylation of ERK2 (Chernova et al, 2009). Binding to the full-length isoform leads to phosphorylation of PKCδ at threonine-505 (Lai et al, 2008).

SP treatment caused an intracellular calcium release in differentiated THP-1 cells, but only enhanced a CCL5-mediated calcium release in undifferentiated THP-1 cells exclusively displaying the truncated NK1R isoform (Lai et al, 2006). In cells expressing full-length NK1R, binding of SP induced intracellular calcium release, phosphorylation of PK; this result is not seen in cells expressing only the truncated isoform (Lai et al, 2008)

Glutamate Homeostasis

Glutamate is a prominent excitatory neurotransmitter, accounting for up to 70% of synaptic transmissions within the central nervous system (Guo et al, 2009). HIV infection of astrocytes reduces their ability to degrade intercellular glutamate and other potentially neurotoxic substances. Glutamate binds to the N-methyl-D-Aspartate (NMDA) receptor, and leads to neuronal excitement. Excess glutamate in the extracellular space, coupled with inefficient degradation by astrocytes, leads to excitatory stimuli and ultimately results in neural apoptosis (DiStefano, 2005).

In a study by Hill et al. (1993), neonatal rats intracranially injected with HIV envelope protein gp120 displayed mental retardation similar to HIV-associated dementia. Gp120 has been linked to neurotoxity in several studies that indicate dysregulation of glutamate homeostasis, reactive astrocytosis, and neuronal excitement and apoptosis through multiple signaling pathways. Gp120 stimulates the release of pro-inflammatory cytokines interleukin1-beta (IL-1beta) and tumor necrosis factor alpha (TNF-alpha). Injection of Gp120 into rat hippocampal regions leads to neuronal apoptosis in an N-methyl-D-aspartate receptor mediated fashion. In activated macrophages, viral Nef stimulates production of quinolic acid, a kynurenine metabolite that acts specifically upon NMDA receptors and contributes to neuronal excitement and apoptosis. High levels of Nef mRNA have been associated with multinucleated giant cells and reactive astrocytes in patients with HIV-associated dementia (Di Stefano, 2005).

Little is known about the relationship between substance P and glutamate signaling, but some recent studies have begun to provide evidence that substance P and NK1R may play a role in glutamate regulation and homeostasis. A study by Teuchner et al. (2010) showed that glutamate may down regulate substance P expression in rats. Another study by González-Flores et al. (2011) showed that substance P may modulate glutamate-AMPA excitation via an NK1R-dependent pathway.

iNOS and Oxidative Stress

Activation of iNOS leads to production of nitric oxide, which binds to several enzymes in the electron transport chain, inhibits mitochondrial respiratory burst, and diminishes the cellular ability to cope with oxidative stress (Di Stefano et al, 2005). In a study by Adamson et al (1996), HIV-associated dementia severity and rate of progression significantly and positively correlate with neural gp41 levels; further, there was a strong correlation between gp41 and iNOS in the brains of severely demented individuals, indicating that gp41 exposure may initiate a cascade of events that results in increased oxidative stress in brain tissue (Adamson et al, 1996). A synthetic peptide containing the N-terminal domain of gp41 was found to elicit neuronal death in an iNOS-dependent manner, indicating that the amino-terminus of gp41 is responsible for its neurotoxic effects (Adamson et al, 1999).

A study by Kraft-Terry et al. (2010) of HIV-infected Puerto Rican women from the Hispanic/Latino Longitudinal NeuroAIDS cohort revealed decreased levels of several antioxidant proteins in monocytes isolated from peripheral blood mononuclear cells of 5 HIV-infected women with HAD when compared to 4 infected women with normal cognitive functioning, indicating a correlation between decreased ability to cope with oxidative stress and severity of neurocognitive impairment.

Expression of iNOS is observed only during immune response and not in healthy tissue; iNOS produces nitric oxide that reacts with superoxide anion to yield peroxynitrite, which increases cellular oxidative stress, damages DNA and RNA and ultimately to cell death via apoptosis. In vitro studies have shown that cultured neurons die 5-7 days following induction of iNOS. Nitric Oxide formation has been demonstrated to be greater in the brains of demented HIV-infected individuals. Similarly, there is a correlation between iNOS expression and rate and severity of HIV dementia progression (Di Stefano, 2005).

Beta-amyloid deposition

As seen in the neurodegenerative disorder Alzheimers' Disease, inefficient processing of amyloid-beta precursor protein is suspected to play a role in progression to HIV-associated dementia. Beta amyloid deposition in the brain of HIV-infected individuals is most common in the hippocampus and frontal lobes, and severity positively correlates with years of infection (Levy, 2007). Amyloid accumulation is typically intraneuronal and amyloid-beta immunoreactivity is greater in individuals with HIV-E compared to HIV-infected controls without pathology (Levy, 2007).

In human tissues from Alzheimer's patients, Substance P immunoreactivity is observed surrounding beta-amyloid plaques, and depleted within the hippocampus (Armstrong et al, 1989 and Quigley and Kowall, 1991), indicating that substance P signaling may be associated with beta-amyloid deposition in HIV infection as well. Substance P expression was similarly altered in mouse models of Alzheimers' Disease. A study using transgenic mice that constitutively over express the beta-amyloid protein found Substance P expression to be increased in astrocytes surrounding B-amyloid plaques (Willis et al, 2007). Interestingly, a portion of the beta-amyloid protein is virtually homologous to tachykinins, and is found to induce MAPK signaling in astrocytes as substance P does.

Dopamine Regulation

Finally, dompaminergic systems appear to play a role in HIV-associated neurocognitive impairment. SIV-infected rhesus macaques have decreased levels of dopamine and dopamine signaling factors even in early infection as compared to uninfected controls (Rumbaugh and Nath, 2009). Dopamine metabolites, specifically homovanillic acid, are decreased in the cerebrospinal fluid of asymptomatic HIV-positive individuals when compared to healthy controls, with levels further depressed in individuals with HIV-associated Dementia (Rumbaugh, 2009). Finally, the presence of gait slowing in >50% of infected individuals and impaired motor skills, indicates dopaminergic dysfunction is prominent in HIV/AIDS.

A study by Whitty et al (1997) found that in the substantia nigra, SP-containing nerve endings are found adjacent to dendrites and dopamine-containing neurons, indicating that substance P and its cognate receptor may play a role in dopamenergic signaling (Nath, 2000).

NeuroAIDS and Substance Abuse

Drug abuse independently affects cognitive and systemic function, and results in physiological changes within the brain. Numerous studies have confirmed increased incidence of neurocognitive impairment, HAD and HIV-encephalopathy in HIV- positive individuals who also abuse drugs (Anthony, 2008). A study by Bouwman et al. (1998) found that neurological and cognitive impairment advanced at a significantly faster rate in HIV-1 infected drug users. Pathologically, drug abusers have greater levels of cytolytic T-cell infiltration, increased microglial activation and inflammation, astrocytosis, and axonal damage (Anthony, page 2008).

One mechanism by which illicit drugs lead to neurotoxicity is by altering the permeability of the blood brain barrier, allowing chemotaxis of infected cells into brain tissues. Cocaine specifically alters expression of cell adhesion molecules and tight junction proteins in brain microvascular endothelial cells, and in doing so, increases permeability of the blood brain barrier to transmigration of HIV-infected macrophages. Cocaine upregulates expression of ICAM-1, VCAM-1, and PECAM-1, which facilitates chemotaxis (Gras, 2010). Further, cocaine increases expression of DC-SIGN, a CD4 independent HIV receptor expressed on the surface of dendritic cells, which increases chemotaxis and infection of dendritic cells (Rumbaugh, 2009). Morphine and methamphetamines activate the release of pro-inflammatory cytokines, leading to a calcium-dependent activation of myosin light chain kinase, and increased permeability of the BBB (Gras, 2010).

Dopamenergic systems and regulation of dopamine signaling play an essential role in the development of addiction, and more recently it has been suggested that they play a significant role in neurocognitive function . The role of several drugs on dopamenergic cells has been studied. An in vivo study found that methamphetamine and Tat protein affect the dopamine system synergistically; administration of each caused 7-8% reduction in striatal dopamine levels when administered alone, but led to a decrease of 65% in striatal dopamine levels and a significant decrease in dopamine release when administered together (Cass et al, 2003). MDMA, commonly called ecstasy, down-regulates dopamenergic receptors, but the effect of MDMA use in HIV-positive individuals has not been sufficiently studied (Rumbaugh, 2009). Opioids alter expression of dopamine receptors on cell surfaces, and cocaine abuse is linked to neurotoxicity of neurons expressing dopaminergic receptors (Rumbaugh, 2009).

Significant evidence exists that that certain drugs interact, and in some cases synergize, with HIV-encoded proteins to enhance pro-inflammatory responses, oxidative stress, and neurotoxic effects. Methamphetamine has been shown to interact with Tat protein to disrupt mitochondrial metabolism, resulting in increased oxidative stress (Rumbaugh, 2009). Additionally, in vivo experiments using a murine model suggest a synergistic and pro-inflammatory neurological role Tat protein and methamphetamines that upregulates expression of ICAM-1, TNFa, and IL1B (Flora et al, 2003). Like HIV, opiate use suppresses immune function, and long-term use disrupts cellular signaling pathways leading to altered gene expression and increased oxidative stress (Rumbaugh, 2009). It has been proposed that HIV and opiates act synergistically in what Donahue and Vlahov (1998) termed the “opiate cofactor hypothesis”. Chronic morphine exposure in SIV-infected rhesus macaques resulted in significant increases in plasma and CSF viral loads, and increased propensity towards AZT-resistance development (Rumbaugh, 2009). Opiates that act on Mu-opioid receptors, including heroin and morphine, increase expression of HIV co-receptors CCR5 and CXCR4, thus increasing HIV infectivity; stimulation of Kappa-opioid receptors (Salvia) decreases CCR5 expression, leading to decreased infectivity of R5-tropic viruses, but increases expression of CCR2, a chemokine responsible for monocytic chemotaxis (Rogers and Peterson, 2003). Murine and human models have demonstrated the neurotoxic synergy of HIV Tat protein and morphine on neurons via direct stimulation of neural targets in an opioid-receptor dependent pathway; the neurotoxic effects of Tat and opioids leads to increased astrocyte death and microglial dysfunction, as well as increased inflammatory responses and chemotaxis. Opiate use has been associated with increased severity of HIV-encephalopathy characterized by increased microglial activation in the thalamus and astrocyte infection in the brains of HIV-positive drug users as compared to non-users.

Unlike other drugs of abuse, cannabinoids interestingly have demonstrated neuroprotective effects. Cannaboids increase in vitro expression of tight junction proteins in HBMECs in the presence of HIV-1 gp120. Cannabinoids decrease BBB permeability and subsequent infiltration of infected macrophages (and lymphocytes) into the brain (Gras, 2010). HIV-positive individuals who utilized medical marijuana reported decreases in muscle pain, nausea, anxiety, and depression. Cannibinoids' inhibition of reactive oxygen species, glutamate, and TNFa activity suggests a protective role in off-setting the neurotoxic effects of HIV-associated proteins (Rumbaugh, 2009).

Similar to many drugs, excessive alcohol use may enhance the neurotoxic effects of HIV. SIV-infected rhesus macaques administered alcohol daily had over 30-fold increases in CSF and plasma viral load (Kumar et al, 2005). Chronic alcohol use has been associated with immunotoxocity and nutritional deficits, as well as changes in cellular metabolism that increase oxidative stress. In vitro studies have shown chronic alcohol exposure to cortical and hippocampal neurons increases sensitivity and total numbers of NMDA receptors (Rumbaugh, 2009). Recall that gp120 and Tat proteins act on NMDA receptors to induce Calcium-dependent glutamate release, leading to neuronal excitability and apoptosis, suggesting that chronic alcohol exposure may exacerbate neurotoxicity of these two viral proteins. Indeed, an in vitro study by Chen et al. (2005) found that gp120 apoptosis in neurons was enhanced by treatment with clinically relevant concentrations of ethanol. Still other studies have demonstrated a protective effect of alcohol consumption. Moderate alcohol consumption was linked to NMDA-receptor antagonism and neuroprotection against beta-amyloid deposition (Mitchell, 2009). In vitro studies utilizing rat hippocampus-entorhinal cortex complex and cerebellar slices pre-conditioned for >4 days with 15 to 30mM ethanol showed blockage of gp120-induced neurodegeneration. After 6 days of alcohol pre-conditioning, HEC cells up-regulate expression of heat shock proteins hsp27 and hsp70, which have neuroprotective effects. These results indicate a neuroprotective effect of limited alcohol consumption (<7 drinks per week), and a neurotoxic effect of binge drinking and alcohol dependence.

Substance P, NK1R, and NeuroAIDS: A potential point of Intervention

Despite the introduction of HAART, HIV-associated neurocognitive disorders persist as a common comorbidity in HIV-infected individuals. As discussed, binding of substance P to NK1R increases infectivity of HIV in vitro, and initiates a pro-inflammatory response that promotes increased oxidative stress, glutamate dysregulation, and NFkB expression, resulting in neurotoxicity. The changes in localization of HIV-E lesions to the temporal cortex and hippocampus, regions associated with memory and higher functions, and subsequent higher correlations between HIV-E and advanced neurocognitive impairment in the post-HAART era suggest that the same molecular mechanisms which lead to neurotoxicity also contribute significantly to development of neurocognitive disorders. As such, disruption of the host pro-inflammatory response offers a substantial opportunity for intervention in NeuroAIDS.

Aprepitant, marketed under the name Emend by Merck, is an FDA approved drug for the treatment of nausea in patients receiving chemotherapy; it acts by blocking binding of SP to NK1R. NK1R antagonism by Aprepitant or CP-96,345 inhibits HIV-1 R5 isolate infectivity in monocyte-derived macrophages and microglial cells in vitro by decreasing CCR5 expression (Lai et al, 2001 and Wang et al, 2007). Further, antagonism of NK1R with aprepitant decreased SP and CCR5 expression in microglial cells. Treatment of MDM with CP-96,345 corresponded to a statistically significant decrease in CCR5 expression and TNFalpha production, but did not alter IL-6 production (Lai et al, 2001). Aprepitant enhances the activity of several antiretroviral medications, including efavirenz, AZT, indinavir, and enfuvirtide (Wang et al, 2007, 2008). In another study, aprepitant was found to act synergistically with saquinavir and ritonavir, two protease inhibitors, to increase the anti-HIV activity of these two antiretroviral drugs (Manak et al, 2010).

To date, no studies have been conducted in human brain tissues to examine the relationship between increasing levels of neurocognitive impairment and neuropathology in HIV-infected individuals. Further, the relationship between substance P, NK1R and CXCR4 has not previously been examined. Finally, this study will provide insight into any synergistic effects of drug use and neuroinflammation modulated by substance P. If substance P and NK1R indeed play a role in neuropathogenesis of HIV and potentiate its toxicity through pro-inflammatory responses in vivo, aprepitant could provide a point of intervention to reduce incidence and prevalence of HIV-associated neurocognitive disorders and neuropathology.

CHAPTER 3. METHODS

Specific Aim 1: To determine if expression of substance P, full-length NK1R and truncated NK1R in three regions of the brain (cerebellum, cingulate cortex, and frontal cortex) are significantly different in HIV-infected individuals by neuropathology or neurocognitive impairment status.

> **Sub Aim 1a:** To determine if levels of expression of substance P, truncated NK1R, and full-length NK1R in each of three brain regions (cerebellum, cingulate cortex, or frontal cortex) are different between individuals by pathology status.
>
> **Hypothesis:** Levels of Substance P, full-length NK1R, and truncated NK1R expression in each of three brain regions will be different in HIV-infected individuals with pathology at autopsy and those without.
>
> **Sub Aim 1b:** To determine if patterns of expression of substance P, full-length NK1R, and truncated NK1R between the cerebellum and cingulate cortex within an individual subject differ by neurocognitive impairment status or neuropathology status.
>
> **Hypothesis:** Differences in within-subject expression of substance P, full-length, or truncated neurokinin-1 receptor between the cerebellum and cingulate cortex will be attributable to neuropathology status or presence of symptomatic HIV-associated neurocognitive disorder.

Specific Aim 2: To determine if expression levels of NK1R correlate with expression levels of CCR5 and CXCR4 within each of three brain regions (cerebellum, cingulate cortex, and frontal cortex) in HIV-infected individuals.

Sub Aim 2a: To determine if CCR5 expression positively correlates with full-length and truncated NK1R expression.

Hypothesis: A positive and significant correlation will exist (in each brain region) between CCR5 expression and expression of full-length and truncated NK1R expression in the same brain region.

Sub Aim 2b: To determine if a correlation exists between CXCR4 expression and full-length or truncated NK1R.

Hypothesis: Truncated and full-length NK1R expression will correlate with CXCR4 expression in each respective region of the brain, although the direction of the correlation is not known.

Specific Aim 3: To determine if prevalence of symptomatic neurocognitive impairment (MCMD or HAD) is significantly affected by diagnosis before HAART, CNS penetration of anti-retroviral regimen, drug and alcohol use, increased age, and substance P expression in the cerebellum and cingulate cortex.

Study Participants

Study participants were chosen from the larger National NeuroAIDS Tissue Consortium (NNTC) cohort. The NNTC, which operates through four local brain banks in Texas, California, and New York, is an NIMH/ NINDS- funded, prospective cohort responsible for the collection, storage, and distribution of tissue and plasma samples from HIV+ individuals and uninfected controls. As of January 2011, tissue samples had been collected from 736 HIV+ individuals, of which 489 had longitudinal medical record data prior to death. The NNTC cohort is predominantly male (81%), with a mean age of 43.3 years (IQR=37-49) (NNTC.org). From this larger cohort, demographic and medical record data and tissue samples were obtained for 63 HIV+ individuals. Individuals were included if they were HIV+. Individuals were excluded if either neurocognitive diagnosis prior to death or neuropathological assessment at autopsy were incomplete.

Demographic Information and Medical Record Data

Gender, date of birth, diagnosis, and death, plasma CD4 and viral counts (with date of blood draw) were reported by the NNTC for each participant based upon medical record review.

Antiretroviral Therapy and CNS Penetration Effectiveness

Antiretroviral Therapy

Data on antiretroviral therapy was provided by the NNTC from medical chart review. CNS penetration effectiveness (CPE) scores were assigned to each drug (as shown below) based on its ability to enter the CNS (low=0, intermediate=0.5, high=1), consistent with previous studies (Letendre *et al, 2008)*.

CPE Score of 0: amprenavir , didanosine, enfuvirtide, nelfinavir, ritonavir, saquinavir, tenofovir, zalcitabine, saquinavir/ritonavir, tipranavir/ritonavir

CPE score of 0.5: efavirenz, lamivudine, stavudine, amprenavir/ritonavir, fosamprenavir/ritonavir, atazanavir, and atazanavir/ritonavir

CPE score of 1: abacavir, delavirdine, emtricitabine, indinavir, nevirapine, zidovudine, indinavir/ritonavir, and lopinavir/ritonavir.

Composite CPE scores were compiled for each reported date based on the current regimen at time of report. Average composite scores were calculated for each study participant by taking the mean of their individual composite scores. Average composite score was converted to an ordinal variable with 3 categories: average composite CPE scores were categorized as low if less than two, intermediate if between two and four, and high if greater than four.

Neurocognitive Impairment

Pre-mortem assessment of neurocognitive status was made by professional neuropathologist at each of the four NNTC study sites using uniform diagnostic criteria. Presence of opportunistic infections and co-morbidities that could account for neurocognitive observations was also performed at time of diagnosis. Each subject was given a diagnosis and a rank according to the Memorial-Sloan Kettering criterion for HIV-AIDS Dementia Complex. Based on neurologist diagnosis, participants were given a neurocognitive impairment score between one and four, as outlined in Table 3.1. Neurocognitive function was further dichotomized as the presence of symptomatic impairment (MCMD or HAD, NCI Score=3 or 4) or absence of symptomatic impairment (NCI Score=1 or 2).

Table 4. Neurocognitive Score Criteria

Neurocognitive Function	NCI Score	Neurologist Diagnosis
No impairment or Asymptomatic Impairment	1	Neurocognitive impairment not detected
	2	Sub-syndromic Neuropsychological Impairment, not meeting criteria for HAND
Symptomatic Impairment	3	Possible or Probable Minor Cognitive Motor Disorder (MCMD)
	4	Possible or Probable HIV-associated Dementia (HAD)

Diagnosis of Clinical Neuropathology

Histological assessment of brain specimens for diagnoses of HIV-associated neuropathology were made at each of the four NNTC collection sites by board-certified neuropathologists. Standardized protocols and data collection forms were utilized to increase diagnostic reliability between different pathologists. Histological findings for study participants were dichotomized as follows:

0: No parenchymal HIV-associated neuropathology

1: Parenchymal HIV-associated neuropathology, including HIV encephalopathy (HIV-E), HIV leukoencephalopathy, and microglial nodular encephalitis. Diagnostic criteria were based upon the 1991 consensus report by Budka *et al*. Diagnosis of HIV-E was based upon histological findings of astrocytosis, multiple disseminated foci of microgliosis, presence of multinucleated giant cells, and immunohistochemistry for HIV p24 antigen. Diagnosis of HIV leukoencephalopathy included histological findings of microgliosis, astrocytosis, and myelin loss, with or without a presence of multinucleated giant cells.

NeuroAIDS Severity

As a measure of severity of neuroAIDS, a combined measure of neurocognitive impairment and pathology called NeuroAIDS Group will be used. Individuals will be categorized into the following groups for comparisons:

NeuroAIDS Group 1=1 if NCI Score=1 or 2, no neuropathology

NeuroAIDS Group 2=2 if NCI Score=3 or 4, no neuropathology

NeuroAIDS Group 3=3 if neuropathology at autopsy

Alcohol Use

Assessment of alcohol dependence or abuse was made for each participant using one of two evaluative tests: the Psychiatric Research Interview for Substance and Mental Disorders (PRISM, Morgello et al, 2006) or the Composite International Diagnostic Interview (CIDI, Wittchen et al, 1991). Independent measures of alcohol abuse and dependence were reported as current, past, or never. For this analysis, alcohol dependence and alcohol abuse were combined as a measure of abnormal alcohol use; abnormal alcohol use for each participant was dichotomized as ever or never affected. Individuals were classified as ever having abnormal alcohol use if current alcohol abuse or dependence was reported at any time on PRISM/ CIDI evaluation, or if past alcohol dependence or abuse was reported on >50% of PRISM/ CIDI evaluations. If neither of these criteria were met, the individual was classified as never affected.

Drug Use

Drug use was assessed by urine toxicology and PRISM/CIDI self-report prior to death. Eight different variables of drug use were established: Self- report opiate use, urine toxicology opiate use, self- report cocaine use, urine toxicology cocaine use, self-report amphetamine use, urine toxicology amphetamine use, self- report cannabinoid use, and urine toxicology cannabinoid use. Individuals were classified as users (if current or past use was reported) or non-users (if there was no record of current or past use).

Tissue processing, RNA Extraction, cDNA preparation, and qPCR Assay

From the larger NNTC cohort, three site-specific brain specimens (cerebellum, cingulate cortex, and frontal cortex) were obtained for 63 HIV+ positive individuals. Frozen brain samples were shipped directly from the four NNTC sites to the laboratory of

Dr. Steven Douglas at the Children's Hospital of Philadelphia (CHOP). Tissue homogenization, RNA extraction, cDNA preparation, and real-time PCR assays were performed by the laboratory of Dr. Steven Douglas as previously described (Lai et al, 2006). Quantitative PCR assessment was performed using primers designed for CCR5, CXCR4, truncated and full-length NK1R, Fractalkine, TNF-alpha, IL-6, CD-4, Substance P, and GAPDH. All copy numbers for test variables were normalized to GAPDH copy numbers.

Statistical Analysis

Specific Aim 1: To determine if expression of substance P, full-length NK1R and truncated NK1R in three regions of the brain (cerebellum, cingulate cortex, and frontal cortex) are significantly different in HIV-infected individuals by neuropathology or neurocognitive impairment status.

> **Sub Aim 1a:** To determine if levels of expression of substance P, truncated NK1R, and full-length NK1R in each of three brain regions (cerebellum, cingulate cortex, or frontal cortex) are different between individuals by pathology status and neurocognitive impairment.
>
> **Analysis:** Histograms and Shapiro-Wilkes tests were employed to assess the distribution of raw and log10 transformed expression levels of Substance P, full-length NK1R and truncated NK1R in each brain region. For each region of the brain, Kruskal- Wallis tests with two-sided alphas were employed to test to for significant differences in each variable (NK1R truncated, NK1R full-length, and SP expression) by NeuroAIDS combined score. A p value of 0.02 (adjusted for 3 independent measurements) was utilized to determine significance. Where

significant differences were found ($p<0.02$) Wilcoxin rank sum tests were utilized to compare the raw expression levels of SP, truncated NK1R and full length NK1R in individuals with HIV-associated neuropathology to HIV-infected individuals without pathology.

Sub Aim 1b: To determine if patterns of expression of substance P, full-length NK1R, and truncated NK1R between the cerebellum and cingulate cortex within an individual subject differ by neurocognitive impairment status or neuropathology status.

Analysis: Repeated measures regression was utilized to assess the between-subject differences attributable to neuropathology or neurocognitive impairment of within-subject variance in expression levels of Substance P, truncated NK1R, and truncated NK1R between the cerebellum and cingulate cortex. Two measurements were entered for each variable per subject: expression in the cerebellum and expression in the cingulate cortex. Expression in the cerebellum was utilized as the reference for simple comparison. A backwards step-wise regression model was run to test for between-subject effects of neuropathology status (presence or absence), neurocognitive function (asymptomatic or symptomatic), age, Nominal CPE score on differences in within-subject expression patterns. A significance level of $p=0.05$ was used to identify significant within-subject and between-subject variations.

Specific Aim 2: To determine if expression levels of NK1R correlate with expression levels of CCR5 and CXCR4 within each of three brain regions (cerebellum, cingulate cortex, and frontal cortex) in HIV-infected individuals.

Sub Aim 2a: To determine if CCR5 expression positively correlates with full-length and truncated NK1R expression.

Analysis: Spearman's non-parametric rho was used to examine the correlation between CCR5 expression in the cerebellum, cingulate cortex, and frontal cortex and full-length and truncated NK1R expression in the respective region. Correlations were stratified by brain region and NeuroAIDS group. Using one-sided alphas, adjusted for two comparisons (NK1R full-length and truncated), correlations with p<=0.5 were flagged as significant.

Sub Aim 2b: To determine if a correlation exists between CXCR4 expression and full-length or truncated NK1R.

Analysis: Spearman's non-parametric rho was used to examine the correlation between CXCR4 expression in the cerebellum, cingulate cortex, and frontal cortex and full-length and truncated NK1R expression in the respective region. Correlations were stratified by brain region and NeuroAIDS group Using two-sided alphas, adjusted for two comparisons (NK1R full-length and truncated), correlations with p<=0.025 were flagged as significant.

Specific Aim 3: To determine if prevalence of symptomatic neurocognitive impairment (MCMD or HAD) is significantly affected by diagnosis before HAART, plasma log viral load or CD4 count, CNS penetration of anti-retroviral regimen, drug and alcohol use, increased age, and substance P expression in the cerebellum and cingulate cortex.

Analysis: A backwards, step-wise logistic regression was utilized to identify significant predictors of symptomatic neurocognitive impairment. The dependent variable was neurocognitive function, dichotomized as asymptomatic or symptomatic as previously described. Substance P expression in the cerebellum and cingulate cortex, age, plasma log viral load and CD4 counts were entered into the model as covariates; cannibinoid use, opiate use, cocaine use, diagnosis before or after 1996 (introduction of HAART), measure of abnormal alcohol use, and nominal average CPE score were entered as factors into the model.

CHAPTER 4: RESULTS

Brain samples from the cerebellum, cingulate cortex and frontal cortex of 63 HIV-infected individuals were received by the Children's Hospital of Philadelphia from the National NeuroAIDS Tissue Consortium. Of these 63 individuals, three were excluded due to insufficient assessment of neurocognitive status prior to death or of neuropathology at autopsy. Scores of neurocognitive impairment (NCI Score, from 1 to 4) were assigned to each study participant based upon reports of clinical evaluation from NNTC staff. Of the 60 subjects, seven had no neurocognitive problems (NCI score=1, 11.7%), thirteen had neuropsychological impairment that did not meet the criteria for HIV associated neurocognitive disorder (NCI score=2, 21.7%), eighteen were diagnosed as having minor cognitive motor disorder (NCI score=3, 30%), and twenty-two were diagnosed as having HIV-associated dementia (NCI Score=4, 36.7%) (See Supplemental Fig.1). NCI Scores correlated with high significance to Memorial-Sloan Kettering AIDS Dementia Complex Ratings ($R=0.787$, $p<0.0005$). Individuals with NCI scores of 1 and 2 were classified as negative for neurocognitive disorder ($n=20$), while subjects with NCI scores of 3 and 4 were classified as having neurocognitive disorder ($n=40$). At autopsy, individuals were classified by presence or absence of neuropathology; of the 60 subjects, 19 had neuropathology at autopsy (31.7%) and 41 lacked pathology (68.3%).

From neuropathology status and neurocognitive impairment score, individuals were classified into the following NeuroAIDS groups. Three individuals had neuropathology findings at autopsy, but did not have neurocognitive disorder prior to death (5%). Due to power considerations, all individuals with neuropathology were combined into NeuroAIDS Group 3, regardless of neurocognitive disorder status.

NeuroAIDS Group 1, n= 17 (28.3%): NCI Score=1 or 2, no neuropathology

NeuroAIDS Group 2, n=24 (40%): NCI Score=3 or 4, no neuropathology

NeuroAIDS Group 3, n=19 (31.7%): Neuropathology at autopsy

Table 5 describes the demographic and medical chart data by NeuroAIDS group. Using tests for analysis of variance, no significant differences existed between NeuroAIDS groups by age, sex, drug use, diagnosis before HAART, Nominal CPE score, years living with HIV, or substance abuse.

Normality Distributions

Shapiro-Wilkes tests were performed to test the normality of distribution of raw expression (in copy number/ GAPDH) and $\log_{10}$-transformed expression of full-length NK1R, truncated NK1R, and Substance P in the cerebellum and cingulate and frontal cortices; the results are shown in Table 6. In this statistical test, a p-value >0.05 indicates that the distribution of data points for a specific variable is not significantly different from a normal distribution, and thus can be considered normally distributed. We found a normal distribution ($p>0.05$) in the $\log_{10}$-transformed values for three of the nine variables- Substance P expression in the cingulate and frontal cortices, and full-length NK1R expression in the cerebellum. Substance P expression in the cerebellum, truncated NK1R expression in all three brain regions, and full-length NK1R expression in the cingulate and frontal cortex were not normally distributed before or after $\log_{10}$ transformation. For all analysis, $\log_{10}$- transformed values were utilized.

Table 5. NNTC Reported Demographic and Medical Chart Data by NeuroAIDS Group

	Group 1 N=17	Group 2 N=24	Group 3 N=19	Total N=60
Age	47.94(8.481)	42.54(8.124)	43.11(8.205)	44.25
Male	88.2%(n=15)	70.8%(n=17)	89.4%(n=17)	81.7%(n=49)
CPE Nominal Score				
1	5 (29.4%)	8 (33.3%)	5 (26.3%)	18 (30.0%)
2	10 (58.8%)	8 (33.3%)	7 (36.8%)	25 (41.7%)
3	1 (5.9%)	4 (16.7%)	7 (36.8%)	12 (20.0%)
Missing Data	1 (5.9%)	4 (16.7%)	0 (0.0%)	5 (8.3%)
Diagnosis before HAART	10 (58.8%)	17 (70.8%)	17 (89.4%)	44 (73.3%)
Alcohol Abuse/ Dependence				
Never	8 (47.1%)	6 (25.0%)	6 (31.6%)	20 (33.3%)
Ever	6 (35.3%)	8 (33.3%)	8 (42.1%)	22 (36.7%)
Missing Data	3 (17.6%)	10 (41.7%)	5 (26.3%)	18 (30.0%)
Cannabinoid Use				
Never	3 (17.6%)	7 (29.2%)	7 (36.8%)	17 (28.3%)
Ever	9 (52.9%)	3 (12.5%)	6 (31.6%)	18 (30.0%)
Missing Data	5 (29.4%)	14 (58.3%)	6 (31.6%)	25 (41.7%)
Opiate Use				
Never	7 (41.2%)	6 (25.0%)	7 (36.8%)	20 (33.3%)
Ever	5 (29.4%)	4 (16.7%)	6 (31.6%)	15 (25.0%)
Missing Data	5 (29.4%)	14 (58.3%)	6 (31.6%)	25 (41.7%)
Cocaine Use				
Never	3 (17.6%)	8 (33.3%)	11 (57.9%)	22 (36.7%)
Ever	9 (52.9%)	2 (8.3%)	2 (10.5%)	13 (21.7%)
Missing Data	5 (29.4%)	14 (58.3%)	6 (31.6%)	25 (41.7%)
Years Living w/HIV	10.35 (5.90)	9.04 (4.73)	12.26(4.24)	10.43(5.05)
Plasma CD4				
Mean	139.64	103.54	117.83	119.93
Median	(150.7)	(161.9)	(113.5)	(140.9)
	93.4	44.8	64.3	65.1
NNTC Site				
UCLA	7 (41.2%)	14 (58.3%)	10 (52.6%)	31 (51.7%)
Texas	5 (29.4%)	8 (13.3%)	3 (15.8%)	16 (26.7%)
CNTN	5 (29.4%)	2 (8.3%)	6 (31.6%)	13 (21.7%)

Table 6. Shapiro-Wilkes Results: Normality Test for Raw and Log_{10}-transformed Expression of Full-length NK1R, Truncated NK1R, and Substance P

	Shapiro-Wilk		
	Statistic	df	Sig.
Log_{10} Substance P (Cerebellum)	.944	53	.015
Log_{10} Substance P (Cingulate Cortex)	.967	53	.150**
Log_{10} Substance P (Frontal Cortex)	.977	53	.406**
Log_{10} Full-length NK1R (Cerebellum)	.982	53	.589**
Log_{10} Full-length NK1R (Cingulate Cortex)	.950	53	.028
Log_{10} Full-length NK1R (Frontal Cortex)	.901	53	.000
Log_{10} Truncated NK1R (Cerebellum)	.928	53	.003
Log_{10} Truncated NK1R (Cingulate Cortex)	.880	53	.000
Log_{10} Truncated NK1R (Frontal Cortex)	.887	53	.000
Substance P (Cerebellum)	.595	53	.000
Substance P (Cingulate Cortex)	.480	53	.000
Substance P (Frontal Cortex)	.893	53	.000
Full-length NK1R (Cerebellum)	.733	53	.000
Full-length NK1R (Cingulate Cortex)	.880	53	.000
Full-length NK1R (Frontal Cortex)	.947	53	.020
Truncated NK1R (Cerebellum)	.796	53	.000
Truncated NK1R (Cingulate Cortex)	.784	53	.000
Truncated NK1R (Frontal Cortex)	.815	53	.000

**$p>0.05$ indicates a normal distribution

Substance P, Full-length and Truncated Neurokinin-1 Receptor Expression-

Specific Aim 1: To determine if expression of substance P, full-length NK1R and truncated NK1R in three regions of the brain (cerebellum, cingulate cortex, and frontal cortex) are significantly different in HIV-infected individuals by neuropathology or neurocognitive impairment status.

Table 7 displays mean and median expression levels of full-length NK1R, truncated NK1R, and substance P by brain region for each of the three NeuroAIDS groups. Of these, only mean Substance P expression in the cingulate cortex was significantly different by NeuroAIDS group (F=4.707, df=2, p=0.013).

Table 7. Expression of Substance P, Full-length NK1R, and Truncated NK1R (in log transformed copy number/ GAPDH) by Brain Region and NeuroAIDS Group

		Group 1 N=17	**Group 2 N=24**	**Group 3 N=19**	**Kruskal-Wallis/ ANOVA**
Full Length NK1R (copy number/ GAPDH)					
Cerebellum	*Mean*	0.61 (5.15)	0.48 (3.42)	0.36 (2.61)	*p*=0.625
	Median	0.26	0.22	1.37	
Cingulate Cortex	*Mean*	1.07 (1.01)	1.15 (1.03)	1.10 (1.07)	*p*=0.785
	Median	0.86	1.03	0.90	
Frontal Cortex	*Mean*	1.10 (1.03)	1.22 (1.01)	1.18 (0.91)	*p*=0.235
	Median	1.00	1.19	1.11	
Truncated NK1R (copy number/ GAPDH)					
Cerebellum	*Mean*	2.30 (2.32)	2.24 (2.37)	2.51 (2.51)	*p*=0.183
	Median	2.20	1.49	2.23	
Cingulate Cortex	*Mean*	2.25 (2.21)	2.34 (2.43)	2.11 (2.26)	*p*=0.761
	Median	2.29	1.51	1.26	
Frontal Cortex	*Mean*	2.30 (2.29)	2.28 (2.40)	2.28 (2.40)	*p*=0.971
	Median	2.13	1.60	1.83	
Substance P (copy number/ GAPDH)					
Cerebellum	*Mean*	1.47 (1.61)	1.39 (1.52)	1.20 (1.17)	*p*=0.542
	Median	1.34	1.03	1.00	
Cingulate Cortex	*Mean*	2.05 (2.30)	1.96 (1.74)	1.70 (1.67)*	*p*=0.013*
	Median	1.79	1.88	1.47	
Frontal Cortex	*Mean*	1.79 (1.68)	1.89 (1.63)	1.82 (1.69)	*p*=0.371
	Median	1.71	1.84	1.78	

* indicates p<0.05

Sub Aim 1a: To determine if levels of expression of substance P, truncated NK1R, and full-length NK1R in each of three brain regions (cerebellum, cingulate cortex, or frontal cortex) are different between individuals by pathology status.

NKIR assessment: There were no significant differences observed in expression of the full-length NK1R isoform by NeuroAIDS group in any region of the brain (F=0.473, df=2, p=0.625 in the cerebellum, Table 8; χ^2=0.483, df=2, p=0.785 in the cingulate cortex, Table 9; and χ^2=2.894, df=2, p=0.235 in the frontal cortex, Table 9). Similarly, we observed no significant differences in truncated NK1R expression in any brain region by NeuroAIDS group (χ^2=3.394, df=2, p=0.183 in the cerebellum; χ^2=0.547, df=2, p=0.761 in the cingulate cortex; and χ^2=0.058, df=2, p=0.971 in the frontal cortex, Table 9). Because no significant differences were observed in expression of either NK1R isoform by NeuroAIDS group, further tests for significant differences between individuals with or without pathology were not performed.

Substance P Assessment: Mean $\log_{10}$-transformed expression of Substance P in the cingulate cortex varied significantly by NeuroAIDS group (F=4.707, df=2, p=0.013, Table 8) and is plotted in Figure 2. Expression of substance P ($\log_{10}$ transformed) is significantly less in NeuroAIDS Group 3 than in Group 1 (t=2.298, df=31, p=0.028) and Group 2 (t=2.805, df=38, p=0.008), but expression in Groups 1 and 2 do not differ significantly from each other (t=-0.230, df=37, p=0.820) (Table 10). On average, Substance P expression in the cingulate cortex was 20% less in individuals with neuropathology at autopsy than those without pathology (t=3.088, df=54, p=0.003), as shown in Figure 2B.

Table 8. One-way ANOVA for Differences in Log_{10}-transformed Expression of Substance P (Cingulate and Frontal Cortices) and Full-Length NK1R (Cerebellum) by NeuroAIDS Group

		Sum of Squares	Df	Mean Square	F	Sig.
Log10-Substance P (Cingulate Cortex)	Between Groups	1.374	2	.687	4.707	.013*
	Within Groups	7.736	53	.146		
	Total	9.110	55			
Log 10-Substance P (Frontal Cortex)	Between Groups	.177	2	.089	1.013	.370
	Within Groups	4.995	57	.088		
	Total	5.172	59			
Log 10- Full-length NK1R (Cerebellum)	Between Groups	.251	2	.126	.473	.625
	Within Groups	14.062	53	.265		
	Total	14.313	55			

*p<0.05 indicates a significant finding

Table 9. Kruskal-Wallis Non-Parametric Tests for Differences in Expression of Substance P, Full-Length NK1R, and Truncated NK1R by NeuroAIDS Group

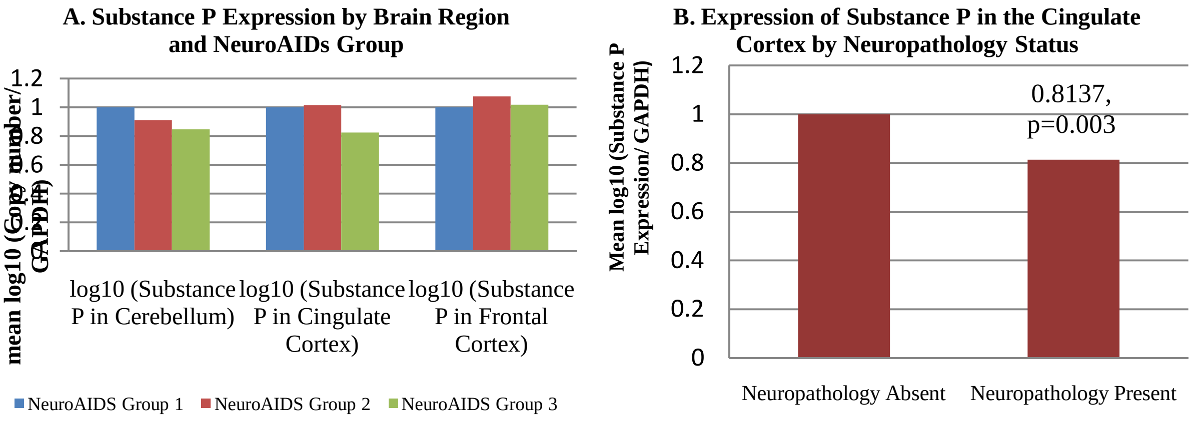
	Chi-square	df	Asymp. Sig.
Full-length NK1R (Cingulate Cortex)	.483	2	.785
Full-length NK1R (Frontal Cortex)	2.894	2	.235
Truncated NK1R (Cerebellum)	3.394	2	.183
Truncated NK1R (Cingulate Cortex)	.547	2	.761
Truncated NK1R (Frontal Cortex)	.058	2	.971
Substance P (Cerebellum)	1.224	2	.542

*p<0.05 indicates a significant finding

From Figure 2A, it appears that there is a trend toward decreasing Substance P expression in the cerebellum with increasing severity of NeuroAIDS. However, taken alone, the normalized mean values are misleading as a result of the non-parametric distribution of the data; a Kruskal-Wallis rank test shows that there is no significant difference in expression of substance P in the cerebellum by NeuroAIDS group (Chi-Square=1.224, df=2, p=0.524, Table 9). Normalized Substance P expression in the frontal cortex does follow a normal distribution, and appears similar between the three NeuroAIDS groups (Figure 2A); one-way analysis of variance concludes that substance

P expression does not differ significantly amongst the three groups in the frontal cortex (F=1.013, df=2, p=0.370, Table 8).

Figure 2. Expression of Substance P by NeuroAIDS Group and Neuropathology Status



Note: Values are reported in $\log_{10}$*-transformed expression normalized to reference categories: NeuroAIDS Group 1 (A) and individuals without pathology (B).*

Table 10. Independent t-test for Differences in Log_{10}-transformed Substance P Expression (Cingulate Cortex) by NeuroAIDS Group and Pathology Status

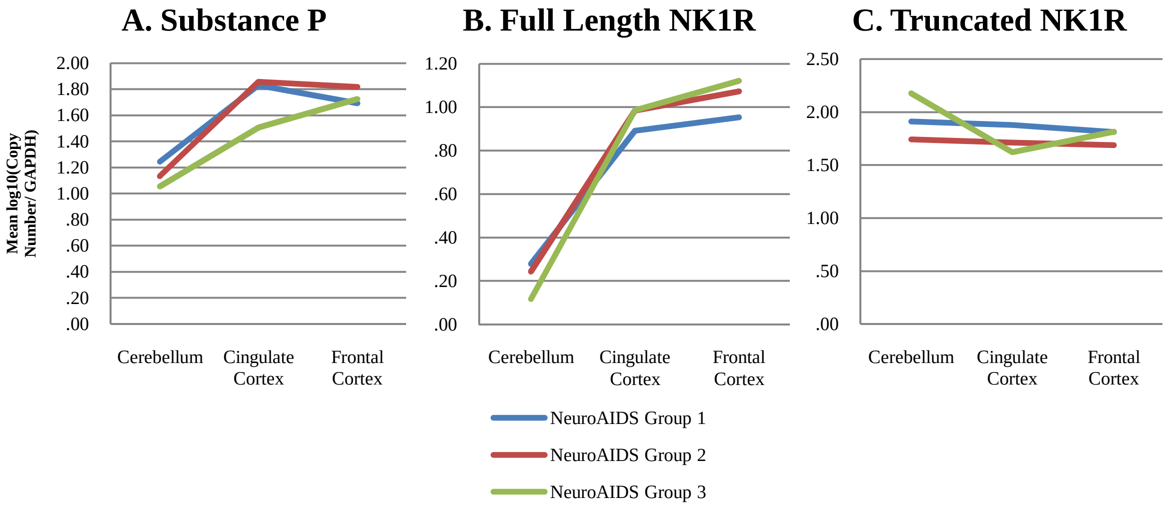
	t	df	*p*
NeuroAIDS Group 1 vs. Group 3	2.298	31	0.028*
NeuroAIDS Group 1 vs. Group 2	-0.230	37	0.820
NeuroAIDS Group 2 vs. Group 3	2.805	38	0.008**
Neuropathology vs. No Neuropathology	3.088	54	0.003**

*p<0.05 indicates significant finding, **p<0.01 indicates very significant finding

Sub Aim 1b: To determine if patterns of expression of substance P, full-length NK1R, and truncated NK1R between the cerebellum and cingulate cortex within an individual subject differ by neurocognitive impairment status or neuropathology status.

There is often significant variation in baseline gene expression between individuals, which complicates detection of differences in gene expression related to specific diseases, particularly in smaller sample sizes where power is limited. For this reason, within-subject differences may be helpful in identifying specific patterns of expression within an individual that may provide clues about disease state or mechanisms of disease progression than between subject comparisons alone. For this reason, a within-subject repeated measures analysis was used to assess differences in expression of Substance P, full-length NK1R, and truncated NK1R in the cerebellum and cingulate and frontal cortices. Figure 3, discussed below, shows average expression trends for Substance P, full-length NK1R, and truncated NK1R by brain region for the three NeuroAIDS groups. The two-way interactions between the within-subject variable of brain region and the between-subject variables of age at death, CPE Nominal Score, neurocognitive disorder, neuropathology, ever cocaine, ever opiate, and ever cannabis use were examined in relationship to significant differences in the slope of the line between cerebellar and cingulate cortex expression.

Figure 3. Expression of Substance P, Full-length NK1R, and Truncated NK1R by Brain Region and NeuroAIDS Group



Within-Subject Model of Substance P Expression: In Figure 3A, we see that Substance P expression is lowest in the cerebellum for all three NeuroAIDS groups, and does not significantly differ by NeuroAIDS group in this region (previously reported, Table 9). In NeuroAIDS groups 1 and 2, a similar 4-fold increase in substance P expression from the cerebellum to the cingulate cortex is observed; in NeuroAIDS group 3, expression of substance P is significantly less in the cingulate cortex than in groups 1 and 2 (previously reported, Table 8), and is, on average, only 3-fold greater than expression within the cerebellum (Fig. 3A).

A repeated measures regression model shows that there is a significant within-subject difference in substance P expression between the cerebellum and cingulate cortex (F=54.897, df=1, p<0.0005, Table11B). Neither neuropathology (non-significant at final model, Table 11B, F=1.490, df=1, p=0.228) nor neurocognitive disorder (removed after step 2, F= 0.331, df=1, p=0.568) contribute significantly to within-subject differences in Substance P expression between the two regions (p=0.209 and p=0.941, respectively, Table 11). Age at death (removed after initial step, F= 0.217, df=1, p=0.644), nominal CPE score (removed after step 3, F=0.957, df=2, p=0.392), and drug use (no significant findings, Table 11C) did not contribute significantly to within-subject differences in expression of substance P in the cingulate cortex and cerebellum; only region changes contributed to the within-subject differences in expression between the two regions. (Table 11).

Table 11. Within-subject Contrasts of Substance P Exp between the Cingulate Cortex and Cerebellum

A. Initial Model: Log10 Substance P

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 2 vs. Level 1	.102	1	.102	.774	.384
region * FinalNeuropathology	Level 2 vs. Level 1	.319	1	.319	2.413	.128
region * NCIYesNo	Level 2 vs. Level 1	.046	1	.046	.350	.557
region * CPENominal	Level 2 vs. Level 1	.248	2	.124	.938	.400
region * AgeatDeath	Level 2 vs. Level 1	.029	1	.029	.217	.644
Error(region)	Level 2 vs. Level 1	5.561	42	.132		

*p<0.05 indicated significant finding; Cerebellum is the reference category (Level 1)

B. Final Model: Log10 Substance P

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 2 vs. Level 1	14.028	1	14.028	54.897	.000*
region * FinalNeuropathology	Level 2 vs. Level 1	.381	1	.381	1.490	.228
Error(region)	Level 2 vs. Level 1	11.754	46	.256		

*p<0.05 indicated significant finding; Cerebellum is the reference category (Level 1)

C. Initial and Final Within-subject effects of Drug use on log 10 Substance P

Initial Model: Drug Use and Log10 Substance P

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Linear	.015	1	.015	.153	.700
region * EverOpiate	Linear	.041	1	.041	.409	.529
region * EverCannabinoid	Linear	.701	1	.701	6.962	.015
region * EverCocaine	Linear	.193	1	.193	1.920	.180
region * CPENominal	Linear	.317	2	.158	1.572	.231
region * AgeatDeath	Linear	.130	1	.130	1.292	.268
Error(region)	Linear	2.114	21	.101		

Final Model: Drug Use and Log10 Substance P

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Linear	5.750	1	5.750	48.192	.000*
region * EverCannabinoid	Linear	.154	1	.154	1.288	.266
Error(region)	Linear	3.222	27	.119		

*p<0.05 indicates significant finding, cerebellum is reference category

Within-subject Model of Full-length NK1R Expression: Expression of full-length NK1R is lowest in the cerebellum and follows a similar pattern of increased expression in the cingulate cortex for all three NeuroAIDS groups (Figure 3B). Within-subject contrasts, with significance testing, are shown in Table 12. Similar to Substance P expression, the repeated measures regression model verifies that while there is a significant within-subject difference by brain region (Table 12B, F=62.815, df=1,p<0005), neither presence of neuropathology (non-significant in final model, F=1.503, df=1, p=0.227) nor neurocognitive disorder (removed after initial model, F=0.079, df=1, p=0.781) contributes significantly to between-subject differences in within-subject patterns of full-length NK1R expression between the cerebellum and cingulate cortex (Figure 3B). Additionally, there was no significant effect of age at death (removed after step 3, F=0.625, df=1, p=0.433) or nominal CPE score (removed after step 2, F=0.833, df=2, p=0.442) on within-subject differences in expression of the full-length isoform of NK1R in the cerebellum and cingulate cortex.

Table 12. Within-subject Contrasts of Full-length NK1R Expression between the Cingulate Cortex and Cerebellum

A. Initial Model: Log 10 Full-Length NK1R

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	.086	1	.086	.207	.652
region * FinalNeuropathology	Level 1 vs. Level 2	.503	1	.503	1.202	.279
region * NCIYesNo	Level 1 vs. Level 2	.033	1	.033	.079	.781
region * CPENominal	Level 1 vs. Level 2	.715	2	.357	.854	.433
region * AgeatDeath	Level 1 vs. Level 2	.483	1	.483	1.154	.289
Error(region)	Level 1 vs. Level 2	17.567	42	.418		

*p<0.05 indicates a significant finding, Cerebellum is reference category (Level 1)

B. Final Model: Log 10 Full-Length NK1R

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	25.312	1	25.312	62.815	.000*
region * FinalNeuropathology	Level 1 vs. Level 2	.605	1	.605	1.503	.227
Error(region)	Level 1 vs. Level 2	18.536	46	.403		

*p<0.05 indicates a significant finding, Cerebellum is reference category (Level 1)

A second repeated measures regression model was built, entering ever cannabis, cocaine, and opiate use as between-subject factors; the within-subject contrasts are presented in Table 13. It was found that cocaine significantly contributed to within-subject differences in expression of full-length NK1R (F=4.795, df=1, p=0.037), but neither opiate use (F=1.392, df=1, p=0.249) nor cannabis use (F=2.479, df=1, p=0.127) significantly contributed to within-subject differences in expression of the full-length NK1R isoform between the cerebellum and cingulate cortex. Cocaine use did not contribute significantly to between subject differences in full-length NK1R in the cerebellum or cingulate cortex when taken alone, but rather altered the pattern of expression within users, distinct from non-users (Figure 4).

Table 13. Within-subject Contrasts of Full-length NK1R Expression between the Cingulate Cortex and Cerebellum with Drug Use

A. Initial Model: Log10 Full Length NK1R by Drug use

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	3.765	1	3.765	20.124	.000
region * EverOpiate	Level 1 vs. Level 2	.260	1	.260	1.392	.249
region * EverCannabinoid	Level 1 vs. Level 2	.464	1	.464	2.479	.127
region * EverCocaine	Level 1 vs. Level 2	.638	1	.638	3.408	.076
region * FinalNeuropathology	Level 1 vs. Level 2	.469	1	.469	2.508	.125
Error(region)	Linear	4.864	26	.187		

*indicates p<0.05, Cerebellum is reference category (Level 1)

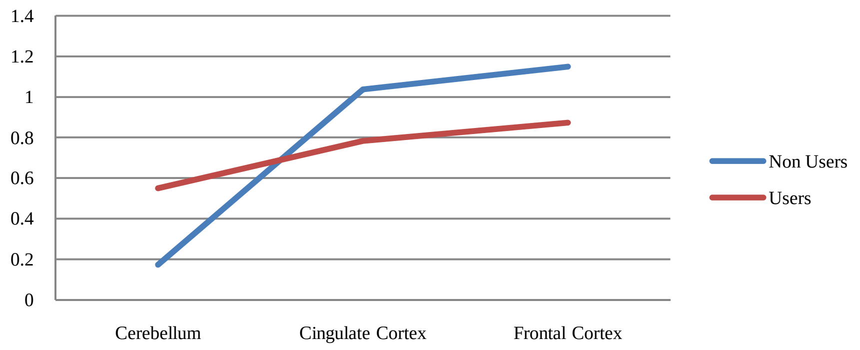
B. Final Model : Log10 Full-length NK1R by Drug Use

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	3.102	1	3.102	15.100	.001*
region * EverCocaine	Level 1 vs. Level 2	.985	1	.985	4.795	.037*
Error(region)	Level 1 vs. Level 2	5.958	29	.205		

*indicates p<0.05, Cerebellum is reference category (Level 1)

Full-length NK1R expression by brain region, and stratified by cocaine use, was plotted and shown in Figure 4. Cocaine users show a 2-fold increase in full-length NK1R expression from the cerebellum to the cingulate cortex, while expression of full-length NK1R is 10-fold greater in the cingulate cortex compared to the cerebellum in non-users.

Figure 4. Full-length NK1R Expression by Brain Region by Cocaine Use



Within-subject Model of Truncated NK1R Expression: In individuals without neuropathology (NeuroAIDS Groups 1 and 2), average expression levels of truncated NK1R are almost identical in the cerebellum and cingulate cortex (Fig. 3C); individuals with neuropathology (NeuroAIDS Group 3), however, have a distinct pattern of expression, with a dramatic 50% decrease in expression levels from the cerebellum to the cingulate cortex.

The within-subject contrasts for the repeated measure model of truncated NK1R expression are shown in Table 14. There is a significant within-subject difference between expression of truncated NK1R in the cingulate cortex and cerebellum (Table 14B, F= 6.335, df=1, p=0.015) A significant interaction between brain region and neuropathology was found in expression of truncated NK1R (Table 14B, F=5.305, df=1, p=0.026). Indeed, the ratio of log10-transformed truncated NK1R in the cingulate cortex compared to the cerebellum is approximately 20% lower in individuals with neuropathology than controls without pathology, and is significant when using a one-sided t-test (Figure 5, t=1.696, df=51, p=0.096). Neurocognitive impairment (removed after step 3, F=0.137, df=1, p=0.721), age at death (removed after step 2, F=0.051, df=1, p=0.822), and nominal CPE score (Table 14A, removed after step 1, F=0.158, df=1, p=0.854) did not contribute significantly to the within-subject variability in truncated NK1R expression.

Table 14. Within-subject Contrasts of Truncated NK1R Expression between the Cingulate Cortex and Cerebellum

A. Initial Model: log10- transformed NK1R Truncated

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	.025	1	.025	.042	.838
region * FinalNeuropathology	Level 1 vs. Level 2	3.022	1	3.022	5.152	.028*
region * NCIYesNo	Level 1 vs. Level 2	.091	1	.091	.155	.696
region * CPENominal	Level 1 vs. Level 2	.185	2	.093	.158	.854
region * AgeatDeath	Level 1 vs. Level 2	.044	1	.044	.076	.785
Error(region)	Level 1 vs. Level 2	24.638	42	.587		

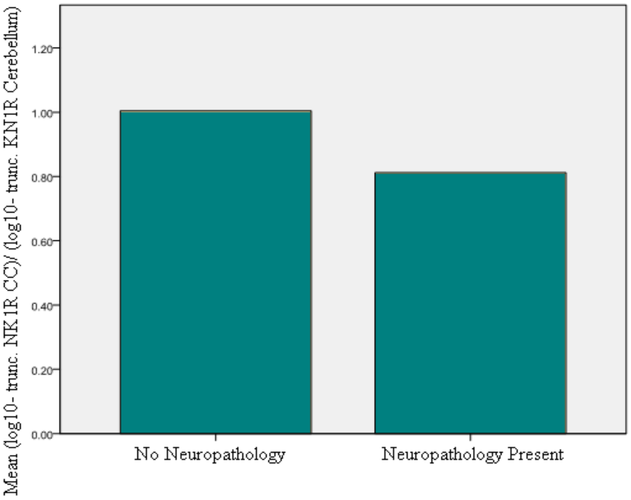
*p<0.05 indicates significant finding. Cerebellum is reference category (Level 1)

B. Final Model: Log10 transformed NK1R Truncated

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	3.433	1	3.433	6.335	.015*
region * FinalNeuropathology	Level 1 vs. Level 2	2.875	1	2.875	5.305	.026*
Error(region)	Level 1 vs. Level 2	24.928	46	.542		

*p<0.05 indicates significant finding. Cerebellum is reference category (Level 1)

Figure 5: Ratio of Truncated NK1R Expression in the Cingulate Cortex to the Cerebellum



*Mean Values normalized to mean of reference group without neuropathology

We ran a second repeated measures regression for truncated NK1R expression including neuropathology, cocaine use, opiate use, and cannabis use as between-subject factors; the within-subject contrasts are shown in Table 15. We found that an interaction between region and cannabis use significantly contributed to between subject differences (in users and non-users) of within-subject variation in expression levels of truncated NK1R between the cerebellum and cingulate cortex (F=5.195, df=1, p=0.030). Opiate use and cocaine use did not contribute significantly to differences in patterns of truncated NK1R expression between the cerebellum and cingulate cortex.

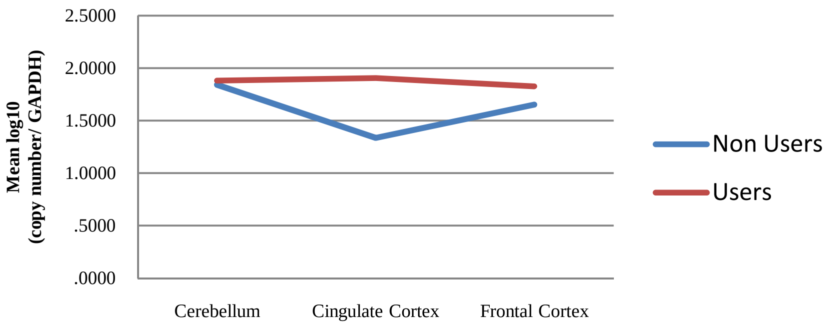
Table 15. Within-subject Contrasts of Truncated NK1R Expression between the Cingulate Cortex and Cerebellum by Drug Use

Log10 transformed Truncated NK1R

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	2.136	1	2.136	13.097	.001*
region * EverCannabinoid	Level 1 vs. Level 2	.847	1	.847	5.195	.030*
region * FinalNeuropathology	Level 1 vs. Level 2	1.768	1	1.768	10.838	.003*
Error(region)	Level 1 vs. Level 2	4.567	28	.163		

*P<0.05 indicates a significant finding; reference category is cerebellum (level 1)

Figure 6: Truncated NK1R Expression by Cannabis Use



Specific Aim 2: To determine if expression levels of Substance P and NK1R correlate with expression levels of CCR5 and CXCR4 within each of three brain regions (cerebellum, cingulate cortex, and frontal cortex) among HIV-infected individuals.

Sub Aim 2a: To determine if CCR5 expression positively correlates with substance P, full-length and truncated NK1R expression.

Because of the non-normal distribution of the data, Spearman's rho was reported for correlations between CCR5 expression and Substance P, full-length NK1R, and truncated NK1R expression in this study population; correlation coefficients were stratified by brain region and NeuroAIDS group, and findings presented in Table 16.

There is a significant ($p<0.01$) positive, almost 1:1 correlation between expression of CCR5 and truncated NK1R in all three regions of the brain examined. Expression of CCR5 strongly and positively correlates with Substance P expression in the cerebellum of individuals in NeuroAIDS groups 1 and 3 (rho=0.729, p=0.002 and rho=0.769, $p<0.0005$, respectively); a non-significant trend towards a negative correlation between CCR5 and Substance P is observed in the frontal and cingulate cortices for all NeuroAIDS groupings. Full-length NK1R expression negatively correlates with CCR5 expression in the frontal and cingulate cortices of individuals with neurocognitive impairment but lacking pathology (NeuroAIDS Group 2, rho=-0.619, p=0.001 and rho=-0.640, p=0.001, respectively); there was not a significant correlation between CCR5 expression and full-length NK1R expression in any brain region for NeuroAIDS groups 1 and 3. Of note, there was a trend towards positive correlations between CCR5 and substance P and full-length NK1R in the cerebellum, and a trend towards negative correlations between CCR5 expression and Substance P and full-length NK1R in the cingulate and frontal cortices.

Table 16. Correlation between CCR5 Expression and Substance P, Full-length NK1R, and Truncated NK1R by Brain Region and NeuroAIDS Group

Brain Region		Substance P	Full Length NK1R	Truncated NK1R
Cerebellum	Group 1	0.729** (P=0.002)	0.139 (p=0.621)	0.893** (p<0.0005)
	Group 2	0.414 (p=0.050)	0.216 (p=0.321)	0.913** (p<0.0005)
	Group 3	0.769** (p<0.0005)	0.179 (p=0.478)	0.938** (p<0.0005)
Cingulate Cortex	Group 1	-0.409 (p=0.116)	-0.135 (p=0.617)	0.968** (p<0.0005)
	Group 2	-0.146 (p=0.506)	-0.640** (p=0.001)	0.930** (p<0.0005)
	Group 3	-0.096 (p=0.715)	-0.426 (0.088)	0.956** (p<0.0005)
Frontal Cortex	Group 1	-0.473 (p=0.055)	-0.409 (p=103)	0.833** (p<0.0005)
	Group 2	-0.293 (p=0.165)	-0.619** (p=0.001)	0.785** (p<0.0005)
	Group 3	-0.133 (p=0.586)	-0.421 (p=0.073)	0.740** (p<0.0005)

*indicates p<0.05, **indicates p<0.01

Sub Aim 2b: To determine if a correlation exists between CXCR4 expression and full-length or truncated NK1R.

Similar to CCR5 expression, a strong, positive, highly significant correlation between CXCR4 expression and expression of the truncated isoform of NK1R exists in all three brain regions (Table 17). Of note, this correlation is stronger in individuals without neuropathology (NeuroAIDS Groups 1 and 2) than those with neuropathology (NeuroAIDS Group 3) in all three brain regions (in the cingulate cortex, rho=0.676, p<0.0005 in Group 3, while rho=0.835 and 0.884, p<0.0005 in Groups 1 and 2, respectively, Table 17).

As with CCR5, there is a trend toward a positive correlation between CXCR4 and Substance P and full-length NK1R expression in the cerebellum. In the cingulate and frontal cortices, however, there is a trend towards a negative correlation between CXCR4 expression and Substance P and full-length NK1R expression.

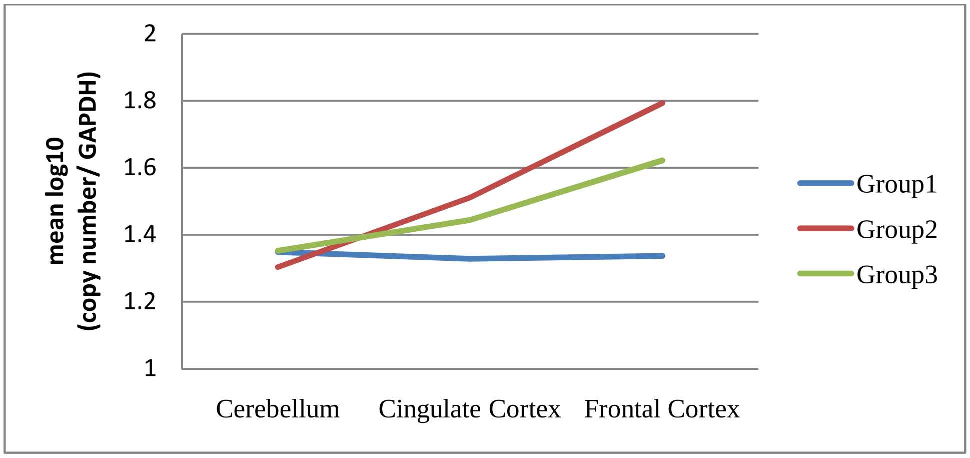
Table 17: Correlation between CXCR4 Expression and Substance P, Full-length NK1R, and Truncated NK1R by Brain Region and NeuroAIDS Group

Brain Region		Substance P	Full Length NK1R	Truncated NK1R
Cerebellum	Group 1	0.793** (p<0.0005)	0.429 (p=0.111)	0.839** (P<0.0005)
	Group 2	0.411 (p=0.051)	0.214 (p=0.326)	0.891** (p<0.0005)
	Group 3	0.554* (p=0.017)	0.366 (p=0.135)	0.738** (p<0.0005)
Cingulate Cortex	Group 1	-0.582* (p=0.018)	-0.415 (p=0.110)	0.835** (p<0.0005)
	Group 2	-0.205 (p=0.349)	-0.615** (p=0.002)	0.884** (p<0.0005)
	Group 3	-0.439 (p=0.078)	-0.150 (p=0.567)	0.676** (p=0.003)
Frontal Cortex	Group 1	-0.473 (p=0.055)	-0.409 (p=103)	0.833** (p<0.0005)
	Group 2	-0.293 (p=0.165)	-0.619** (p=0.001)	0.785** (p<0.0005)
	Group 3	-0.133 (p=0.586)	-0.421 (p=0.0.073)	0.740** (p<0.0005)

*indicates p<0.05, **indicates p<0.01

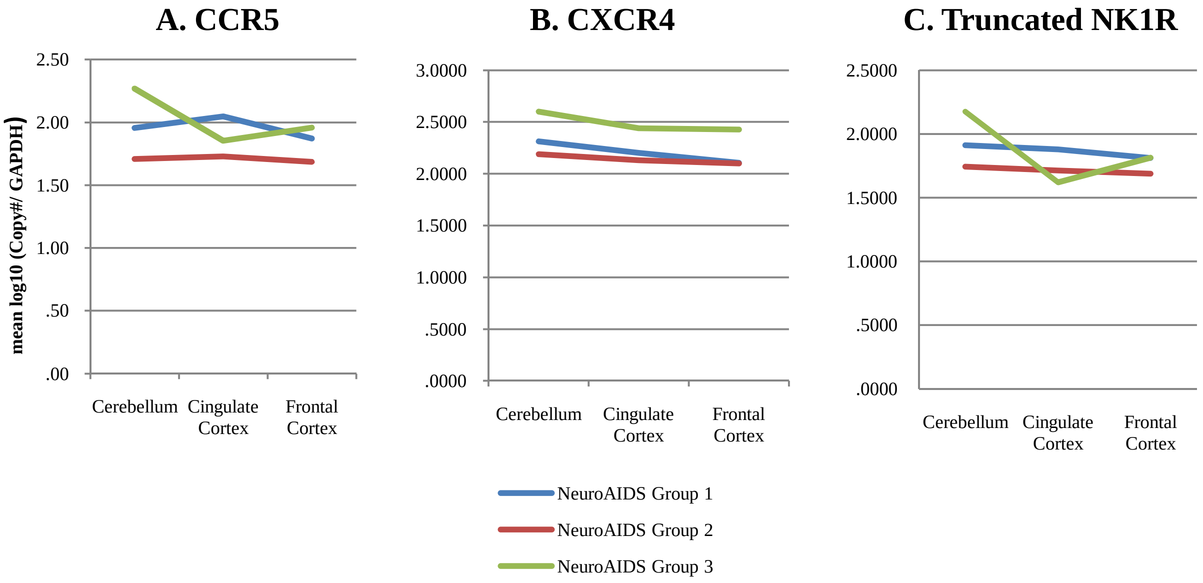
To gain a better understanding of the relationship between truncated NK1R and CXCR4 expression in each of the three NeuroAIDS groups, the ratio of $\log_{10}$-transformed CXCR4 to truncated NK1R by brain region was stratified by NeuroAIDS group and plotted in Figure 7. In NeuroAIDS Group 1, expression of CXCR4 and truncated NK1R remain at a constant ratio in all regions of the brain. Interestingly, in individuals with neurocognitive impairment, the ratio of CXCR4 to truncated NK1R is increased from the cerebellum to the cingulate and frontal cortices, indicating that expression of CXCR4 in relationship to NK1R expression may be elevated in these individuals.

Figure 7: Ratio of CXCR4 Expression to Truncated NK1R Expression by Brain Region and NeuroAIDS Group



Because of the strong correlations between truncated NK1R and CCR5 and CXCR4, plots of the mean log10 transformed (copy number/ GAPDH) were prepared by brain region (Figure 8). As suggested by the correlation values in Table 17, CCR5 expression follows an almost identical pattern of expression in the three brain regions as truncated NK1R in those with pathology (Group 3) and those without (Groups 1 and 2). Expression patterns of CXCR4 did not mirror as closely the patterns of expression of truncated NK1R in those with pathology.

Figure 8: Expression of CCR5, CXCR4, and Truncated NK1R by Brain Region and NeuroAIDS Group



A. CCR5

B. CXCR4

C. Truncated NK1R

Within Subject CCR5 Expression: As CCR5 expression correlated strongly with truncated NK1R expression in all regions of the brain and almost identically mirror it in Figure 8, a within-subject repeated measures analysis of CCR5 expression between the cingulate cortex and cerebellum was performed to determine if neuropathology status or drug use contributed significantly to patterns of CCR5 expression between these two regions. Within-subject contrasts for the regression model are reported in Table 18. Similar to truncated NK1R expression, we found a significant difference in within-subject expression of CCR5 between the cerebellum and cingulate cortex by neuropathology status (Figure 4.5A, F=9.108, df=1, p=0.005). When drug use was incorporated into the analysis, it was found that cannabis use (F=6.509, df=1, p=0.017) and opiate use (F=4.633, df=1, p=0.040) both contributed significantly to differences in within-subject CCR5 expression between the cerebellum and cingulate cortex, but cocaine did not (F=0.593, df=1, p=0.448).

Table 18. Within-subject Contrasts of CCR5 Expression between the Cingulate Cortex and Cerebellum

A. Initial Model: log10 CCR5

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	1.180	1	1.180	6.647	.016*
region * EverCannabinoid	Level 1 vs. Level 2	1.106	1	1.106	6.228	.019*
region * FinalNeuropathology	Level 1 vs. Level 2	1.638	1	1.638	9.229	.005*
region * EverOpiate	Level 1 vs. Level 2	.913	1	.913	5.143	.032*
region * EverCocaine	Level 1 vs. Level 2	.105	1	.105	.593	.448
Error(region)	Level 1 vs. Level 2	4.615	26	.178		

*P<0.05 indicates significant finding; Cerebellum is reference category (Level 1)

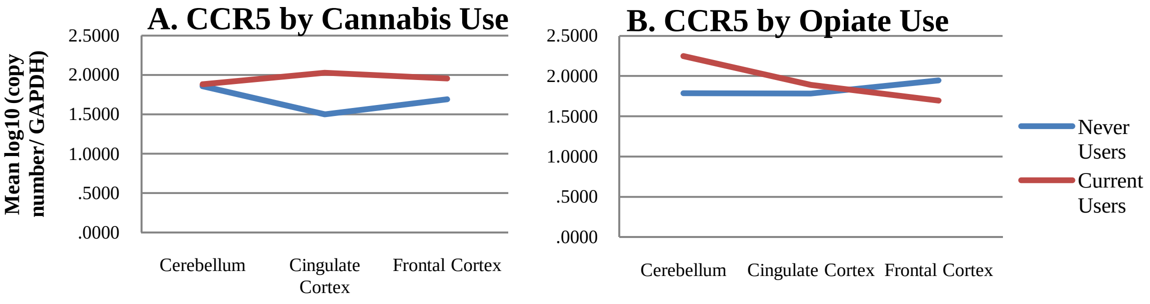
B. Final Model: log10 CCR5

Source	region	Type III Sum of Squares	df	Mean Square	F	Sig.
region	Level 1 vs. Level 2	1.219	1	1.219	6.970	.014*
region * EverCannabinoid	Level 1 vs. Level 2	1.138	1	1.138	6.509	.017*
region * FinalNeuropathology	Level 1 vs. Level 2	1.592	1	1.592	9.108	.005*
region * EverOpiate	Level 1 vs. Level 2	.810	1	.810	4.633	.040*
Error(region)	Level 1 vs. Level 2	4.721	27	.175		

*P<0.05 indicates significant finding; Cerebellum is reference category (Level 1)

Mean $\log_{10}$ transformed expression of CCR5 by brain region and drug use was plotted, and shown in Figure 9. Interestingly, opiate use results in a decrease in expression of CCR5 from the cerebellum to the cingulate cortex, while cannabis use results in an increase in expression from the cerebellum to the cingulate cortex.

Figure 9: CCR5 Expression by Brain Region and Illicit Drug Use



Predictors of Neurocognitive Impairment

Specific Aim 3: To determine if prevalence of symptomatic neurocognitive impairment (MCMD or HAD) is significantly affected by diagnosis before HAART, plasma CD4 count, CNS penetration of anti-retroviral regimen, drug and alcohol use, increased age, and substance P expression in the cerebellum and cingulate cortex.

Using backwards stepwise elimination logistic regression, with symptomatic neurocognitive impairment as the outcome, no significant main effects were found for any of the independent variables entered. Coefficients and significance values are presented for the initial model in Table 19; the log-likelihood ratio for this initial model was non-significant ($\chi 2=12.891$, df=11, p=0.300), indicating that inclusion of the between subject factors and covariates does not improve modeling of neurocognitive disorder better than an intercept-only model. Diagnosis before or after HAART, average plasma CD4, CNS penetration effectiveness of HAART, drug (opiate, cannabinoid, and cocaine) and alcohol use, age, and Substance P expression in the cingulate cortex and cerebellum do not significantly contribute to neurocognitive impairment, and do not differ significantly by neurocognitive disorder status. Using Backwards step-wise regression, the order of removal was: nominal CPE score, Substance P expression in cerebellum (log10 copy #/ GAPDH), ever cocaine use, average plasma CD4, age at death, diagnosis before HAART, ever opiate use, substance P expression in the cingulate cortex (log10 copy #/ GAPDH), ever cannabis use, and ever abnormal alcohol use.

Table 19: Binary Logistic Regression for outcome of Neurocognitive Disorder

Parameter			95% Wald Confidence Interval		Hypothesis Test		
	B	Std. Error	Lower	Upper	Wald Chi-Square	df	Sig.
(Intercept)	-12.149	8.6844	-29.170	4.873	1.957	1	.162
[EverAlcohol=1.00]	5.564	2.9150	-.150	11.277	3.643	1	.056
[EverAlcohol=2.00]	0[a]	.	.	.	.	.	.
[CPENominal=1.00]	-.050	2.4797	-4.910	4.810	.000	1	.984
[CPENominal=2.00]	2.657	2.2173	-1.689	7.003	1.436	1	.231
[CPENominal=3.00]	0[a]	.	.	.	.	.	.
[EverOpiate=1.00]	-5.322	2.8717	-10.951	.306	3.435	1	.064
[EverOpiate=2.00]	0[a]	.	.	.	.	.	.
[EverCannabinoid=1.00]	-.753	1.7822	-4.246	2.740	.178	1	.673
[EverCannabinoid=2.00]	0[a]	.	.	.	.	.	.
[EverCocaine=1.00]	-.255	2.1401	-4.449	3.940	.014	1	.905
[EverCocaine=2.00]	0[a]	.	.	.	.	.	.
AverageCD4	.002	.0055	-.008	.013	.204	1	.651
logSPCB	1.741	2.6385	-3.430	6.912	.435	1	.509
logSPCC	2.151	2.8249	-3.386	7.687	.580	1	.446
diagnosisbeforeHAARTLinear	.263	.2204	-.169	.695	1.420	1	.233
AgeatDeath	.143	.1248	-.102	.388	1.312	1	.252
(Scale)	1[b]						

Dependent Variable: NCIYesNo

Model: (Intercept), EverAlcohol, CPENominal, EverOpiate, EverCannabinoid, EverCocaine, AverageCD4, logSPCB, logSPCC, diagnosisbeforeHAARTLinear, AgeatDeath

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

CHAPTER 5: DISCUSSION

Primary Aim:

Decreased Expression of Substance P in subjects with Neuropathology

In HIV-positive individuals with neuropathology at autopsy, there was a 30% reduction in expression of Substance P within the cingulate cortex, but no significant changes in Substance P expression in the cerebellum and frontal cortex when compared to infected individuals lacking pathology. No significant *between-subject* differences were detected in full-length or truncated NK1R expression in any region of the brain by pathology status. No significant differences in expression of Substance P or either isoform of NK1R was observed between individuals with or without neurocognitive disorder.

Substance P is a neurokinin signaling molecule that acts on endocrine and immune cells through its cognate receptor NK1R, and is known to induce pro-inflammatory responses. Douglas et al. (2001, 2008) showed increased expression of Substance P in the plasma of HIV-infected individuals compared to uninfected individuals. Substance P expression was significantly increased within SIV-E lesions in rhesus macaques (Vinet- Oliphant, 2010), and near beta-amyloid plaques in Alzheimer's patients. Given this, the finding that Substance P expression was significantly decreased within the cingulate cortex of individuals with HIV neuropathology was surprising.

In this study, we had the advantage that all participants were HIV-infected. Any differences in substance P expression could thus be examined within the frame of neurocognitive impairment or pathology. The decrease in SP expression in the cingulate cortex of those with neuropathology may result from down-regulation of expression (decrease in copies per cell) or from a reduction in the total number of cells producing substance P. Since substance P expression is increased in HIV-infected individuals, it is possible that prolonged exposure to increased levels of substance P leads to down-regulation of its expression in more advanced stages of disease. Alternatively, decreased Substance P could result from loss of SP-producing cells, possibly from apoptosis or chemotaxis to sites of infection. It is expected that increased expression of Substance P would be localized to HIV-E lesions; unfortunately, the scientists performing RT-PCR were not made aware as to whether the tissue specimens they received contained lesions. Thus, it is possible that increased expression was not observed in the tissue samples from those with neuropathology because there were no lesions within the tissue samples received; further chemotaxis of SP-producing cells to lesion sites could result in a pattern of decreased expression of substance P in tissue where lesions were not present. Future research will be required to understand the mechanism underlying the decrease in expression of substance P in those with neuropathology.

In this study, we did not find a change in expression of the Full-length NK1R isoform associated with neuropathology nor neurocognitive impairment. A study by Douglas et al. (2008) found expression of full-length NK1R, substance P’s cognate receptor, was reduced in the cingulate cortex of individuals with HIV compared to uninfected controls. Caberlotto et al. (2003) reported a 10-fold increase in expression of the full-length isoform in the cingulate cortex compared to the cerebellum in four uninfected subjects (584±169 copies/ ng mRNA in the cingulate cortex vs. 53±41 copies/ ng mRNA in the cerebellum). In this study, mean expression of the full-length NK1R isoform was 7-fold greater in the cingulate cortex than in the cerebellum, suggesting that expression within the cingulate cortex in this HIV-infected population may be lower than in uninfected individuals (different normalization techniques make comparison of absolute values inapplicable). Nevertheless, no significant changes in total copy number or patterns of full-length NK1R expression between brain regions were observed between the HIV-infected individuals with or without neurocognitive impairment or pathology in this study.

Within-subject Differences by Neuropathology and Neurocognitive Impairment

Due to the small sample size in this study (n=60), and to the variability observed in expression levels between individuals, a repeated measures regression was performed to test for significant changes in within-subject patterns of full-length NK1R, truncated NK1R and substance P expression. Using this model, we demonstrated that, proportional to cerebellar expression, there is a significant decrease of within- subject expression of truncated NK1R in the cingulate cortex associated with neuropathology.

Caberlotto et al. (2003) demonstrated that expression levels of truncated NK1R in the cerebellum and cingulate cortex of healthy human subjects were similar, if not slightly elevated in the cingulate cortex (153±57 and 186±42 copies/ ng mRNA, respectively). Consistent with this finding, we observed in Figure 3C that mean expression levels of truncated NK1R (in $\log_{10}$ transformed copy number/ GAPDH) were similar between the cerebellum and cingulate cortex of individuals lacking neuropathology. Individuals with pathology, on the other hand, had a significant, 50% decrease in expression of truncated NK1R from the cerebellum to the cingulate cortex.

This study lacked power to find a significant between-subject difference in expression of truncated NK1R in the cerebellum or cingulate cortex between individuals with and without pathology, but the within-subject patterns of expression differed significantly by pathology status. This finding could indicate that expression of truncated NK1R is truly elevated in the cerebellum, or truly decreased in the cingulate cortex, of HIV- infected individuals with neuropathology, but would require further testing with a greater number of subjects. A previous study by Douglas et al (2008) found that truncated NK1R was expressed at equal levels in the cerebellum of those with and without HIV- infection, suggesting that the cerebellum might be useful as a region for normalization. The findings from this study suggest that more experimentation with larger sample sizes will be needed to determine the validity of using cerebellar expression as a baseline for expression in other regions.

While expression of truncated NK1R was decreased in the cingulate cortex as compared to the cerebellum in individuals with pathology, substance P and full-length NK1R expression were higher in the cingulate cortex than the cerebellum, consistent with the pattern observed in NeuroAIDS Groups 1 and 2. This suggests that the decrease in expression of the truncated isoform in the cingulate cortex of individuals with pathology is independent of the expression pattern of substance P and full-length NK1R. Future research will be needed to understand the mechanism underlying the change in expression patterns for truncated NK1R by region, and to appreciate how it impacts disease progression and treatment opportunities.

Secondary Aim:

CCR5 Expression

Expression of CCR5 had a significant, almost 1:1 correlation with expression of the truncated NK1R isoform in all regions of the brain and in all NeuroAIDS groups. In the cerebellum, CCR5 expression correlated strongly, and positively, with substance P expression, but not with full-length NK1R expression. In the cingulate and frontal cortices, there was a trend towards a negative correlation between expression of CCR5 and both Substance P and full-length NK1R expression.

The finding of significant correlations between CCR5 expression and the truncated, but not full-length, isoform of NK1R lends its way to two postulations regarding the role of NK1R in CCR5 expression: either that 1) substance P signaling through truncated NK1R may be responsible for CCR5 expression, or 2) that truncated NK1R and CCR5 may be co-expressed.

In vitro studies have shown that Substance P signaling through NK1R increases HIV-infectivity through upregulation of CCR5 expression. The trend towards a negative correlation between Substance P and CCR5 expression in the cingulate and frontal cortices suggests that substance P- mediated upregulation of CCR5 expression may not be occurring within these regions, or might be occurring over a period of time that makes it difficult to detect in this cross-sectional model.

Previous studies have yet to identify which isoform of NK1R is responsible for the upregulation of CCR5 expression. The strong positive correlation between truncated NK1R and CCR5 expression found in this study suggests that signaling through truncated NK1R may be responsible for regulating expression of CCR5. However, in the cingulate and frontal cortices, a similar pattern of negative correlation between CCR5 expression and full-length NK1R expression and Substance P expression is observed; this suggests that if SP is altering expression of CCR5, it is likely doing so in a full-length NK1R-dependent manner in these regions.

Given the strong positive correlation found between CCR5 and truncated NK1R in this study, it is extremely likely that the two are being co-expressed. It has been demonstrated that the truncated isoform plays a role in CCR5-mediated calcium release and intracellular signaling cascades, and thus it is likely that the signaling pathways that result in upregulation of CCR5 also result in upregulation of truncated NK1R.

It is known that substance P binds with 10-fold greater affinity to the full-length isoform, suggesting that most substance P activity is likely acting through the full-length isoform when it is present. In this study, expression of NK1R in the truncated isoform is 80-times higher than expression of the full-length isoform in the cerebellum, indicating that Substance P is predominantly acting through the truncated receptor in this region, likely facilitating the action of CCR5. In the cingulate and frontal cortices, it likely has a different role, operating instead through the full-length isoform.

CXCR4 Expression

Expression of CXCR4 also strongly correlated with expression of truncated NK1R in all three regions of the brain examined. Interestingly, this correlation was weakest in individuals with neuropathology. Analysis of expression patterns of CXCR4 (Figure 8B) shows that, within NeuroAIDS group, expression levels were fairly constant between the three brain regions examined; expression was increased in individuals with neuropathology within all three brain regions.

CXCR4 is expressed on both astrocytes and neurons, but not microglia (the macrophage equivalent of the CNS). Exposure to pro-inflammatory cytokines TNF-alpha and IL-1beta up-regulates expression of CXCR4 in astrocytes (Wang and Gabuzda, 2002). In this study, the increase in CXCR4 expression associated with neuropathology at autopsy may be the result of increased expression of CXCR4 in resident astrocytes, or the result of chemotactic migration of astrocytes or other R4+ cells to these regions. Because the CXCR4 expression was consistently increased in Group 3 across all three brain regions examined, it is suspected that upregulation of CXCR4 on resident astrocytes, rather than chemotaxis, is responsible for the observed pattern of expression. Still, it is thought that T-cell infiltration across the brain barrier in those with severe NeuroAIDS may contribute to increased expression of CXCR4.

CXCR4 is a co-receptor utilized by HIV for viral entry; increased expression of CXCR4 facilitates infectivity of R4- tropic viruses into astrocytes and neurons, ultimately leading to apoptosis and death. Because CXCR4 expression is increased in individuals with neuropathology, this may point to a greater presence of X4 or R5X4 viral tropisms in the CNS of individuals with pathology than those without. Figure7 presents an interesting finding, by which CXCR4 expression was trends upward in comparison to truncated NK1R expression not only in individuals with pathology, but also in individuals with neurocognitive disorder lacking pathology. These findings indicate that R4 viruses and CXCR4 expression may be more neurotoxic than R5 viruses and CCR5 expression. Viral tropic switches from R5 to X4 in the CNS may be important to development of neurocognitive impairment, and may present a risk factor for increased severity of NeuroAIDS.

Tertiary Aim:

The final aim of this study sought to identify biological and lifestyle predictors for HIV-associated Neurocognitive Disorder (MCMD or HAD) in a sample of HIV-infected individuals. Using a binary logistic model with an outcome of neurocognitive disease, it was found that neither age at death, diagnosis prior to HAART (1996), CNS penetration effectiveness of antiretrovirals, illicit drug use (cocaine, cannabis, and opiate), or abnormal alcohol use contributed significantly to the outcome of neurocognitive disease. It is possible that with greater sample sizes, between subject measures could prove significant.

Illicit Drug Use and Expression of CCR5, truncated NK1R, and full-length NK1R

Within-subject models were employed to explore the contribution of drug use on patterns of Substance P, full-length NK1R, truncated NK1R, and CCR5 expression in the cerebellum and cingulate cortex.

Cannabis use contributed significantly to within-subject differences in expression of both CCR5 and truncated NK1R between the cerebellum and cingulate cortex. Expression levels were virtually identical in the cerebellum between users and non-users, but decreased significantly in the cingulate cortex in individuals who had no record of cannabis use (Figures 6 and 9A); in cannabis users, truncated NK1R expression in the cingulate cortex was similar to levels in the cerebellum, while CCR5 expression was slightly increased in the cingulate cortex as compared to the cerebellum.

Rogers and Peterson (2003) report that mu-opiates (heroine and morphine) upregulate expression of CCR5, and thus increase infectivity of R5 strains. In this study, average CCR5 expression was greatest in the cerebellum of opiate users; CCR5 expression in the cingulate cortex of opiate users was almost identical to expression in non-users, but in the frontal cortex, expression was actually less in opiate users. In a within-subject model, opiate use resulted in a pattern of decreased CCR5 expression from the cerebellum to the cingulate cortex, while it remained unchanged in non-users (Figure 9B). While no significant conclusion can be drawn about opiate use and CCR5 expression in the individual brain regions from this study, the difference in patterns of CCR5 expression is significant.

Interesting amongst these findings is that stratification by neuropathology status or opiate use showed similar trends in expression of CCR5 between the cerebellum and cingulate cortex. In opiate users and individuals with neuropathology, expression was greater in the cerebellum than in the cingulate cortex. In non-users and individuals without pathology, expression was virtually identical between the two regions. On the other hand, stratification by cannabis use showed an inverse pattern to this, where users had greater expression of CCR5 in the cingulate cortex as compared to non-users. In this case, it was non-users who showed a significant drop in expression of CCR5 from the cerebellum to the cingulate cortex. A similar observation is made when stratifying truncated NK1R expression by cannabis use and neuropathology status. These opposing patterns by opiate use/ neuropathology state and cannabis use are consistent with previous findings that opiate use contributes to neurodegeneration and accelerates progression of NeuroAIDS, while cannabis use offers neuroprotective properties.

Limitations:

One of the most obvious limitations of this study is the cross sectional study design, which makes it impossible to make causal inferences from any associations found in the study.   Since brain tissues can only be extracted from corpses, expression levels in the brain tissue of a living subject cannot be measured over a period of time with current technology. Moreover, the BBB establishes two distinct compartments for infection, allowing it to persist in the brain even while it is controlled peripherally. Hence, it won't do for our purposes to measure biomarker expression in the blood or plasma of living subjects, which often does not correlate well with biomarker expression in the CNS. While cerebrospinal fluid might provide a good medium for repeated measurements, extraction (spinal tap) is highly invasive, and puts individuals (who, in the case of HIV, already have an immune-compromising infection) at greater risk for infection.

Another limitation to this study design was the use of mRNA copy numbers to estimate relative abundance for each biomarker. While mRNA levels provide insight into patterns and levels of gene expression, mRNA expression is not always a good proxy for estimating protein expression. Many mRNAs undergo post-transcriptional modifications that allow a single gene to produce multiple proteins. As a method for regulating protein levels, some mRNA transcripts are more susceptible to degradation, while others are more stable. Hence, one mRNA transcript often does not translate into one protein, and the ratio of transcript number to protein number varies greatly and depends heavily on traits specific to each gene.

A third limitation to this study design is its small sample size, and the absence of a significantly powered group with neuropathology but lacking neurocognitive impairment. The presence of this fourth group would provide insights into which variables are significant in neuropathology but not development of neurocognitive impairment. Further, these individuals could provide information on neuroprotective pathways that preserve cognitive capabilities despite NeuroAIDS infection.

Finally, there was a significant portion of subjects who were missing data, specifically urine toxicology data for analysis of drug use (n=25, 41.7%), which limited the power of models built with these factors. Additionally, some drugs remain detectable in the urine longer than others; where THC remains detectable for several weeks, cocaine is typically not detectable after 48 hours. This means that cocaine users who are aware of their upcoming drug test might forego cocaine use in the couple of days prior to the appointment , thus skewing the results towards the null hypothesis. Indeed, cocaine might have greater effects than is observed in this study.

CHAPTER 6. CONCLUSION

The advent of HAART in 1996 dramatically reduced AIDS-related mortality and improved quality of life in individuals with HIV. Still, HIV-associated neurocognitive disorder persists as a common clinical manifestation in infected individuals, despite controlled peripheral viremia and immune function. This study was addressed at exploring the neurotoxic and neuroprotective effects of Substance P and NK1R expression, life-style choices, and antiretroviral drugs in the context of HIV-infection and NeuroAIDS.

In this study, opiate use produced patterns of expression in CCR5 and truncated NK1R consistent with advanced NeuroAIDS progression (characterized by neurocognitive impairment and neuropathology), while cannabis use produced an inverse effect. This confirms findings from other studies that opiate use may have neurodegenerative effects, but suggests that the mechanism of neurodegeneration may be more complicated than merely increasing expression of CCR5. Along these same lines, this study supports the findings of previous studies that suggest a neuroprotective effect of cannabis use. These findings highlight the importance for management of opiate use in the HIV-infected population, and suggest that medical marijuana may reduce the severity of NeuroAIDS progression.

Second, this study suggests a role for switches in viral tropism in more severe cases of NeuroAIDS, characterized by neuropathology. Individuals with pathology had decreased expression of CCR5 in the cingulate cortex compared to cerebellar expression. CXCR4 expression was trending higher in individuals with neuropathology than those without (Figure 8). Taken together, this suggests a role for X4 tropic viruses in advanced NeuroAIDS, and highlights a need to focus greater attention towards understanding the impact of tropic switches in NeuroAIDS progression. It provides support for research and development of anti-retroviral therapies that specifically inhibit R5 to X4 tropism changes, which could be delivered in conjunction with drugs, such as aprepitant, that inhibit infectivity with R5-tropic strains.

Finally, this study outlines the need for subsequent studies to examine biomarker expression in NeuroAIDS. The significant findings that Substance P expression is significantly decreased in the cingulate cortex of individuals with pathology, and that expression patterns of truncated NK1R are dramatically altered in individuals with pathology suggest that these signaling molecules do indeed play a role in advanced stages of NeuroAIDS, and may provide a therapeutic point of intervention if their mechanism of action can be elucidated.

Furthermore, the findings from this study emphasize the importance of considering within-subject patterns when examining subtle changes in expression of signaling molecules. In general, there is a great degree of variability between subjects in biomarker expression, and as a result "normal levels" often encompass an extremely wide range. This is apparent from routine blood tests to more invasive evaluations of CSF markers, and complicates diagnosis of pre-clinical conditions and implementation of preventative treatment aimed at staving off full-blown disease. While repeated measures over time are ideal, comparison of a "test" region with a "control" region (if one such region can be identified for a particular disease) may provide adequate.

From the repeated measures analysis, we also observed that expression levels were differentially changed in specific regions of the brain. This could have pharmacological implications in the design of drug delivery. In the case of NeuroAIDS, it may not simply be enough to get a drug past the blood-brain barrier- it may be important to ensure it is targeted to specific regions for optimal effect. It is important that future NeuroAIDS research be guided by the idea that not only will differences between individuals be present in overall expression, but that findings from one region of the brain in one sub-population may not be generalized to findings of another region of the brain in a different sub-population. Increased insight into patterns of expression across time and location should provide greater effectiveness in adequate drug-delivery, and thus improve outcomes- not only in NeuroAIDS, but in a multitude of complicated, chronic diseases.

REFERENCES

Anthony IC and Bell JE. (2008) The Neuropathology of HIV/AIDS. *International Review of Psychiatry*, 20(1):15-24.

Anthony IC, Ramage SN, Carnie FW, Simmonds P, and Bell JE. (2005) Does drug use alter microglial phenotype and cell turnover in the context of advancing HIV infection? *Neuropathology and Applied Neurobiology*, 31(3): 325-38.

Anthony IC, Ramage SN, Carnie FW, Simmonds P, and Bell JE. (2005) Influence of HAART on Hiv-related CNS disease and neuroinflammation. *Journal of Neuropathology and Experimental Neurology*, 64:529-536.

Antinori A, Arendt G, Becker JT, Brew BJ, et al. (2007) Updated research nosology for HIV-associated neurocognitive disorders. *Neurology*, 69:1789-99.

Armstrong DM, Benzing WC, Evans J, Terry RD, Shields D, and Hansen LA. (1989) Substance P and somatostatin coexist within neuritic plaques: implications for the pathogenesis of Alzheimer's disease. *Neuroscience*, 31(3):663-71.

Banks W. (2005) The blood-brain barrier: its structure and function. In H. Gendelman et al. (Ed.) *The Neurology of AIDS, second edition* (pp.107-13) New York: Oxford University Press.

Bell JE and Gray F. (2005) Brain pathology in HIV-1 infected drug abusers. In H. Gendelman et al. (Ed.) *The Neurology of AIDS, second edition*. (pp.393-403) New York: Oxford University Press.

Bouwman F, Skolasky R, Hes D, et al, (1998) Variable progression of HIV-associated dementia. *Neurology*, 50:1814-20.

Brack-Werner R. (1999) Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *AIDS*, 13(1):1-22.

Budka H. (2005) The neuropathology of HIV-associated brain disease. In H. Gendelman et al. (Ed.) *The Neurology of AIDS, second edition* (pp. 375-391). New York: Oxford University Press.

Caberlotto L, Hurd Y, Murdock P, et al. (2003) Neurokinin 1 receptor and relative abundance of the short and long isoforms in the human brain. *European Journal of Neuroscience*, 17:1736-46.

Cass W, Harned M, Peters L, Nath A, and Maragos W. (2003) HIV-1 protein Tat potentiation of methamphetamine-induced decreases in evoked overflow of dopamine in the striatum of rat. *Brain Research*, 984: 133-42.

Chang MM, Leeman SE, and Niall HD. (1971) Amino-acid sequence of substance P. *Nature-New Biology*, 232:86-87.

Chen W, Tang Z, Fortina P, et al. (2005) Ethanol potentiates HIV-1 gp120-induced apoptosis in human neurons both the death receptor and NMDA receptor pathways. *Virology*, 334:59-73.

Chernova I, et al. (2009) Substance P (SP) enhances CCL5-induced chemotaxis and intracellular signaling in human monocytes, which express the truncated neurokinin-1 receptor (NK1R). *Journal of Leukocyte Biology*, 85(1):154-64.

Clapham PR and McKnight A. (2001) HIV-1 receptors and cell tropism. *British Medical Bulletin*, 58:43-59.

Cooper PE, Fernstrom MH, Rorstad OP, Leeman SE, and Martin JB. (1981) The regional distribution of somatostatin, substance P and neurotensin in human brain. *Brain Research*, 218:219-232.

Devadas K, Lal RB, and Dhawan S. (2005) Immunology of HIV-1. In H. Gendelman et al. (Ed.) *The Neurology of AIDS, second edition* (pp. 29-47). New York: Oxford University Press.

Di Stefano M, Sabri F, and Chiodi F. (2005) HIV-1 structural and regulatory proteins and neurotoxicity. In H. Gendelman et al. (Ed) *The Neurology of AIDS, Second Edition* (pp. 49-56) New York: Oxford University Press.

Douglas SD and Leeman SE. (2010) Neurokinin-1 receptor: functional significance in the immune system in reference to selected infections and inflammation. *Annals of the New York Academy of Sciences: The Year in Immunology*, 1217(2011):83-95.

Douglas SD, Cnaan A, Lynch KG, et al. (2008) Elevated substance P levels in HIV-infected women in comparison to HIV-negative women. *AIDS Research and Human Retroviruses*, 24(3): 375-8.

Douglas SD, Ho WZ, Gettes DR, et al. (2001) Elevated substance P levels in HIV-infected men. *AIDS*, 15(15): 2043-5.

Epidemiologic Notes and Reports: Pneumocystis Pneumonia --- Los Angeles (1981, June 05) MMWR: Morbidity and Mortality Weekly Report, 30(21):1-3.

Garvey L, Winston A, Walsh J, et al. (2011) Antiretroviral therapy CNS penetration and HIV-1-associated CNS disease. *Neurology*, 76:693-700.

Ghorpade A, Brichacek B, and Bukrinsky M. (2005) The Molecular Miology of HIV-1. In H. Gendelman et al. (Ed.) *The Neurology of AIDS, Second Edition* (pp. 17-28). New York: Oxford University Press.

Gras G and Kaul M. (2010) Molecular mechanisms of neuroinvasion by monocytes-macrophages in HIV-1 infection. *Retrovirology*, 7:30-41.

Green DA, Masliah E, Vinters HV, Beizai P, Moore DJ, and Achim CL. (2005) Brain Deposition of beta-amyloid is a common pathologic feature in HIV positive patients. *AIDS*, 19(4): 407-11.

Guo Y, Wang HL, Xiang XH, and Zhao Y. (2009) The role of glutamate and its receptors in mesocorticolimbic dopaminergic regions in opioid addiction. *Neuroscience and Biobehavioral Reviews*, 33(6): 864-73.

Heaton RK, Clifford D, Franklin DR, et al. (2010) HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. *Neurology*, 75:2087-96.

Heaton RK, Franklin DR, Ellis RJ, et al. (2011) HIV-associated neurocognitive disorders before and during the era of combination antiretroviral therapy: differences in rates, nature, and predictors. *Journal of Neurovirology*, 17:3-16.

Hill J, Mervis RF, Avidor R, Moody TW, and Brenneman DE (1993) HIV envelope protein induced neuronal damage and retardation of behavioral development in rat neonates. *Brain Research*, 603: 222-33.

Janssen RS, Cornblath DR, Epstein LG, et al. (1991) Nomenclature and research case definitions for neurological manifestations of human immunodeficiency virus type-1 (HIV-1) infection. Report of a Working Group of the American Academy of Neurology AIDS Task Force. *Neurology*, 41:778-85.

Kaltschmidt B and Kaltschmidt C. (2009) NF-kappaB in the nervous system. *Cold Spring Harbor Perspectives in Biology*, 1(3):a001271.

Kanazwa I and Jessell T. (1976) Post mortem changes and regional distribution of substance P in the rat and mouse nervous system. *Brain Research*, 117: 362-367.

Kraft-Terry S, Gerena Y, Wojna V, et al. (2010) Proteomic analyses of monocytes obtained from Hispanic women with HIV-associated dementia show depressed antioxidants. *Preotomics Clinical Applications*, 4(8-9):706-14.

Kumar R, Perez-Casanova A, Tirado G, et al. (2005) Increased viral replication in simian immunodeficiency virus/ simian HIV-infected macaques with self-administering model of chronic alcohol exposure. *Journal of Virology*, 78:11425-28.

Kure K, Llena JF, Lyman WD, et al. (1991) Human Immunodeficiency Virus-1 infection of the nervous system: an autopsy study of 268 adult, pediatric, and fetal brains. *Human Pathology*, 22:700-10.

Lai JP, Cnaan A, Zhao H, and Douglas SD (2007) Detection of full-length and truncated neurokinin-1 receptor mRNA expression in human brain regions. *Journal of Neuroscience Methods*, 168(2008):127-33.

Lai JP, et al. (2008) Differences in the length og the carboxyl terminus mediate functional properties of neurokinin-1 receptor. *Proceedings of the National Academy of the Sciences*. 105(34):12605-10.

Lai JP, Ho WZ, Kilpatrick LE, et al. (2006) Full-length and truncated neurokinin-1 receptor expression and function during monocyte/ macrophage differentiation. *Proc. National Academy of the Sciences USA*, 103(20):7771-6.

Lai JP, Ho WZ, Zhan GX, Collman RG, and Douglas SD. (2001) Substance P antagonist (CP-96, 345) inhibits HIV-1 replication in human mononuclear phagocytes. *Proceedings of the National Academy of Science*, 98(7):3970-75.

Lanoy E, Guiguet M, Bentata M, et al. (2011) Survival after neuroAIDS: Association with antiretroviral CNS Penetration-Effectiveness score. *Neurology*, 76:644-651.

Letendre S, Marquie-Beck J, Capparelli E, et al. (2008) Validation of the CNS Penetration-Effectiveness rank for quantifying antiretroviral penetration into the central nervous system. *Archives of Neurology*, 65(1): 65-70.

Levy, Jay A. (2007) *HIV and the Pathogenesis of AIDS, Third Edition*. Washington, DC: ASM Press.

Liner II KJ, Ro MJ, and Robertson KR. (2010) HIV, Antiretroviral Therapies, and the brain. *Current HIV/AIDS Reports*, 7:85-91.

Manak MM, Moshkoff DA, Nguyen LT, et al. (2010) Anti-HIV-1 activity of the neurokinin-1 receptor antagonist aprepitant and synergistic interactions with other antiretrovirals. *AIDS*, 24(18):2789-96.

McArthur JC, McClernon DR, Cronin MF, et al. (1993) Dementia in AIDS patients: incidence and risk factors. Multicenter AIDS Cohort Study. *Neurology*, 43:2245-52.

Mitchell RM, Neafsey EJ, and Collins MA. (2009) Essential involvement of the NMDA receptor in ethanol preconditioning-dependent neuroprotection from amyloid-beta in vitro. *Journal of Neurochemistry*, 111(2):580-8.

MMWR (1987) Revision of the CDC Surveillance Case Definition for Acquired Immunodeficiency Syndrome. 35(15).

Morgello S, Mahboob R, Yakoushina T, Khan S, and Hague K. (2002) Autopsy findings in a human immunodeficiency virus-infected population over 2 decades: influences of gender, ethnicity, risk factors and time. *Archives of Pathology and Laboratory Medicine*, 126(2):182-90.

Nakamuta S, Endo H, Higashi Y, et al. (2008) Human immunodeficiency virus type 1 gp120-mediated disruption of tight junction proteins by induction or proteasome-mediates degradation of zonula occludens-1 and -1 in human brain microvascular endothelial cells. *Journal of Neurovirology*, 14:186-195.

Nath A, Anderson C, Jones M, et al (2000). Neurotoxicity and dysfunction of dopaminergic systems associated with AIDS dementia. *Journal of Psychopharmacology,* 14:222-7.

Navia BA and Price RW. (2005) An overview of the clinical and biological features of the AIDS dementia complex. In H. Gendelman et al. (Ed.) *The Neurology of AIDS, second edition* (pp. 339-56). New York: Oxford University Press.

Neuenburg JK. (2009) The neuropathology of HIV Pre- and Post- HAART. In K. Goodkin et al. (Ed) *The Spectrum of NeuroAIDS Disorders* (pp.181-99) Washington, DC: ASM Press.

Pantaleo G, Graziosi C, and Fauci AS. (1997) Virologic and immunologic events in primary HIV infection. *Springer Seminar on Immunopathology,* 18:257-66.

Pulliam L, Gascon R, Stubblebine M, McGuire D, and McGrath MS (1997) Unique monocyte subset in patients with AIDS dementia. *Lancet*, 349(9053):692-5.

Purohit V, Rapaka R, and Shurtleff D. (2011) Drugs of abuse, dopamine, and HIV-associated neurocognitive disorders/ HIV-associated dementia. *Molecular Neurobiology,* 44:102-110.

Quigley BJ Jr and Kowall NW. (1991) Substance P-like immunoreactive neurons are depleted in Alzheimer's disease cerebral cortex. *Neuroscience*, 41(1)-41-60.

Robertson KR, Fiscus S, Kapoor C, et al. (1998) CSF, plasma viral load and HIV associated dementia. *Journal of NeuroVirology,* 4:90-94.

Robertson KR, Smurzynski M, Parsons TD, et al. (2007) The prevalence and incidence of neurocognitive impairment in the HAART era. *AIDS*, 21:1915-21.

Robertson KR, Su Z, Margolis DM, et al. (2010) Neurocognitive effects of treatment interruption in stable HIV-positive patients in an observational cohort. *Neurology,* 74: 1260-1266.

Rogers TJ and Peterson PK (2003) Opioid G protein-coupled receptors: signals at the crossroads of inflammation. *Trends in Immunology,* 24(3):116-21

Rumbaugh JA and Nath A (2009) Substance use disorders and Neuro-AIDS in the HAART era. In Goodkin et al. (Ed) *The Spectrum of Neuro-AIDS Disorders: Pathophysiology, Diagnosis and Treatmen*t (pp.411-423). Washington, DC: ASM Press.

Satake H and Kwada T. (2006) Overview of the primary structure, tissue distribution, and functions of tachykinins and their receptors. *Current Drug Targets*, 7:963-74.

Sevigny JJ, Albert SM, McDermott MP, et al. (2007) An evaluation of neurocognitive status and markers of immune activation as predictors of times to death in advanced HIV infection. *Archives in Neurology,* 64(1): 97-102.

Teuchner B, Dimmer A, Troger J, et al. (2010) Secretoneurin and the tachykinins substance P and neurokinin-1/B in NMDA-induced excitotoxicity in the rat retina. *Regulatory Peptides*, 165(1): 123-7.

Tozzi V, Balestra P, Bellagumba R, et al. (2007) Persistence of neuropsychologic deficits despite long-term highly active antiretroviral therapy in patients with HIV-related neurocognitive impairment: prevalence and risk factors. *Journal of Acquired Immune Deficiency Syndrome*, 45: 174-82.

Vinet-Oliphant H, Alvarez X, Buza E, et al. (2010) Neurokinin-1 Receptor (NK1-R) Expression in the Brains of SIV-Infected Rhesus Macaques: Implications for Substance P in NK1-R Immune Cell Trafficking into the CNS. *The American Journal of Pathology*, 177(3): 1286-97

Wang X, Douglas SD, Lai JP, Tuluc F, Tebas P, and Ho WZ (2007) Neurokinin-1 receptor antagonist (aprepitant) inhibits drug-resistant HIV-1 infection of macrophages in vitro. *Journal of Neuroimmune Pharmacology*, 2(1): 42-48.

Wang X, Douglas SD, Song L, Wang YJ, and Ho WZ. (2008) Neurokinin-1 receptor antagonist (aprepitant) suppresses HIV-1 infection of microglia/ macrophages. *Journal of Neuroimmune Pharmacology*, 3(4): 257-64..

Whitty CJ, Paul MA, and Bannon MJ. (1997) Neurokinin receptor mRNA localization in human midbrain dopamine neurons. *Journal of Comparative Neurology*, 382:394-400.

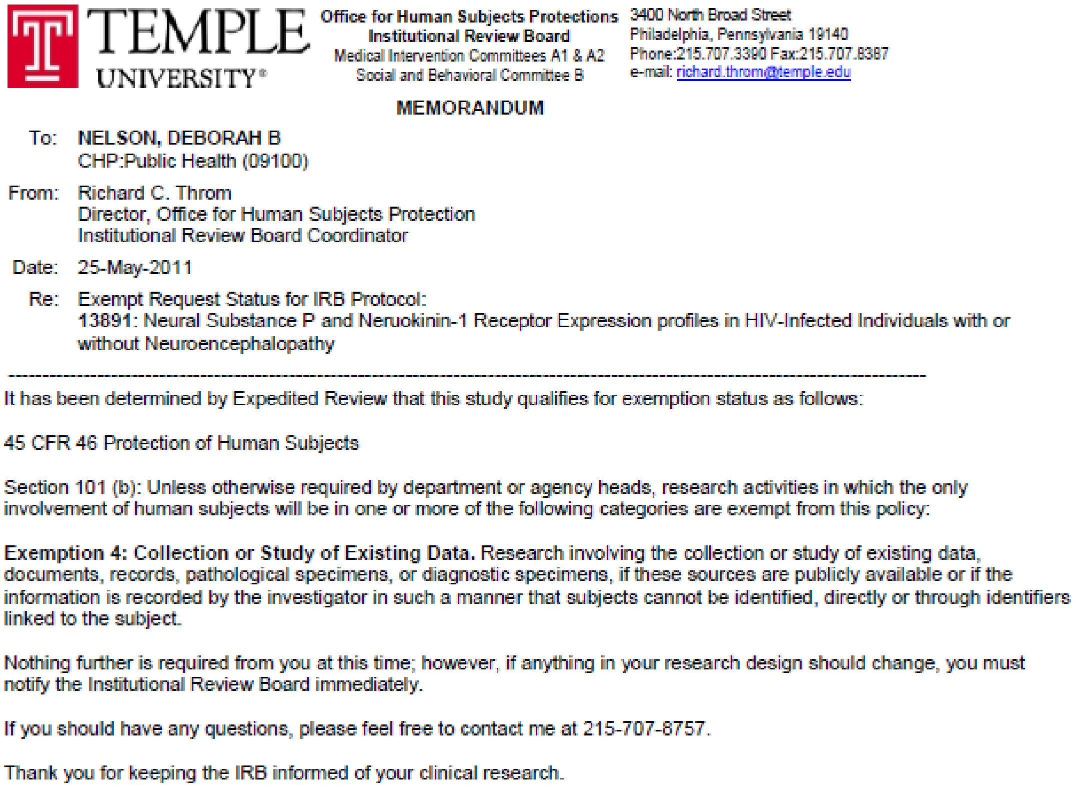
Williams KC, Corey S, Westmoreland SV, et al. (2001) Perivascular macrophages are the primary cell type productiely infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS. *Journal of Experimental Medicine*, 193(8):905-15.

Willis M, Hutter-Paier B, Wetzorrek G, et al. (2007) Localization and expression of substance P in transgenic mice overexpressing human APP751 with the London (V717I) and Swedish (K670M/N671L) mutations. *Brain Research*, 1143:199-207.

Wolcott DL, Fawzy FI, and Pasnau RO. (1985) Acquired immune deficiency syndrome (AIDS) and consultation-liaison psychiatry. *General Hospital Psychiatry*, 7(4):280-93.

-APPENDICES-

APPENDIX A. TEMPLE IRB EXEMPTION



TEMPLE UNIVERSITY®
Office for Human Subjects Protections
Institutional Review Board
Medical Intervention Committees A1 & A2
Social and Behavioral Committee B

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MEMORANDUM

To: **NELSON, DEBORAH B**
CHP:Public Health (09100)

From: Richard C. Throm
Director, Office for Human Subjects Protection
Institutional Review Board Coordinator

Date: 25-May-2011

Re: Exempt Request Status for IRB Protocol:
13891: Neural Substance P and Neruokinin-1 Receptor Expression profiles in HIV-Infected Individuals with or without Neuroencephalopathy

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It has been determined by Expedited Review that this study qualifies for exemption status as follows:

45 CFR 46 Protection of Human Subjects

Section 101 (b): Unless otherwise required by department or agency heads, research activities in which the only involvement of human subjects will be in one or more of the following categories are exempt from this policy:

Exemption 4: Collection or Study of Existing Data. Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subject.

Nothing further is required from you at this time; however, if anything in your research design should change, you must notify the Institutional Review Board immediately.

If you should have any questions, please feel free to contact me at 215-707-8757.

Thank you for keeping the IRB informed of your clinical research.