DYSREGULATION OF NEUROGRANIN, A NEURON SPECIFIC FACTOR, IN HIV-1 POSITIVE SUBJECTS AND ITS IMPLICATIONS IN HIV-1 ASSOCIATED NEUROCOGNITIVE DISORDERS

by

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ABSTRACT

Description of The Problem:

The HIV/AIDS epidemic is an issue of public health relevance due to the widespread prevalence of the disease and the difficulty in treatment. Thirty million individuals worldwide are currently living with HIV, and approximately one million of those infected die each year from AIDS-related illnesses. While improvements in medication and therapy have increased the lifespan of HIV-1 positive individuals and reduced the severity of symptoms, more than half of HIV-1 positive subjects continue to suffer from HIV-associated neurocognitive disorders (HAND). Even individuals undergoing combination anti-retroviral therapy (cART) that have an undetectable viral load suffer from HAND, suggesting cART is unable to prevent neuronal dysfunction. The resulting cognitive deficits can burden the daily functioning of HIV-1 positive subjects and constitute an issue in great need of further research.

Objectives:

Our preliminary studies have shown that the neuron specific protein, Neurogranin (Nrgn), is dysregulated in the brains of HIV-1 positive subjects. Nrgn is a protein important in the Ca^{2+}/calmodulin signaling pathway and long-term potentiation (LTP) in the brain. However, it is not clear how HIV-1 infection alters Nrgn and its function. Based on what is known, we hypothesize that both viral and virus influenced cellular factors contribute in part to the dysregulation of Nrgn and the development of HAND.

Methods Used:

I propose to investigate how viral protein mediated cellular effects may contribute to Nrgn regulation. Using macrophages infected with a wild type virus as well as viruses deficient in Nef, Env, Vpr, or Vif protein expression, I propose to evaluate how these viral proteins and inflammatory factors (cytokines/chemokines) released from HIV-1 infected cells affect Nrgn expression.

Results:

Our results show differences in the secreted concentration of human IL-8, as well as the expression of Nrgn in cells exposed to each HIV-1 virus type studied. HIV-1 wild type virus upregulated IL-8 and dysregulated Nrgn at a higher level compared to other viral mutants. While future studies need to be conducted to confirm these results, our study provides evidence that both viral proteins and secreted cellular factors may contribute to the dysregulation of Nrgn.

Conclusions:

By increasing our understanding of the role of viral proteins and inflammatory factors in Nrgn expression, we can better understand how HIV-1 dysregulates Nrgn in the brains of infected individuals and illuminate potential therapeutic targets to reduce the development of HAND.

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PREFACE

I would like to thank all individuals in the Ayyavoo lab at the University of Pittsburgh Graduate School of Public Health for their assistance and guidance during this project. I would especially like to thank Dr. Jay Venkatachari and Dr. Debjani Guha for helping me learn the techniques used and Dr. Velpandi Ayyavoo, my advisor, for her support throughout my time at Pitt. Completion of this project would not have been possible without each of you all.

1.0 INTRODUCTION

The HIV/AIDS epidemic is an issue of great public health significance due to the widespread prevalence of the disease, the difficulty in treatment, and the approximately 34 million lives that have been lost to the disease and associated complications. HIV/AIDS has far-reaching effects across the globe, with approximately 37 million people currently living with the infection worldwide [1]. Differences in awareness and prevention strategies, as well as access to testing and treatment, play a role in the unequal distribution of HIV-1 cases globally. While there are different rates of HIV-1 prevalence among certain ages, races, and regions, the virus does not discriminate against any single group of individuals. Understanding the disparities around HIV-1 infection can help organizations better target the individuals at greatest risk; this allows for earlier access to testing and treatment for those infected, as well as a more focused prevention campaign.

HIV-1 is most often transmitted through sexual contact, IV drug use, and childbirth. Infection can also occur to lesser degrees from job-related exposure, blood transfusions, and organ transplants. While it is beneficial to prevention efforts to know who is at risk for infection, the natural history of HIV-1 infection is unique and presents many additional problems for effective prevention and treatment measures.

Infected individuals can follow various courses of disease progression that involve the development of a wide array of symptoms. Initial symptoms of an HIV-1 infection, such as

fever, sore throat, and headache, can be nonspecific and may mimic other common infections [2]. In fact, the World Health Organization estimates that approximately 47% of those that are HIV-1 positive are unaware they are infected [1]. Interestingly, noticeable outward symptoms do not always develop that prompt an individual to be tested for HIV-1, even despite knowing they are at risk for infection. It is at this stage of infection that individuals are especially likely to transmit the virus to others. After a short time, the individual enters a stage of clinical latency with minimal symptoms as the virus replicates at much lower levels.

At later stages of HIV-1 infection, AIDS can develop in infected individuals who have become immunocompromised and are now vulnerable to opportunistic infections. While there is no cure for an HIV-1 infection, several elements can impact the prognosis of an individual and delay the onset of AIDS. The initiation and consistent use of antiretroviral therapy early in infection can improve one's prognosis. Additionally, taking part in other behaviors that support a healthy lifestyle such as reducing stress, eating healthy foods, and generally taking care of oneself can help improve the outlook of an HIV-1 infection. [3] While significant advances have been made in our understanding of HIV-1, much is left to learn about the biology of the virus before substantial progress can be made in developing a vaccine or cure.

1.1 BIOLOGY OF HIV-1

The virology of HIV-1 makes it a difficult infection to both treat and prevent within the population. HIV-1 has several structural features that make it successful at infecting immune target cells, while also evading detection by the host's immune system. The HIV-1 virion is roughly 120-145 nm in diameter and contains two nine kb RNA molecules [4]. There are nine

genes in the HIV-1 genome that are cleaved into fifteen protein products; in general, these proteins include viral enzymes, structural proteins, and regulatory proteins (Figure 1).

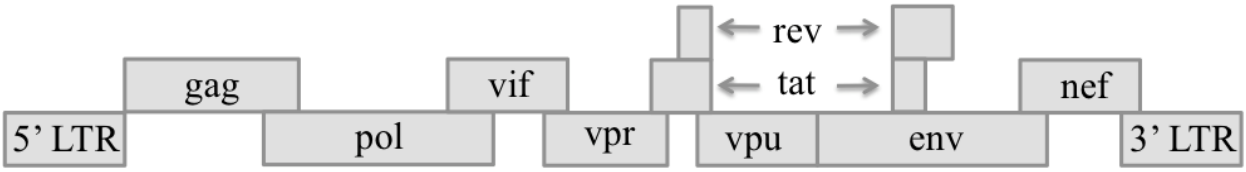

Figure 1. HIV-1 viral genome

The viral enzymes (protease, reverse transcriptase, and integrase) are cleaved from the polymerase (Pol) polyprotein and are responsible for the transcription and maturation of gene products. Structural proteins (matrix, capsid, nucleocapsid and two envelope proteins) are responsible for the entry of the virus upon infection of cells and the budding of new viruses during viral replication. The group-specific antigen (Gag) polyprotein is cleaved into p6, as well as the matrix and capsid proteins, while the Env polyprotein encodes the envelope glycoprotein gp160, that is further processed into gp120 and gp41. Lastly, the accessory and regulatory proteins (Vpu, Vif, Vpr, Nef, Rev, and Tat) serve various functions for the virus that increase infectivity, as well as evasion of the host's immune system. [5]

The RNA molecules at the center of the spherical virion are complexed with a nucleocapsid protein coat, p24, which is responsible for protecting the viral RNA. The p24/RNA core, reverse transcriptase, and integrase are surrounded by structural capsid proteins responsible for the delivery of the viral RNA into the target cell during infection. Reverse transcriptase is utilized to transcribe dsDNA from the RNA in the viral genome. Integrase then takes the newly created dsDNA and inserts the molecule into the genome of the host cell. This allows the viral genome to be copied and replicated along with the host DNA to create many new viral proteins.

Around the capsid coated core resides protease proteins responsible for the cleaving and activation of polypeptides in the HIV-1 virion. The inner structure of the HIV-1 virion is again

enclosed within a matrix protein (p17) coat that plays an integral part of the budding of new HIV-1 viruses during viral reproduction within infected cells. [5, 6] Surrounding the matrix coat is a lipid bilayer envelope consisting of the glycoproteins gp120 and gp41. These proteins assist binding of the virus to surface receptors, namely CD4 and co-receptors, CCR5 (macrophage-tropic viruses) or CXCR4 (T-cell trophic viruses), on target cells and penetration for entry of the viral genome. [4, 6]

In addition to structural and enzymatic proteins, the accessory and regulatory proteins (Vpu, Nef, Vif, Vpr, Rev, Tat) within the viral genome play important roles in the success of the pathogen at evading and manipulating the host's immune system. This allows for a greater propagation of the virus within the host and a higher degree of progression to disease.

Viral protein U (Vpu) increases the budding of new viruses from virally infected cells as well as the degradation of host CD4 molecules to reduce CD4 expression on the surface of infected target cells [6]. Vpu binds to the cytoplasmic tail of gp160-CD4 complexes on the endoplasmic reticulum, leading to the destabilization and degradation of CD4 molecules in proteasomes and a subsequent prevention of display on the cell membrane [7-9]. With a reduced number of CD4 molecules present on the target cell membrane, the virus has a greater chance of evading detection by the host's immune system [10]. Additionally, it has been shown that subjects with Vpu defects have a marked 5-10 fold reduction in the number of viral particles released from infected cells [11]. The protein, tetherin is normally responsible for the inhibition of virion release; however, the presence of Vpu in the cell counteracts the effects of tetherin and increases budding of new virus particles. The specific mechanisms behind this action are not yet known. [12, 13] Vpu aids the functioning of the virus through both the release of new viral particles, as well as protection of the virus against the host's immune system.

The viral protein negative factor (Nef) plays a role in the induction of disease after infection. Research has shown that subjects infected with a Nef-deficient virus tend to demonstrate a slow or non-progressor phenotype and have lower viral loads; this results in a lower pathogenicity of the virus and a delayed progression to AIDS [10, 14, 15]. The primary function of Nef is the down-regulation of virus replication after infection to evade detection by the host immune system. Nef leads to increased endocytosis of CD4, CD28, and MHC-I complexes on the target cell surface, followed by fusion with lysosomes for degradation [10, 16-18]. Nef also induces bystander damage, or apoptosis, of CD4 and CD8 cells by upregulating Fas ligand, either through interacting directly with the zeta chain of the T cell receptor complex or through a pathway involving p38 mitogen-activated protein kinase (MAPK) and activator protein-1 (AP-1) [19, 20]. Collectively, evidence supports Nef having a prominent role in the ability of the virus to evade control by the host immune system and increase the pathogenicity of the virus.

The protein virion infectivity factor (Vif) increases the infectivity of the virus by allowing the infection of non-permissive cells (e.g. lymphocytes) [6, 21]. Vif functions primarily by antagonizing the antiviral effects of the APOBEC3G (A3G) gene through pre- and post-translational modifications to prevent the inclusion of the gene in virus producing cells [10]. The A3G gene is responsible for the inclusion of cytidine to uracil deamination mutations in the viral genome during the process of reverse transcription, essentially terminating the life cycle of the virus and preventing replication [22]. Vif (along with Vpr) has also been shown to play a role in the degradation of interferon regulatory factor 3 (IRF-3) by proteasomes in the early stages of HIV-1 infection. IRF-3 induces an antiviral and inflammatory state in the host by generating type I interferons that stimulate macrophages and natural killer cells to fight off invading pathogens.

[23, 24] The presence of Vif, primarily in acute stages of infection, increases the success of the pathogen by helping to evade the innate immune response of the host [25].

Like other accessory proteins, viral protein R (Vpr) also functions to increase the persistence of infection within the host. Vpr, along with integrase and matrix protein, aids the entrance of the HIV-1 genome into the nucleus of the target cell for induction into the host genome. This gives HIV-1 the ability to infect non-dividing cells that typically have restrictions for nuclear transport, allowing HIV-1 to infect and replicate virtually undetected within macrophages and monocytes. [6, 10, 26-28] Vpr also increases the budding of newly synthesized virion particles through decreased interferon production in target cells and subsequent increases in Env production [29]. In a related manner, Vpr also functions at the G2 checkpoint to reactivate latently infected cells by activating the long terminal repeats (LTR) and other promoters, increasing viral RNA production and persistence within the host [27, 30, 31].

Trans-activating protein (Tat) and regulator of viral expression (Rev) are two regulatory proteins also associated with the HIV-1 genome. Rev regulates the splicing of the viral RNA within the infected cell and increases the transport of unspliced or singly spliced products from the nucleus, via recruitment of nuclear export factors [10, 32]. Tat functions to increase the transcription of new viral RNA through interactions with Sp1 transcription factor, therefore increasing the amount of protein made during translation [5]. Other studies have also shown evidence for a role of Tat in viral RNA splicing, capping, and translation, as well as with functions of reverse transcriptase [9].

Together, the biology of HIV-1 poses a challenge for the successful eradication of the virus from the host population. While there have been significant advances in the development of anti-retroviral therapies, none have yet been successful at elimination of the virus. Individuals

infected with HIV-1 are living longer with advanced treatment options, but continue to suffer from comorbid diseases and opportunistic infections. Furthermore, it is becoming evident that prolonged exposure to the virus can result in several additional disorders within the host. One such condition of increasing concern is the development of neurocognitive disorders.

1.2 HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

With advances in early detection of HIV-1 and improvements in therapeutic drugs to suppress viral loads, individuals infected with HIV-1 have a greater chance of survival and a longer lifespan than those in the past. While diagnosis with HIV-1 in the past often resulted in a shorter lifespan and the development of various opportunistic infections, with treatment options today, many live approximately normal lifespans and experience far less complications [33]. With earlier initiation of antiretroviral therapies, the prognosis is even more promising [34-36]. A recent study analyzed the life expectancies of 22,937 individuals infected with HIV-1 and currently on combination anti-retroviral therapy (cART). Researchers observed an increase in lifespan of 15.3 years between 2000 and 2007, making the average lifespan of an HIV-1 infected individual on cART in the early 70's, nearly the typical lifespan of an uninfected person. [37]

While improvements in drug therapies have been shown to increase the overall lifespan of a person infected with HIV-1, other diseases of aging are beginning to appear and causing further complications. One prominent example is HIV-Associated Neurocognitive Disorders (HAND). There are various degrees of severity associated with HAND that produce differing impacts on cognition and different levels of impairment in daily functioning.

Initial symptoms of HAND typically develop as minor impairments in cognition with little to no impact on the daily lives of those infected. Individuals can continue to maintain the same level of social functioning, live with the same degree of independence, and remain in the same employment. The least severe form of HAND is known as HIV-Associated Asymptomatic Neurocognitive Impairment (ANI). This condition does not interfere with functions of daily living, but does involve deficits in tasks of at least two of the following categories: language, working memory, executive functioning, memory and recall, motor skills, and/or sensory functions. The next, more severe, form of HAND is known as HIV-Associated Mild Neurocognitive Disorder (MND). Individuals with MND experience interruptions in their daily functioning and again have impaired functioning in at least two of the previously mentioned categories. The final and most severe form of HAND is HIV-Associated Dementia (HAD). While HAD does result in the most severe impact on daily functioning, it is also the least common form of HAND among those infected with HIV-1. Symptoms of HAD include impairments in two of the aforementioned categories, significant difficulty in daily functioning, and no presence of delirium or evidence of another cause of dementia. [38] Interestingly, there is currently limited data to support that HAND progresses in a linear fashion from asymptomatic to severe cognitive impairments over the course of an HIV-1 infection. [39] This differs from most other neurocognitive disorders such as Parkinson's or Alzheimer's disease.

It is important for clinicians to recognize these stages of HAND to provide an early diagnosis and link the patient to treatment sooner. Clinician awareness is also important to effectively monitor for changes in the degree of HAND over the course of disease progression and in response to treatment. In addition to the monitoring of disease within patients, understanding the biology of HIV-1 infection and its relation to the central nervous system

(CNS) is important. Increased knowledge of the role of the CNS can shed light on the neurocognitive symptoms associated with infection and to develop new therapeutic techniques to reverse the effects. While most therapies are looking at CD4+ T-cells as a therapeutic target, some research supports the presence of a viral reservoir within the brain that persists despite viral suppression in the blood from highly active antiretroviral therapy (HAART) [40]. A study of sixty-nine participants with an asymptomatic HIV-1 infection and who were also receiving HAART showed that 10% of subjects had HIV-1 RNA present in the cerebrospinal fluid despite having viral suppression in the blood [41]. Post-mortem studies have shown that HIV-1 tends to localize the most in the caudate nucleus of infected individuals, a region important for motor function and learning tasks. Researchers found that the lowest amount of HIV-1 RNA was found in the frontal cortex and in the cerebrospinal fluid (CSF), potentially suggesting that CSF samples may underestimate the amount of viral RNA present. [42] Because of differences in viral load between blood plasma and within the CNS, it is unlikely that HIV-1 infection is persisting in the CNS because of replenishment from circulating plasma [43]. Additional studies have shown a difference in the RNA sequence and decay kinetics of HIV-1 taken from both the CNS and blood plasma, further supporting the presence of a CNS reservoir [44].

Research has shown that HIV-1 enters the CNS soon after initial viral exposure when infected monocytes or lymphocytes cross the blood brain barrier (BBB) into the CNS and begin infecting other cells [45, 46]. Macrophages and microglia are two immune cells that are likely targets for HIV-1 within the CNS. These cells comprise approximately 10-15% of the cells within the CNS. Their functions include presenting antigens to other immune cells, undergoing phagocytosis of unwanted or damaged cells, and regulating the production of cytokines and other inflammatory factors to increase or decrease an immune response against a pathogen. Their

infection results in the dysregulation of their immune functions and the potential for the development of a neurotoxic environment within the CNS. Astrocytes are another cell type targeted by HIV-1 and, like macrophages and microglia, they play an important role in the immune functions of the CNS. Astrocytes function as part of the BBB, participate in the production of cytokines important for cell signaling, and present antigens to T-cells to induce an immune response. [47] While some studies have suggested a relatively low rate of astrocyte infection, it has been shown that a significant portion (16-19%) of astrocytes can become infected with viral DNA upon HIV-1 infection. This proportion correlates with the proximity to macrophages as well as to the severity of pathological changes within the CNS. [48]

While many cells within the brain can become infected with HIV-1, neurons themselves are not infected. Instead, neurons are indirectly affected by neurotoxic mechanisms related to the immune response within the CNS. [39] Upon infection, macrophages and microglia release viral proteins that in turn activate astrocytes. Upon activation, astrocytes increase the surrounding extracellular levels of glutamate, an excitatory neurotransmitter. The increased levels of glutamate, as well as the increased cell signaling from chemokines and cytokines lead to excitotoxicity and injury of surrounding neurons. [49] In addition to damage and loss of neurons, the production of inflammatory cytokines from infected CNS cells is thought to contribute to symptoms associated with HAND. This can produce symptoms such as nerve damage, confusion, forgetfulness, and behavioral changes. Post-infection, the functions of macrophages become impaired, including the production of cytokines for cell signaling and the ability to phagocytose unwanted cells. [46] This further contributes to the pathogenesis of HIV-1 infection.

Some studies have shown that the initiation of HAART has reduced the incidence and prevalence of more severe neurological disorders associated with HIV-1 [50]. However, other

research continues to show a significant prevalence of less severe forms of HAND among HIV-1 infected individuals. Two studies, in particular, have shown a persistence in neuropsychological deficits associated with HIV-1 infection in 52% and 62.8% of individuals receiving HAART [51, 52]. Interestingly, it has been shown that the specific type of neurocognitive impairments present among patients appear to differ between the period of time before cART was used and the current time period with more advanced treatment options. In the pre-cART period, deficits generally involved the speed and fluency of speech, general cognitive tasks, and difficulties with motor skills. In the current era of treatment, deficits more often appear to involve memory and learning impairments. [53] Because of the presence of the BBB and the difficult nature of determining what levels of a drug are needed to penetrate the BBB, more research into HAART options is needed to effectively treat the presence of HIV-1 within the CNS.

Gaining an understanding of HAND and the underlying mechanisms for the associated neurocognitive symptoms not only helps prolong the lifespan of those infected, but also aims to improve their quality of life. A greater investigation into the role of the CNS can aid the development of additional treatment options targeted at improving the cognitive deficits still associated with HIV-1 infection.

1.3 NEUROGRANIN

In order to effectively study the development and progression of HAND, it is important to have an understanding of the changes that occur within the central nervous system (CNS) in response to infection with HIV-1. Knowing which proteins or factors are dysregulated in those suffering from HAND can help to reveal a potential mechanism of action for the decline in cognitive functioning seen in these individuals. One protein that is believed to be dysregulated in those infected with HIV-1 and has the potential to explain deficits in neuropsychological functioning is the protein neurogranin (Nrgn). Nrgn has been shown to be significantly downregulated (50-80%) in cells with a high viral load of HIV-1, as compared to cells with a low viral load and an uninfected control [54].

Nrgn is generally considered a neuron specific protein, as it is expressed most abundantly in the dendritic spines of neurons [55]. However, Nrgn is also found in platelets, B-lymphocytes, and to a lesser extent, in other areas of the body such as bone marrow and the lungs. Within the brain, Nrgn is found in various regions including the cerebral cortex, hippocampus, and the amygdala. These regions are important for various functions such as memory, language, attention, and emotion. On the other hand, Nrgn is essentially absent in the cerebellum, thalamus, and brainstem regions. These regions are important for the coordination of movement, sensory interpretation, and basic bodily functions. [56, 57]

On a cellular level, the gene for Nrgn is about 12.5 kbp in length and results in a protein with a molecular mass of approximately 7.8 kDa [58]. Nrgn is a heat and acid stabilized protein that is comprised of seventy-eight amino acids, several of which are acidic. This feature has been shown to aid in the purification of the protein. [59] The serine 36 residue of neurogranin is a key phosphorylation site that is responsible for modulating its function and its interactions with other

proteins. The Isoleucine-Glutamine (IQ) domain on Nrgn also allows it to interact with the protein calmodulin (CaM) and sequester it beneath the postsynaptic membrane of neurons. This location allows the complex to sense for changes in [Ca^{2+}] during signal transmission. The reversible binding between Nrgn and CaM allow for changing dynamics in their signaling pathway and frequent shifts between bound and unbound states. [60, 61] In this manner, Nrgn plays an important role in modulating the activity of subsequent signaling pathways involving CaM.

In particular, Nrgn functions in the Ca^{2+}/CaM synaptic signaling pathway between neurons. Glutamate released from the pre-synaptic neuron in response to an action potential crosses the synapse and binds to an NMDA receptor on the postsynaptic neuron. NMDA receptors allow the entrance of Ca^{2+} into the postsynaptic neuron through voltage-gated Ca^{2+} channels. Ca^{2+} enters the neuron and binds to the Nrgn/CaM complex, activating CaM and triggering its dissociation from Nrgn. [58, 62] In response to this activating signal, Protein Kinase C (PKC) phosphorylates Nrgn within the soma of the postsynaptic neuron, leading to an additional increase in [Ca^{2+}] and subsequent CaM activation [57, 59, 63, 64]. The influx of Ca^{2+} also increases the affinity of CaM for its associated target proteins. Because CaM alone does not have enzymatic activity, it relies on the binding of Ca^{2+} and a subsequent conformational change to then transduce the signal to other downstream enzymes. CaM activates these additional proteins, including CaMKII, which helps induce long-term potentiation (LTP) via the mobilization of synaptic vesicles. CaMKII also increases the presence of AMPA receptors in the synapse, therefore increasing the postsynaptic response to neurotransmission and further increasing the influx of Ca^{2+} into the neuron. [65, 66] Interestingly, Nrgn has also been shown to significantly increase the rate of dissociation between Ca^{2+} and CaM bound to CaMKII.

However, this is believed to function at the end of the Ca^{2+}/CaM signaling pathway by triggering the release of Ca^{2+} and CaM for use in future signal transmission. [67]

Because of its function in Ca^{2+}/CaM signaling, Nrgn is believed to play a role in long-term potentiation (LTP) and synaptic plasticity. [60, 68] LTP is important for strengthening the connection between the synapses of neurons, aiding in the formation of memories, and improving performance on various cognitive tasks. Research has shown a connection between the phosphorylation of Nrgn and LTP, as well as long-term depression (LTD). With the induction of LTP, the phosphorylation of Nrgn is increased. [69, 70] If an inhibitor for the protein kinase is applied, there is a reversal of Nrgn phosphorylation and a decline in the excitatory post-synaptic potential (EPSP) to baseline, essentially ending LTP [71]. This correlates with what is known on a biological level in the Ca^{2+}/CaM signaling pathway. A function of Nrgn is to sequester CaM in the postsynaptic neuron. Therefore, if more Nrgn is present, more CaM will also be present. During signal transmission, as a result of Nrgn phosphorylation by PKC, the bound CaM is released and available for use in the neuron. CaM then activates many downstream enzymes that contribute to LTP in the cell. This greater availability of unbound CaM also lowers the threshold for Ca^{2+} signaling and increases the likelihood of LTP. [60, 72, 73]

In a related manner, after periods of LTD, levels of phosphorylated Nrgn are decreased, as the level of phosphatases in the cell increases. If a phosphatase inhibitor is added, LTD will be prevented. [74, 75] If Nrgn is not phosphorylated, then it will remain bound to CaM, preventing its activation and the activation of its downstream targets. This can contribute to LTD in the neuron.

In regards to HAND, it is thought that the dysregulation of Nrgn and subsequent changes in the Ca^{2+}/CaM signaling pathway may have an impact on the development of neurocognitive deficits seen in HIV-1 infected individuals. Symptoms of HAND often include difficulties in spatial learning, attention, and memory tasks [53, 76]. In a computerized spatial learning task modeled after the Morris water maze used with animals, Morales et al. demonstrated a significant reduction in learning ability and memory in HIV-1 positive individuals compared to sero-negative controls. HIV-1 infected subjects took longer to find the target in each trial and had a reduced ability to re-locate the target when tested again. [77] Declines in cognitive functioning such as this could be due to decreased expression of Nrgn present in the brains of infected individuals. HIV-1 has been shown to indirectly impact neurons, resulting in neuronal injury and death [49]. A decrease in the amount of Nrgn present in the brains of individuals with HAND can impact the process of signal transmission in the CNS and have significant impacts on those suffering from all degrees of HAND.

Studies with Nrgn knockout mice have provided evidence for a link between Nrgn and cognitive functioning [78, 79]. Van Dalen et al. observed altered Ca^{2+} signaling pathways in samples from Nrgn knockout mice [80]. In mice deficient in Nrgn, researchers observed a higher resting [Ca^{2+}] and a decreased amount of Ca^{2+} transiently present during periods of neurotransmission. This can be associated with a greater likelihood of LTD and a smaller likelihood of LTP. From their work, it appears that these changes are not due to any morphological changes in the neurons, but rather from changes in the availability of the Nrgn protein. [80] Likewise, samples of hippocampal neurons from Nrgn knockout mice showed a smaller postsynaptic response of Ca^{2+} to NMDA receptors that could be explained by the altered regulation of CaM availability in the neuron. [80, 81] NMDA receptors have been shown to be

negatively controlled by CaM. Therefore, in a neuron with reduced levels of Nrgn and subsequent higher levels of active CaM, it is more likely there will be an inactivation of NMDA receptors and therefore, a lower level of Ca^{2+} influx into the postsynaptic neuron. [82] This can reduce the levels of synaptic transmission within the neuron and lead to many additional downstream effects that alter additional signaling pathways.

Studies of behavior in mice also support the role of Nrgn in cognitive tasks. Pak et al. demonstrated that while developmentally and anatomically normal, mice deficient in the Nrgn protein demonstrate severe deficits in spatial learning and neuronal plasticity. [83] In the Morris water maze task, wild type (WT), heterozygous (HET), and Nrgn knockout (KO) mice were able to perform comparatively when the target platform was visible, indicating no difference in motor or sensory abilities. However, when the platform was hidden, the Nrgn KO mice had significantly longer escape latencies than the other two groups of mice, meaning Nrgn KO mice took longer to find the platform successfully. Additionally, to test for spatial memory, another trial was run in which there was no platform present. Mice with high degrees of spatial memory spend more time in the region where the hidden platform was located previously. Investigators found that WT mice had the strongest spatial memory, followed by HET mice and then Nrgn KO mice. [83] On a molecular level, the study also showed that Nrgn KO mice had changes in neuronal plasticity and deficiencies in the activation of CaMKII. In terms of LTP, there were observed differences in the initiation of potentiation between WT and Nrgn KO mice. Nrgn KO mice produced lower initial excitatory postsynaptic potentials (EPSPs) that increased in intensity only upon simultaneous stimulations. WT mice, in comparison, reached near maximum EPSPs upon initial stimulation. Additional analysis of short-term plasticity demonstrated that upon successive administrations of stimuli, both groups experienced a depression in EPSP, however,

this was more severe in Nrgn KO mice. [83] These results suggest that the absence of Nrgn interferes with the proper functioning of signaling pathways in the brain and leads to outward behavioral deficits.

Because of its role in various cognitive tasks, Nrgn shows potential as a factor in the development of HAND. More research is needed to evaluate the effects of HIV-1 on Nrgn levels and elucidate a potential mechanism for the dysregulation of Nrgn in neurons. With increased knowledge of the specific HIV-1 viral proteins and associated immune factors that impact the expression and function of Nrgn, a pathway for its dysregulation can be deduced. With this information, more targeted therapies can be given to individuals infected with HIV-1 to prevent and/or treat cognitive decline. Together, this has the potential to reduce the occurrence of HAND in the population and improve the quality of life for the millions of individuals infected with HIV-1.

2.0 PROJECT AIMS

Neurons themselves are not infected by HIV-1; therefore, the neurotoxic effects observed in HAND must indirectly result from infected cells within the CNS and the host's neuroimmune response. Because of the role of Nrgn in cognition, we propose to study the role of Nrgn regulation in response to HIV-1 infection and its implications in the development of HAND. Specifically, we assess how HIV-1 viral and immune factors alter Nrgn expression.

By comparing wildtype HIV-1 virus with four viral protein deletion mutants, we will be able to determine if specific viral proteins play a role in the regulation of Nrgn. Likewise, through an analysis of the cytokines released upon infection of macrophages with each virus, we will be able to determine if cytokines are also likely to impact the regulation of Nrgn.

We hypothesize that both viral proteins and secreted immune factors from HIV-1 infected macrophages contribute to the dysregulation of Nrgn and subsequent neuronal dysfunction. From this research, we will be better able to extrapolate the potential link between Nrgn down regulation and infection with HIV-1. This information can be used to increase awareness of HAND among patients and health care providers, develop therapies for the neurocognitive symptoms seen in those with HAND, and help develop strategies to prevent the development of HAND in others. With the large number of individuals infected with HIV-1 and the significant proportion that simultaneously suffer from neurocognitive disorders, this area of research has an unquestionable public health significance.

3.0 METHODS

3.1 HIV-1

An NL43 enhanced green fluorescent protein (EGFP) HIV-1 virus was used in this study. A wildtype (WT) virus as well as four viral mutants, each deficient in a particular protein (ΔVif, ΔVpr, ΔNef, ΔEnv), were used to compare results and analyze the role that each viral protein plays in the pathways of interest. Virus mutants were prepared in the lab, as previously described [84, 85]. For each part of the study, in addition to HIV-1, an uninfected control was also used.

3.2 CELL CUTLURE

All cells used in this study were grown in a monolayer at 37°C and 5.0% carbon dioxide. Unless otherwise stated, cells were cultured in vented polystyrene T-75 flasks (Falcon, Corning Inc., Corning, NY) in a media consisting of Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, and 1% L-glutamine. These factors provide nutrients for cellular growth and aid in cellular proliferation.

3.2.1 293T Cells

293T cells obtained from the NIH, AIDS Reagent Program were utilized for the initial culture of the viruses used in the study (NIH, Bethesda, MD). 293T cells are human embryonic kidney cells used in studies such as this for their tendency to become transfected with viral plasmids. The growth of virus in 293T cells allowed for the acquisition of a high viral titer for each virus used.

Initially, a NanoDrop (ThermoFisher Scientific, Waltham, MA) was used to verify the concentration of DNA in each stock proviral DNA plasmid to ensure that equal amounts of viral DNA was added to the 293T cells. Vesicular stomatitis virus (VSV) and PolyJet (SignaGen Laboratories, Rockville, MD) were also added to aid in the ability of the virus to infect cells and increase viral titer. 293T cells were grown in 10cm plates and exposed to each virus type (WT, ΔVif, ΔVpr, ΔNef, and ΔEnv) for a period of forty-eight hours. After forty-eight hours, the supernatant was removed and centrifuged at 4,000 rpm for ten minutes at 4°C. The viral supernatant was removed as 1.5 mL aliquots and stored at -80°C. A buffer (RIPA buffer) containing 50mM Tris (pH 7.5), 150mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1.0 mM phenylmethyl-sulfonylfluoride, 0.05% deoxycholate, 10% SDS, 0.07 trypsin inhibitor units/mL aprotinin, and protease inhibitors Leupeptin, Chymostatin, and Pepstatin (1 μg/mL) was added to the pellet. The sample was kept at 4°C, spun on a rotor for forty-five minutes, and centrifuged at 13,000 rpm for ten minutes. The supernatant containing the cellular contents of the 293T cells was saved and stored at -80°C until later use.

3.2.2 TZM-bl Cells

TZM-bl cells obtained from the NIH, AIDS Reagent Program were also used in the study as a method for analyzing the titer of each virus (NIH, Bethesda, MD). TZM-bl cells are derived from the HeLa cell line originating from human carcinoma cells. These cells have been developed to express CXCR4, CCR5 and CD4 receptors and are able to be infected with HIV-1. As a result, TZM-bl cells are often used as part of infectivity assays to assess the viral titer of virus samples. The protocol for the culture of these cells is described below (Section 3.3 Infectivity Assay).

3.2.3 SH-SY5Y Cells

SH-SY5Y cells were differentiated into neurons using a protocol based on previous studies (NIH, AIDS Reagent Program, Bethesda, MD) [86]. SH-SY5Y cells are derived from human neuroblastoma cells and are often used to study neuronal function. The protocol for the culture and use of these cells is described below (Section 3.6 SH-SY5Y Differentiation).

3.3 INFECTIVITY ASSAY

3.3.1 293T Infectivity Assay

An infectivity assay was conducted to determine the titer of each HIV-1 virus type used. This was performed to adjust the amount of 293T viral supernatant added to the macrophages in order to allow for equal opportunity for infection between virus types.

TZM-bl cells were used for the assay and grown in a T75 flask in D-10 media until they became 80-90% confluent. Cells were then plated on a 96-well plate with 2 x 10^4 cells per well in D-10 media. Cells were infected with varying concentrations of each virus type ranging from 100ul virus (no D-10 media), to 0.1 uL virus (+ 99.9 uL of D-10 media). The cells were left exposed to the virus undisturbed for a period of forty-eight hours. Two days post-infection, the plates were observed under a fluorescent microscope and the number of infected green fluorescent-positive cells was counted and recorded. Each concentration of virus used was compared to determine which concentration would provide the most accurate cellular count. For example, for the wildtype virus, the well at a concentration of 1:100 (1 uL of virus in 99 uL of media) was used to count the number of green fluorescent-positive cells. From the number of cells counted, taking into account the concentration used, the number of infectious particles per mL was calculated. From these viral titer results, the multiplicity of infection (MOI) was determined for the wild type and each viral protein deletion mutant.

3.3.2 Macrophage Supernatant Infectivity Assay

TZM-bl cells were again used for this infectivity assay. TZM-bl cells were grown as described above (Section 3.3.1). Twenty-four hours after plating the cells, the D-10 media was removed and viral supernatant samples previously harvested from macrophages were added (Section 3.4). Samples were added for all virus types (WT, ΔNef, ΔEnv, ΔVpr, ΔVif) as well as the uninfected control for both time points (day eight and day sixteen post-infection). 100 uL (with 100 uL D-10) and 50 uL (with 150 uL D-10) of each sample were used. 100 uL additions of viral supernatant were run in duplicate. Cells in the plate were exposed to the viral macrophage supernatant, undisturbed, for a period of forty-eight hours. After forty-eight hours, cells were

observed under a fluorescent microscope to determine the number of infected green fluorescent-positive cells. From this information, the infectivity of each sample was determined.

3.4 MONOCYTE ISOLATION

Heparinized blood samples were obtained with consent from two healthy HIV-1-negative donors (Red Cross Blood Bank, Pittsburgh, PA). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque gradient centrifugation (GE Healthcare, United Kingdom). A Beckman Coulter Vi-Cell machine was used to estimate the number of cells present in each sample and determine the number of viable cells (Beckman Coulter, Brea, CA). This provides a more accurate determination of the number of cells to plate than manually counting with a hemocytometer. Cells were suspended in a buffer solution of 0.5% Bovine Serum Albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA) in 1X phosphate-buffered saline (PBS). Using a MACS Multistand device and a magnetic column, $CD14^+$ monocytes were isolated using antibody-coated magnetic microbeads specific for human $CD14^+$ cells (MACS Miltenyi Biotec, Bergisch Gladbach, Germany). CD14 receptors are typically expressed in large numbers on human monocytes and were therefore selected as the marker for monocyte isolation [87]. Monocytes typically comprise about 10% of the total number of cells in a whole blood sample. Therefore, a comparison of the number of collected monocytes to the total number of cells in the PBMC sample allowed for an estimation of the efficiency of monocyte isolation.

Isolated monocytes were plated at 2×10^6 cells per well in 6-well plates along with 2mL of D-10 media, MCSF (50 ug/uL) in a 1:10,000 dilution, and GM-CSF (Leukine, sargramostim, SANOFI, France) in a 1:1,000 dilution to aid in the differentiation into macrophages. The media

in the plates was changed every three days for six days and differentiation from monocytes to macrophages was observed visually using a light microscope. When changing the media, the removed media was centrifuged at 1,000 rpm for five minutes. The cell pellet was then resuspended with fresh media and added back to the plates to prevent the removal of cells.

After seven days of differentiation, HIV-1 wildtype (WT) or one of the four viral protein mutants (ΔNef, ΔEnv, ΔVpr, ΔVif) was added to each well with 1.5 mL of additional D-10 media. The viral titer calculated from the previous TZM-bl infectivity assay (Section 3.3.1) was used to determine the amount of each virus type to add. A multiplicity of infection (MOI) of 0.5 was desired, implying that with the presence of two million cells in each well, the desired amount of added virus is approximately one million infectious particles per well. An uninfected control was also used, in which only D-10 media was added to the wells. The media containing the virus was removed after twenty-four hours and fresh D-10 media with MCSF and GMCSF was added. After this, half of the volume of media was changed every three days to provide sufficient amounts of differentiation factors for the continued period of differentiation.

On day eight post-infection, half of the media comprising the viral supernatant (1mL) was removed from each well. These samples were centrifuged at 1,000 rpm for five minutes to remove any cellular debris, and the supernatant was stored at -80°C. The pellet was then resuspended in fresh media and added back to the plates. For an additional seven days, the macrophages remained exposed to the viral supernatant, and the media was not changed.

On day sixteen post-infection, half of the media in each well was again removed, centrifuged, and the supernatant was stored at -80°C. The pellet was resuspended in media and added back to the plates.

On day twenty-one post-infection, the macrophage cell lysate was harvested by lysing the cells in RIPA buffer. The media containing the lysed cells was removed from each well and centrifuged at 13,000 rpm for ten minutes at 4°C. The cell pellet was discarded as unwanted cellular debris and the supernatant containing the cellular proteins was saved and stored at -80°C.

3.5 CYTOKINE PROFILE

An IL-8 cytokine profile was performed to determine the presence of IL-8 released by HIV-1 infected macrophages used during the study. This was completed using an IL-8 ELISA (DuoSet ELISA Development System, R&D Systems; Minneapolis, MN). Selection of IL-8 was based on previous studies that evaluated the relationship between a pro-inflammatory environment in the presence of HIV-1 and neuronal cell death [88].

With the ELISA, human IL-8 (CXCL8) levels were measured in the supernatant harvested during the infection period of macrophages (Section 3.4). This provides information on the amount of this particular chemokine present in the environment surrounding the cells. IL-8 is a chemokine produced by macrophages that plays a role in chemotaxis. It has been shown that IL-8 levels are increased in individuals with HIV-1 infection [89, 90]. Studies have also demonstrated that IL-8 stimulates the growth of HIV-1 in macrophages [91].

To perform the ELISA, a 96-well plate was coated with a capture antibody specific for IL-8 and incubated at room temperature overnight. The next day, the wells were blocked using a 1% BSA solution in PBS (1X) and washed with a 0.05% Tween 20 (Fisher Scientific, Pittsburgh, PA) solution in PBS. A serial dilution was performed with the IL-8 standard and was run in duplicate. 1ul of each macrophage supernatant sample was added (WT, ΔNef, ΔEnv, ΔVpr,

ΔVif, and uninfected control for both day eight and day sixteen post-infection collection). All samples were run in triplicate. After a two-hour incubation at room temperature, the plates were washed, and an IL-8 detection antibody (biotinylated goat anti-human IL-8) reconstituted with a reagent diluent (0.1% BSA and 0.05% Tween 20 in 1X PBS) was added to each well. Following another incubation period and wash, Streptavidin-HRP conjugated to horseradish-peroxidase in a 1:200 dilution with the reagent diluent was added to each well. This allowed for the appropriate enzymatic activity to occur to aid in the subsequent detection of IL-8. After another wash, a substrate solution containing equal proportions of a hydrogen peroxide solution and tetramethylbenzidine (TMB) was then added to the plate (BD OptEIA TMB Substrate Reagent Set, BD Biosciences, San Jose, CA). After a short incubation period, H_2SO_4 was added to cease all reactions, and the plate was read using a microplate reader (BioTek, Winooski, VT). Data on optical density was exported to EXCEL (Microsoft, Redmond, WA) and analyzed to determine the average human IL-8 concentration (pg/mL) present in each sample at each time point.

3.6 SH-SY5Y DIFFERENTIATION

SH-SY5Y cells were grown in a T-75 flask until 80-90% confluent. Plates were coated with Poly-D-Lysine and incubated at 37°C for one hour prior to cell plating. This was done to improve the binding of cells to the plate to aid in the growth and elongation of neurons. SH-SY5Y cells were plated on the coated 6-well plates (1×10^5 cells/well) and allowed to grow and differentiate for a period of five days. D-3 media was used, instead of D-10, to reduce the proliferation of SH-SY5Y cells and encourage, instead, their differentiation into neurons. D-3 media was prepared using DMEM with 3% FBS, 1% penicillin, 1% streptomycin, and 1% L-

glutamine. To also aid in the differentiation of the cells, retinoic acid (RA) was added at a concentration of 10uM to cells. During the differentiation period, the media was changed and fresh D-3 media with RA was added every two to three days. After five days, 700 uL of D-3 media and 300 uL of macrophage supernatant exposed to each virus (Section 3.4) was added for a period forty-eight hours. After forty-eight hours, the media in the plates was discarded, and RIPA buffer was added to collect the neuronal cell lysate. The plates were kept on ice and placed on a shaker for fifteen minutes to allow for the lysing of the cells. The supernatant was then removed and centrifuged at 13,000 rpm for ten minutes at 4°C. The supernatant containing the lysed content from the neuronal cells was saved at -80°C until use.

3.7 WESTERN BLOT

Over the course of the project, a series of western blots were performed to confirm and compare the presence of specific proteins in samples. The same procedure outlined below was performed for all western blots, with only the antibodies used varying.

The protein in each sample was first quantified using a Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA). A 1:50 dilution of reagent B and A, respectively, was added to each well in a 96-well plate. A BSA standard (New England BioLabs, Ipswich, MA, 10 mg/mL) was diluted to 2 mg/mL and used to make a serial dilution. Next, 1ul of each sample to be tested was added to the plate in duplicate. The plate was allowed to incubate on a shaker at 37°C for thirty minutes. The plate was then read using a microplate reader (BioTek, Winooski, VT), and the data was exported to EXCEL for analysis (Microsoft, Redmond, WA). The volume of samples to load into the gel was calculated from this data and adjusted appropriately to ensure

the presence of equal amounts of protein (30 ug) for each virus type and the control. In addition to the sample, PBS (1X) and a loading dye were added to have a final volume of 31ul in each well. 15 uL of a protein ladder was also added to the gel (Prestained Protein MW Marker, ThermoScientific, Waltham, MA).

SDS-PAGE gels were made using a Bis-Acrylamide 40% solution (Fisher Scientific, Fair Lawn, NJ), Electrophoresis Grade Tetramethylethylenediamine (TEMED) (MP Biomedicals, LLC, Solon, OH), 10% ammonium persulfate (APS), 10% sodium dodecyl sulfate (SDS), and 1.5 M Tris HCl buffer (lower gel) or 0.5 M Tris HCl buffer (upper gel).

Gels were run using a Bio-Rad PowerPac at a constant voltage between 45V and 80V (Bio Rad, Hercules, CA). Transfer of the gels to a membrane was performed overnight at 4°C at a constant voltage of 15V to ensure complete transfer of protein to the membrane. Membranes were blocked using a 5% milk solution or a 5% BSA solution in phosphate buffered saline Tween-20 (PBST). Membranes were incubated in a primary antibody overnight, as described below. After washing with PBST, the membranes were exposed to the appropriate secondary antibody and washing was repeated. The membranes were exposed to a WesternBright enhanced chemiluminescence (ECL) solution (Advansta, Menlo Park, CA) and developed on film (Konica Minolta, Japan).

3.7.1 Western blot of 293T cell lysate

A western blot was first conducted to confirm the presence of HIV-1 in transfected 293T cells. This was done to confirm previous visualization of green fluorescence in cells, prior to continuing with additional experiments.

293T cells were grown and transfected with WT, ΔNef, ΔEnv, ΔVpr, or ΔVif virus (Section3.2.1). The cell lysate for each virus type as well as an uninfected control was harvested. A mouse monoclonal p24-gag antibody was used as the primary antibody in a 1:750 dilution (Cell Signaling Technology, Danvers, MA). A horse anti-mouse IgG antibody conjugated to horseradish peroxidase was used as the secondary antibody in a 1:5,000 dilution (Cell Signaling Technology, Danvers, MA).

3.7.2 Western blot of macrophage cell lysate

A western blot was also performed to confirm the presence of virus in the isolated macrophage cell lysate prior to exposing the macrophage supernatant to differentiated SH-SY5Y cells. This was done using a mouse monoclonal p24-gag primary antibody in a 1:750 dilution (Cell Signaling Technology, Danvers, MA). The secondary antibody used was a horse anti-mouse IgG antibody conjugated to horseradish peroxidase in a 1:5,000 dilution (Cell Signaling Technology, Danvers, MA).

A second antibody was used on the membrane to confirm the presence of cellular contents in the cell lysate. The blot was stripped using a solution of 0.5% SDS, 1% Tween-20, 1.5% glycine, and HCl, and then blocked in a 5% milk solution. It was then incubated in a mouse monoclonal α-tubulin primary antibody in a 1:1,000 dilution (Cell Signaling Technology, Danvers, MA). The secondary antibody used was a horse anti-mouse IgG antibody conjugated to horseradish peroxidase in a 1:5,000 dilution (Cell Signaling Technology, Danvers, MA).

3.7.3 Western blot of neuronal cell lysate

A final western blot was conducted to characterize the changes in neurogranin present in neurons that have been differentiated from SH-SY5Y cells. A rabbit polyclonal α-neurogranin primary antibody was used in a 1:5,000 dilution (EMD Millipore, Billerica, MA). The secondary antibody used was a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase in a 1:2,000 dilution (Cell Signaling Technology, Danvers, MA). A negative control was included that consisted of loading dye, PBS (1X) and the RIPA buffer used to lyse the neuronal cells. A positive control was also used that contained frontal cortex matter from an HIV-1 negative individual that has previously been demonstrated in the lab to contain Nrgn.

The blot was stripped after developing and reprobed with a mouse monoclonal α-tubulin primary antibody in a 1:1,000 dilution (Cell Signaling Technology, Danvers, MA). The secondary antibody used was a horse anti-mouse IgG antibody conjugated to horseradish peroxidase in a 1:5,000 dilution (Cell Signaling Technology, Danvers, MA). This was done to confirm the presence of cellular contents in the harvested neuronal cell lysate.

After developing the film for the Nrgn western blot, ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, MD) was used for analysis. Results were exported to EXCEL for quantification (Microsoft, Redmond, WA). Densitometry results for Nrgn were normalized for the expression of tubulin between each viral type studied.

4.0 RESULTS

4.1 INFECTIVITY ASSAY

4.1.1 293T Infectivity Assay

Table 1 depicts the results of the infectivity assay performed for the wildtype (WT) virus and each of the four viral protein deletion mutants (ΔVif, ΔVpr, ΔNef, ΔEnv). Data is displayed as the number of infectious particles per mL. As expected, the wildtype virus has the highest viral titer, with the greatest number of infectious particles present. ΔVif and ΔNef have the next highest titers and ΔEnv and ΔVpr have the two lowest. This information was taken into account in subsequent stages of the experiment when determining the amount of virus needed to infect macrophages.

Table 1. Estimation of virus titer

Virus	Titer (Infectious particles/mL)
WT	0.92×10^6
Δ Vif	0.82×10^6
Δ Vpr	0.50×10^6
Δ Nef	0.78×10^6
Δ Env	0.65×10^6

4.1.2 Macrophage Supernatant Infectivity Assay

After exposure of the TZM-bl cells to the macrophage supernatants, green fluorescent-positive infected cells were observed under a fluorescent microscope. The number of infectious particles observed in the macrophage supernatant was too low to determine an accurate viral titer. It is likely that there are viral proteins present in the supernatant, as the macrophages themselves were infected with HIV-1; however, there were an undetectable number of infectious virus particles present in the supernatant.

4.2 CYTOKINE PROFILE

Figure 2 displays the results of an ELISA specific for human IL-8 performed on the macrophage supernatants harvested day eight and day sixteen post-infection. The macrophage supernatants exposed to the WT virus and ΔVif virus have the highest concentrations of IL-8 in both day eight post-infection collection as well as day sixteen. For all of the virus types studied, the concentration of human IL-8 decreased between day eight and day sixteen collection, indicating that the concentration of the IL-8 cytokine in the macrophage supernatant decreased during a prolonged exposure to each of the HIV-1 types used. This is likely from the macrophages becoming overstressed or damaged from viral exposure.

Additionally, the uninfected control displayed the lowest initial concentration of human IL-8. This would be expected, as there is no virus present to stimulate an inflammatory response and the release of cytokines. However, in the supernatant collected on day sixteen of the study, the concentration of human IL-8 increased. This is the reverse of the trend observed with each of

the HIV-1 types. While a small increase in the amount of IL-8 would be expected as a basal accumulation of cytokines in an uninfected macrophage, we would not expect this large increase to be typical.

Results of the ELISA for IL-8 were comparable between both donors studied. The only difference observed was ΔVif having a lower IL-8 concentration in donor 2, to a level similar to the other viral mutants for both time points.

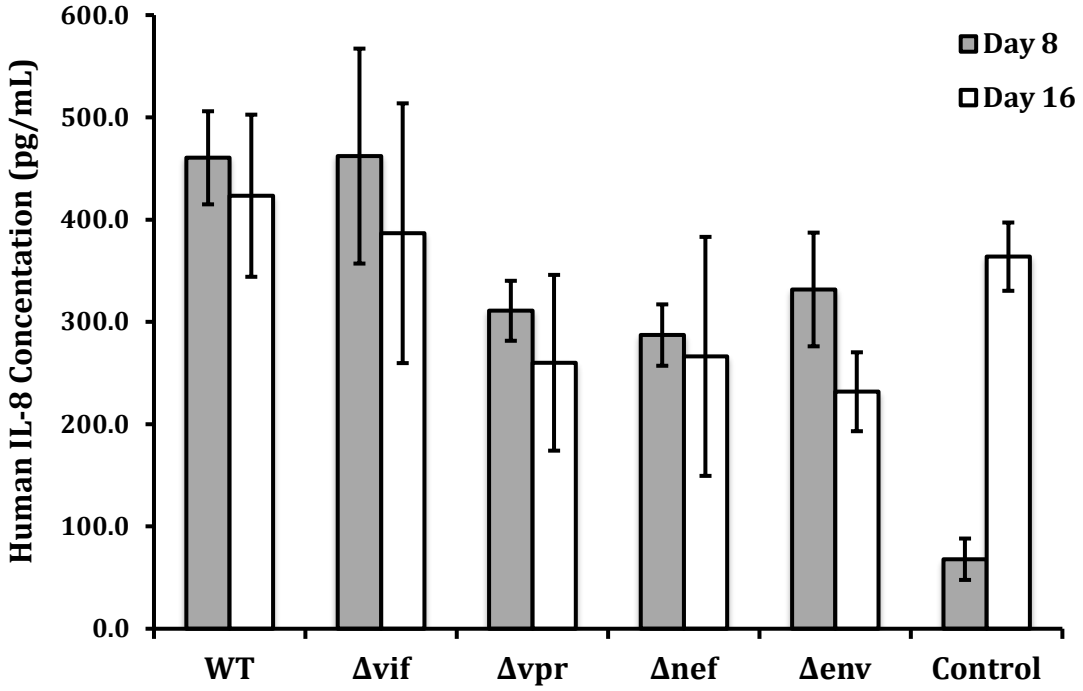

Figure 2. Concentration of human IL-8 in macrophage supernatant (Donor 1)

4.3 WESTERN BLOT

4.3.1 Western blot of 293T cell lysate

Figure 3 depicts the results of the western blot performed on 293T cell lysate to confirm the presence of HIV-1 in each sample prior to continuing with the experiment. The antibody used was specific for both the p24 and p55 viral proteins in HIV-1. The presence of bands for the two viral proteins in each of the virus types studied indicates the presence of HIV-1 in the samples. There were no bands present in the lane with the uninfected control cell lysate. This western blot confirmed the presence of HIV-1 in each virus sample and was sufficient evidence to continue with the experiment and utilize the virus samples in the infection of macrophages.

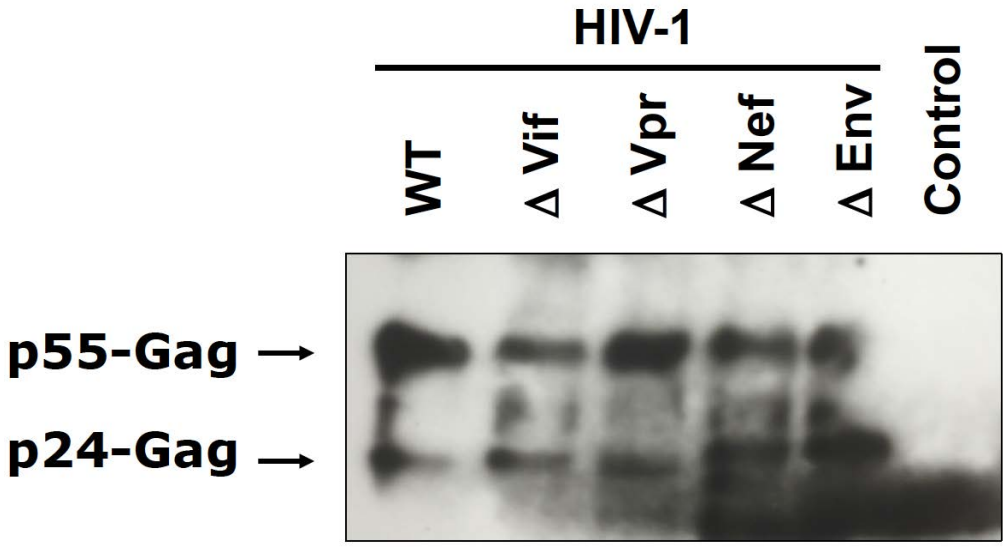

Figure 3. Western blot of 293T cell lysate for Gag

4.3.2 Western blot of macrophage cell lysate

Two western blots were performed on the harvested macrophage cell lysate after infection with each virus type. An uninfected control was also used in which macrophages were not exposed to HIV-1.

Figure 4 shows the results of the western blot performed with a p24-gag primary antibody. This was done to confirm the presence of the virus in the macrophage cell lysate. The bands are clearly visible in the lanes for each virus type (WT and all four viral protein deletion mutants) and not present in the lane with the uninfected control. These results were sufficient to indicate that the virus was able to infect the macrophages upon exposure. Similar results were observed in both donors.

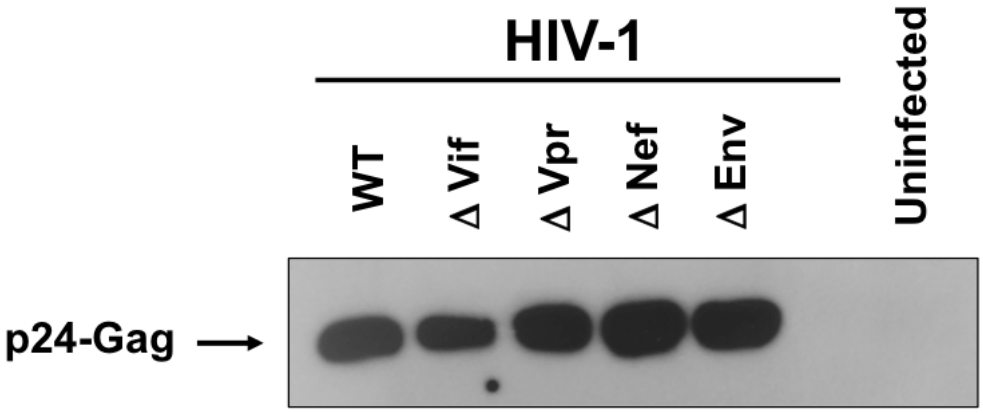

Figure 4. Western blot of macrophage cell lysate for p24-Gag

Figure 5 depicts the results of the blot probed with an α-Tubulin primary antibody. This was performed to confirm the presence of cellular contents in the samples harvested from the macrophage cellular lysate. From the developed blot, it is clear that tubulin is present in all viral samples as well as in the uninfected control. These results allowed us to conclude that the

harvesting of the macrophage cell lysate was successful and that the viral protein (p24) visualized in Figure 4 is in fact present in the macrophage cell lysate. Again, results were similar in the blot developed with donor 2 samples.

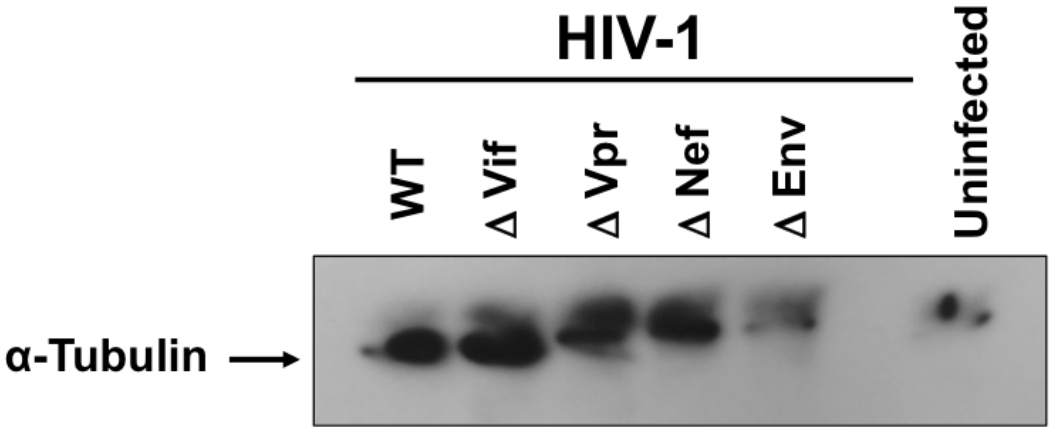

Figure 5. Western blot of macrophage cell lysate for tubulin

4.3.3 Western blot of neuronal cell lysate

The result of the western blot of neuronal cell lysate for Nrgn is depicted below (Figure 6). There appear to be visible differences in the concentration of Nrgn present in the neuronal cell lysate samples between different viral protein deletion mutants used. Specifically, the least amount of Nrgn is present in the neuronal cells exposed to the macrophage supernatant infected with the WT virus. This would be expected, as the WT virus has all viral proteins present and is likely the most effective at infection. HIV-1 ΔVpr appears to have the most Nrgn present as it has the darkest and largest band. This could indicate that Vpr is an important viral protein for the ability of HIV-1 to cause the dysregulation of Nrgn.

Additionally, the positive control used was positive for the presence of Nrgn and likewise, no Nrgn appeared with the negative control used. This validated the presence of Nrgn

in the neuronal cell lysate samples and confirmed that there was no Nrgn present already in the RIPA buffer used to lyse the neuronal cells.

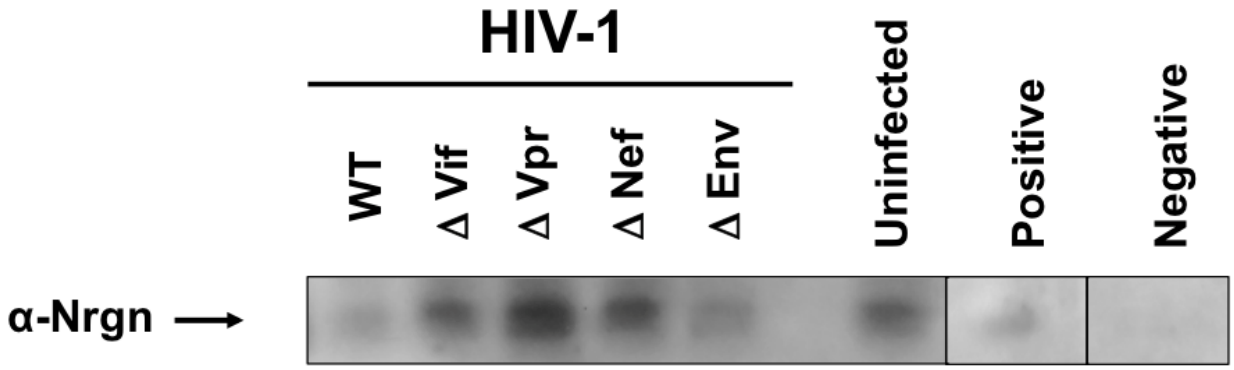

Figure 6. Western blot of neuronal cell lysate for neurogranin (Nrgn)

Figure 7 depicts the results of the western blot of the neuronal cell lysate after stripping and reprobing with an α-Tubulin primary antibody. Bands are present for each virus type as well as the uninfected control. This confirms the presence of cellular contents in the cell lysate harvested and shows that the Nrgn previously seen in Figure 6 was present in the neuronal cells.

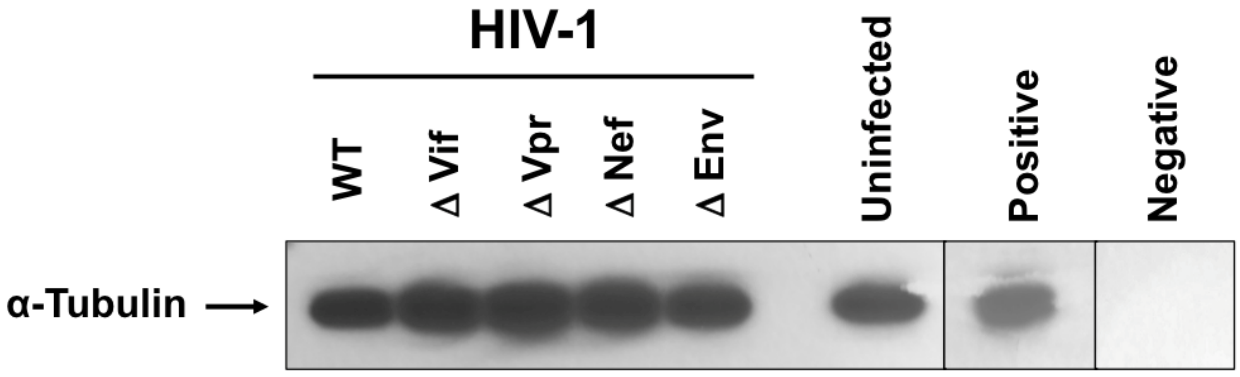

Figure 7. Western blot of neuronal cell lysate for tubulin

To obtain a more quantitative result of the western blot for Nrgn, ImageJ software was used to produce the graph below (Figure 8). The areas of the bands in the blots above were analyzed and their intensities were compared. To normalize the amount of Nrgn present between samples, the amount of tubulin in each sample was also evaluated. The uninfected control had the largest amount of Nrgn present and the WT virus had the least amount of Nrgn present. In Figure 6 above, the band for ΔVpr appeared to be larger and darker than the control band, which would imply that there is more Nrgn present in the ΔVpr sample than the uninfected control. However, when analyzing the amount of tubulin present, it was found that there was a larger amount of tubulin, and therefore cell lysate, in the ΔVpr sample. Once the data was normalized, taking into account the amount of tubulin, the relative amount of Nrgn in the ΔVpr sample was less than the uninfected control.

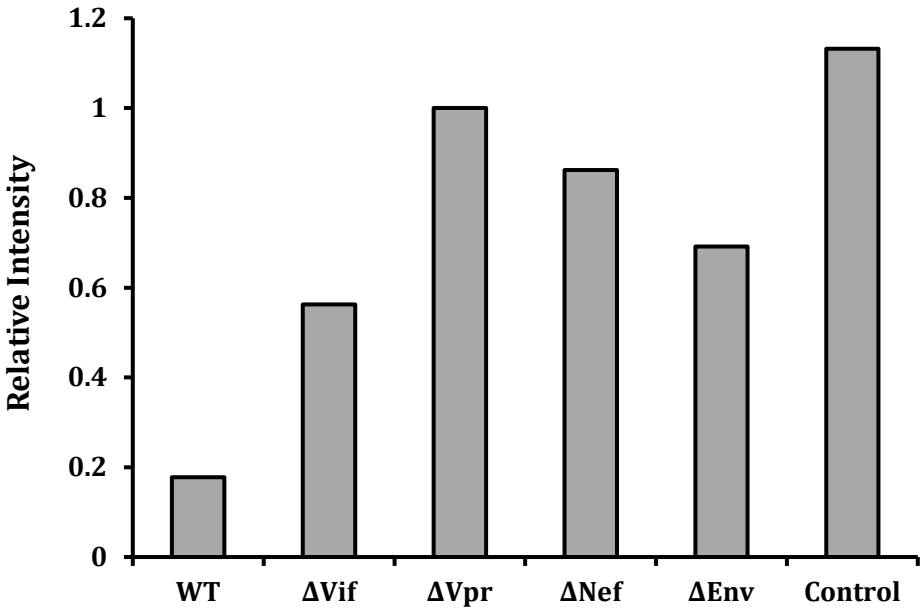

Figure 8. Neurogranin band intensities normalized to tubulin

Using the same output data from the ImageJ software, the percent reduction in Nrgn levels with each viral sample was determined (Figure 9). This was accomplished by comparing the area of the Nrgn band in each lane with that of the uninfected control, taking into account the amount of tubulin present in each sample. As expected, the WT virus had the largest degree of reduction in the amount of Nrgn present.

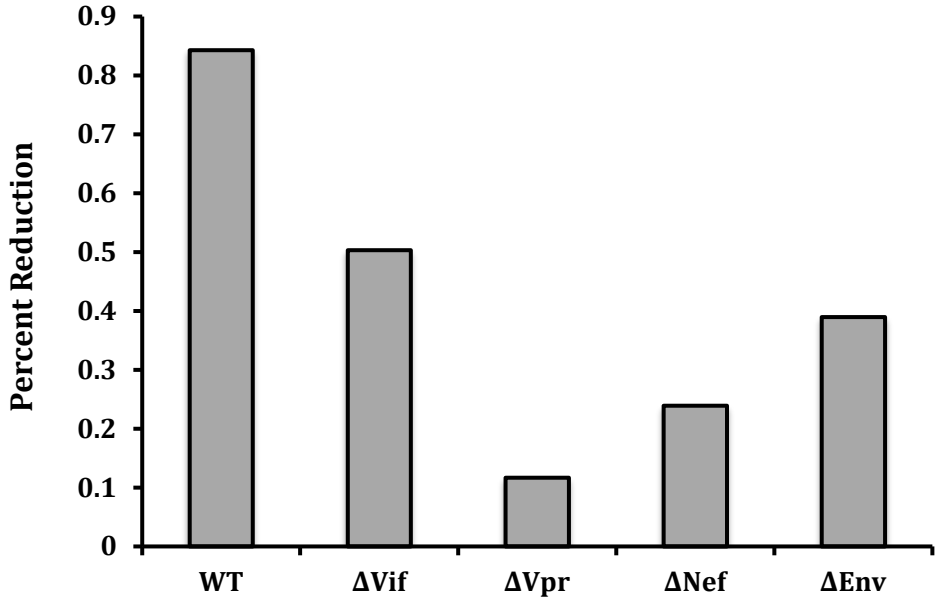

Figure 9. Reduction in neurogranin compared to uninfected control

The western blots developed with samples obtained from donor 1 at day sixteen and donor 2 at both time points showed inconclusive results. Nrgn was not present in the uninfected control lane as well as some of the viral mutants. The absence of Nrgn in the control lane invalidated the results of these particular western blots. Therefore, future studies are needed to repeat these tests and confirm the results depicted above.

5.0 CONCLUSION

The results of this study show promising evidence that Nrgn may be dysregulated in the neuronal cells of individuals infected with HIV-1. There are differences in the amount of Nrgn present in neuronal cells after exposure to HIV-1 WT as well as ΔVif, ΔVpr, ΔNef, and ΔEnv viruses. This implies that these viral proteins may have a direct role in the dysregulation of Nrgn and that some, in particular, may be responsible for a greater degree of dysregulation than others.

Likewise, there appear to be differences in the amount of the inflammatory cytokine IL-8 released from macrophages in response to infection by each of the virus types studied. This difference is likely a direct consequence of the specific viral proteins present or absent in each virus. The correlation between IL-8 levels present and the dysregulation of Nrgn can lead to potential conclusions about the impact of this particular cytokine on the protein Nrgn. The highest concentration of IL-8 was released in response to the WT and ΔVif viruses. Correspondingly, the largest degree of dysregulation of Nrgn was observed in the neuronal cells exposed to the WT and ΔVif macrophage supernatants. The differences in IL-8 concentrations between viral protein mutant types also indicate a potential indirect role of the viral proteins studied in the dysregulation of Nrgn.

Together, our results show evidence that Nrgn is dysregulated in neuronal cells exposed to HIV-1. This dysregulation appears to at least partially depend on the viral proteins present and the inflammatory cytokine, IL-8, released from macrophages in response to infection. However,

additional studies are needed to examine the effect of other cytokines, such as IL-1β, to add to these results.

While more research is needed to confirm the results of this study, these findings add to previous research and further demonstrate a link between the presence of HIV-1 and the dysregulation of Nrgn. This information provides additional evidence that Nrgn dysregulation should be studied to a greater degree, as a potential source of cognitive deficits seen in HIV-1 positive individuals.

6.0 DISCUSSION

Current diagnostic criteria for HIV-1 infection relies on blood tests to assess $CD4^+$ cell numbers and levels of HIV-1 RNA. These factors are used to determine the presence of infection, guide treatment decisions, and assess the effectiveness of treatment measures. There has been significant success with the advent of cART and HAART in achieving viral suppression in the plasma and prolonging the lifespan of individuals infected with HIV-1. [33-37] However, it is apparent that more focus needs to be placed on the associated comorbidities in HIV-1 CNS infection in both diagnosis and treatment plans. While there is evidence to show a decline in the presence of HIV-Associated Dementia, a more severe form of neurocognitive impairment, studies have shown that there is a continued presence of milder forms of neuropsychological deficits that continue to have devastating effects on those infected with HIV-1. More than half of HIV-1 positive subjects suffer from HAND. Even individuals well managed with cART and who have an undetectable viral load experience HAND, suggesting cART is unable to prevent neuronal dysfunction. [50-53]

An analysis of cognitive function should play a role in the determination of when to initiate treatment, what therapeutic drugs should be given, and the effectiveness of the prescribed treatment options. Additionally, therapies should be developed to target CNS reservoirs of virus and the inflammatory factors believed to play a role in cognitive morbidities associated with HIV-1 infection. As individuals infected with HIV-1 are living longer due to prescribed

HAART, cognitive deficits generally seen in an aging population are becoming more prevalent among individuals infected with HIV-1. It is likely that long-term and persistent infection with HIV-1 will only exacerbate these cognitive deficits and result in further impairments to the daily functioning of these individuals.

This study aims to provide support for the need of targeted therapeutics to help overcome the neurocognitive deficits seen in many individuals infected with HIV-1. By showing evidence for changes in neural proteins upon infection with HIV-1, it is clear that a heavier focus needs to be placed on the CNS in the context of HIV-1 infections. By increasing our understanding of the role of viral proteins and inflammatory factors in Nrgn expression, we will have a better understanding of how HIV-1 dysregulates Nrgn in the brains of infected individuals and may illuminate potential therapeutic targets to reduce the development of HAND.

6.1.1 Limitations

While the results of this study do show a potential dysregulation in Nrgn levels upon infection with HIV-1, repeat studies need to be performed before these results can be confirmed. Two donor PBMC samples were used in this study, however the final results of the second donor were inconclusive. In the western blot for Nrgn, bands for Nrgn were present for some virus types, but not others. Likewise, a Nrgn band did not appear in the uninfected control. The reason for this is unknown and is likely the result of an error in the lab techniques used. For this reason, this study will need to be repeated with additional blood donor samples to confirm these results.

With additional studies, a more intensive investigation into the surrounding inflammatory environment should be performed to evaluate the levels of additional cytokines present (e.g. IL-1β). This would allow us to further evaluate the role of inflammatory factors in the dysregulation

of Nrgn and the correlation between viral proteins and the levels of cytokines and chemokines. In these repeat studies we will also evaluate the levels of cytokines in the uninfected control macrophages to confirm or disprove the increase in IL-8 seen in the uninfected control between day eight and day sixteen post-infection collections.

6.1.2 Future Aims

After the identification of viral and immune factors that alter Nrgn expression in HIV-1 infected cells, we seek to overexpress Nrgn using gene therapy approaches. This will help to reduce neuronal dysregulation, preserve neuronal plasticity, and reduce the risk of HAND development. This work will further contribute to the growing field of knowledge surrounding Nrgn and will help in the development of more targeted therapeutic techniques for HIV-1 treatment.

From a public health perspective, in addition to advancing our scientific knowledge related to neurogranin and other potential markers for the development of HAND, it is important to also focus on improving methods of testing for cognitive impairments and education of clinicians about the symptoms of HAND. This is especially important for lower resource regions that do not have access to advanced diagnostic techniques and do not have a sufficient number of highly skilled clinicians to perform intensive cognitive testing.

Together, an increased background of knowledge related to HIV-1 CNS infection and changes in the practices of health care providers, it will be possible to bring increased awareness to the presence of HAND and work towards developing thorough diagnostic and treatment plans.

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