CHARACTERIZATION OF A NOVEL HOST CELLULAR FACTOR INVOLVED IN HIV-1 NEUROPATHOGENESIS

by

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ABSTRACT

HIV-1 causes progressive neurological disorders collectively known as HIV-associated neurocognitive disorders (HAND). HAND is considered an umbrella term that encompasses three major stages: Asymptomatic neurocognitive impairment (ANI), mild/ moderate neurocognitive disorder (MND), and HIV-associated dementia (HAD). The prevalence of HAND has increased despite the use of combined antiretroviral therapy (cART) and still affects 20 – 40% of HIV-1 infected individuals. The underlying cause of HAND observed in a particular set of subjects has not yet been fully elucidated nor have there been any ways to determine if an infected individual will eventually progress to HAND.

Previously we have shown that Neurogranin (NRGN), is significantly downregulated in patients with high viral load at the mRNA level. NRGN is involved in the protein kinase C pathway and binds to calmodulin (CaM) and decreases the threshold for long-term potentiation (LTP), which is involved in learning and memory. We hypothesize that dysregulation of NRGN may be in part associated with development of HAND. To further understand the role of NRGN, we propose to delineate the role of NRGN and the mechanism(s) involved in HIV-1 induced degradation of NRGN.

The public health significance is in determining contributors to the degradation of cognition in HIV-1 infected individuals that progress to HAND, treatments can be developed to combat the effects on HIV-1 on proteins and cells pertinent to normal brain function.

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PREFACE

I would like to thank the University of Pittsburgh. It seems weird, but by choosing to come to Pitt, I gained a lot more than just a couple of degrees. I learned what I liked about the general world of science and what I don't like. I'm sure I could have found that out at any university, but I feel that Pitt has such a vast array of avenues in science with people of all ages doing so many different things and willing to teach younger generations of researchers, medical doctors, and health and research lawyers that I think here is the perfect place to be if you're not exactly sure of what impact you want to make in the science world.

I also want to thank one of my favorite scientists that I know – my advisor, Dr. Velpandi Ayyavoo. Many of you that have heard me rant about her may think that I dislike her, but she is probably one of the best advisors I've ever had. If it weren't for her slight kicks in the butt (figuratively) every couple of months, I probably would have given up on this project and wanted to do something else. She is truly like my lab mother – much like my real mother – she doesn't take crap and will make you work until you have something worth showing that you're actually proud of. Anytime I had a question or had new ideas on my project, she was there to listen or would immediately respond to my e-mails. What I think makes her an amazing advisor is that she won't hold your hand – either you want to do something and get it done or you don't – just don't waste her time. I honestly must say that is probably the best thing I super appreciate about her.

Next, I absolutely have to thank my lab. Jeez, my lab – Leah Walker, Jessica Sparks, Allison Mancini, Dr. Debjani Guha, Dr. Lily Francis and Dr. Jay Venkatachari – when everything went wrong they saw my tears, my anger, my frustration, and me hiding from

VA. I feel so much more confident as a researcher because of the new techniques I learned as well as the fine-tuning to the lab techniques I already knew. Thanks Jess for the viruses! These are very likely the people I will talk about for ages when I think about my old labs.

A massive thanks to my family and especially my parents! Mary and Andrew are the best! They may not always get what I'm talking about or what I'm trying to do with things, but they support me with all of their might!

And finally, I want to thank the many friends I've made since I came to Pitt in 2006. Between the restaurants, the gyms, the retail stores and many other places I've worked, I've made so many friends along the way and they are all so different that it has definitely kept me open and welcoming to those from all career paths and walks of life.

Thank you guys, you're all too awesome for words, honestly.

1.0 INTRODUCTION

Human immunodeficiency virus type 1 (HIV) infection affects an estimated 40 million people worldwide, and the virus is associated with disruption of the central nervous system (CNS) in roughly 30% of individuals infected with the virus[1]. HIV-1 virus enters the CNS from the peripheral circulation via infected macrophages. This infection of the CNS is an early event that occurs during the acute phase of the infection[2]. This results in neurological complications in a significant number of individuals with HIV-1[3]. The primary cells that are infected with HIV-1 within the CNS are macrophages, microglia and a small proportion of astrocytes, however the astrocytes do not support productive infection of the virus[4].

HIV-1-associated Neurocognitive Disorders (HAND) affects ~40 – 60% of HIV-1 patients who are not on cART and ~20%-40% of those on cART. HAND occurs when HIV enters the CNS and impacts the integrity and health of specific cells within the brain. This impact of HIV-1 on the neuronal cell lineages in CNS can impair the activity of cells involved in attention, memory, language, problem solving, and decision-making – activities collectively known as cognition[5]. There are 3 stages of HAND: Asymptomatic neurocognitive impairment (ANI), mild/moderate neurocognitive disorder (MND), and HIV-associated dementia (HAD). Of these three, HAD is the most severe but MND is the most common type of HAND among HIV-1 patients[3, 6-9].

Host cellular gene expression as a response to viral infection is directly correlated with disease patterns. Previous studies in our laboratory have shown that one of the host cellular factor that was found to be affected by HIV-1 infection (significantly downregulated) in patients with high viral load was neurogranin (NRGN)[10].

Neurogranin (NRGN) is a synaptic gene that spans 7.3kb of genomic DNA and contains four exons that transcribe a protein of 78 amino acids, which translated to about 17kDa protein [11]. NRGN is present primarily in neurons and cells of neuronal lineages and accumulates predominantly in the dendritic shafts and spines of neurons. It is abundantly expressed in the cerebral cortex, hippocampus, basal ganglia, amygdala, and the hypothalamus[12]. NRGN is a cellular factor that plays a role in the calcium-calmodulin signaling pathway and has been suggested to play a role in learning and memory and acts as a substrate for protein kinase C (PKC) [13-16]. Currently, detection of NRGN in cerebral spinal fluid (CSF) is used as a biomarker for Alzheimer's Disease (AD)[17].

NRGN is involved in synaptic plasticity and long-term potentiation (LTP) and is currently characterized as a synaptic gene[18, 19]. LTP is an important process involved in learning and memory, which are key parts of cognition that are affected by HIV-1 infection in the brain and synaptic degeneration [19, 20]. However, the interaction between HIV-1 and NRGN and the downstream effects of HIV-1 infection on the expression and degradation of NRGN is not well defined. The work performed here is to define the role of HIV-1 - NRGN interaction and its effects using an *in vitro* model.

2.0 BACKGROUND

2.1. VIRAL GENOME ORGANIZATION

The HIV viral genome consists of structural and enzymatic genes (*gag*, *pol*, and *env*), two regulatory elements (*tat* and *rev*), and several accessory genes (*vif*, *vpr*, *vpu* and *nef*) that are flanked by long terminal repeats (LTRs)[21, 22]. The 5'LTR serves as a promoter to signal for viral gene expression. The signals include sequences that allow for transcription initiation, termination, and poly-adenylation, and elements that direct reverse transcription.

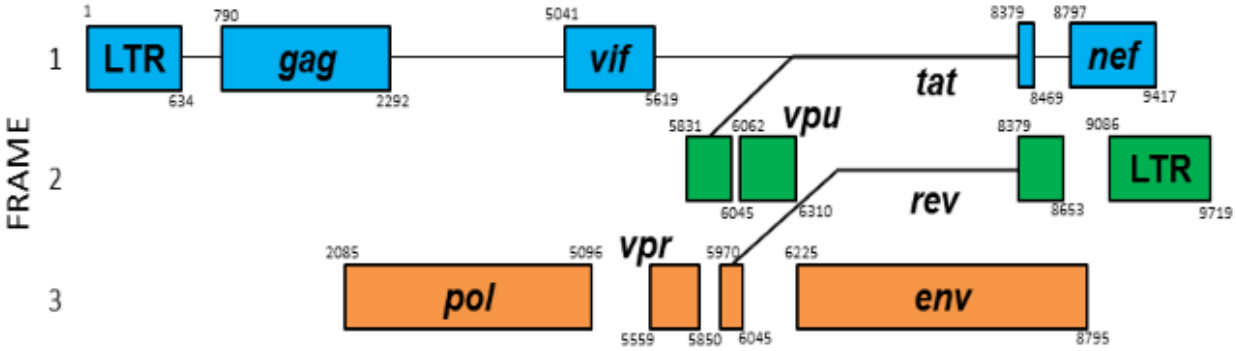


Figure 1. HIV gene organization.

The genomic organization schematic in Figure 1 shows reading frames necessary for the production of the viral proteins[23]. The largest genes – *gag*, *pol*, and *env* – encode structural proteins of the virus particles. *gag* transcription makes the Gag poly-protein, which is cleaved to produce the matrix (MA), the capsid (CA), and the nucleocapsid (NC) proteins that encapsulate the virion. *pol* transcription results in enzymes that have multiple functions, including integrase (IN), protease (PR), and reverse transcriptase (RT) with RNase H activity[24]. Transcription of *env* leads to the formation of the envelope protein present in the viral membrane. The transcribed proteins form a hetero-trimer that binds to

the host CD4 glycoprotein and the chemokine co-receptor (CXCR4 or CCR5) to allow for viral entry into the cell. A *gag-pol* poly-protein is also produced in low levels, indicated by the presence of pol enzymes in the virion structure.

Tat and Rev are required for viral transcription and protein translation. After viral integration, low levels of viral RNA transcripts are primarily produced, which produces low levels of Tat. Tat enhances the rate and efficiency of viral transcription via binding to a RNA hairpin structure at the 5' end of viral transcripts – the trans-activating response element (TAR). When bound to TAR, Tat then interacts with RNA Polymerase II, therefore upregulating polymerase efficiency[25]. Rev controls the nuclear export of unspliced and partially spliced viral transcripts, regulating the production of viral genomes and structural proteins. Without Rev, viral transcripts remain in the nucleus and are fully spliced – only producing Rev, Tat, and the accessory protein Nef. Rev works by binding to the Rev response element (RRE), a 351-nucleotide RNA sequence encoded within the unspliced *env* gene[26, 27]. When Rev binds to the RRE, it exports unspliced and partially-spliced viral transcripts into the cytosol before complete splicing, allowing for the translation of structural proteins and viral RNA genome for virion incorporation[28].

The accessory genes of HIV-1, which include *nef*, *vif*, *vpr*, and *vpu* are not completely necessary for viral infection but do play a major role in augmentation of infection and replication efficiency. *Nef* aids in the degradation of CD4 glycoprotein, in addition to interruption of cellular signal transductions; *Vif* interrupts antiviral activity of APOBEC3G, a host restriction factor; *Vpr* regulates nuclear import and is present in the pre-integration complex; and *Vpu* aids in the degradation of CD4 and tetherin in infected cells[29-32].

2.2. HIV-1 VIRAL ENTRY AND ESTABLISHMENT OF INFECTION

The human immunodeficiency virus-1 (HIV-1) belongs to the *Lentivirus* genus of the Retroviridae family. The HIV-1 virion contains structural proteins, two single-stranded RNA molecules, and several enzymatic proteins necessary to establish infection. The capsid of the virion is enveloped with a lipid bilayer, which also contains the viral envelope proteins. HIV-1 infects its target cells through the use of gp120, the surface viral envelope glycoprotein. gp120 interacts with the CD4 antigen present on target cells, along with one of two potential chemokine co-receptors: CCR5 or CXCR4. The interactions between host CD4 and viral gp120 stabilizes the virus-host binding to facilitate secondary interactions between gp120 and the co-receptors. These interactions facilitate a conformational change in the viral transmembrane protein gp41 that allows for fusion to the membrane and entry of the capsid into the host cell [33-36].

Upon entry of the capsid into the cytosol of the host, the preintegration complex (PIC) is formed. This complex is composed of accessory protein viral protein R (Vpr), reverse transcriptase, capsid gag proteins (matrix proteins and p24), integrase, and two copies of the single-stranded RNA viral genome[37]. When the PIC is released into the host cytosol the process of reverse transcription begins, resulting in a double-stranded proviral DNA product that is capable of integration. The PIC then moves through the nuclear envelope and enters into the nucleus by way of active transport through the nuclear pore complex. When the PIC enters the nucleus, the proviral DNA is integrated into the host genome, allowing for initiation of virus production and establishing infection[38, 39].

2.3. AIDS EPIDEMIC AND HAND PROGRESSION

Globally, there are currently an estimated 35.3 million individuals infected with HIV -1 and millions of new cases reported yearly[40]. HIV-1 virus primarily infects two target cells of the immune system – macrophages and CD4+ T cells. CD4+ T cell count is used as a marker for HIV-1 progression[41].

Natural HIV infection is characterized by three major stages – acute infection (Stage 1), clinical latency (Stage 2), and Acquired Immune Deficiency Syndrome (AIDS) (Stage 3). Presently, most infected individuals infected with HIV-1 are treated with cART and therefore progression to AIDS is minimum[42-44]. The acute infection stage occurs 2-4 weeks after infection and during this time, the virus replicates and as the viral load increases, the CD4+ T cell count decreases until the viral set point is reached. At the viral set point, the immune system responds and stabilizes the level of the virus and increases the CD4+ T cell count minimally. The clinical latency stage, based on the infected individual's disease progression, lasts from 8 – 20 years and is the stage at which the virus replicates at low levels and the infected individual can show a healthy CD4+ T cell count and undetectable viral load levels. It is towards the end of this period in which the virus begins to rapidly replicate, causing a decrease in bystander CD4+ T cells[45]. When the CD4+ T cell count decreases below 200 cells/mm^3, the infected individual is then diagnosed with AIDS. The individual will in time succumb to opportunistic infections that a healthy individual would normally be able to fight off[42, 46].

Currently, there is no cure for HIV-1, but there are treatments available to combat viral replication within the body in the form of antiretroviral treatment. Antiretrovirals are separated into six different classes based on the way the drugs interfere with certain stages

of HIV-1 replication within the body[47]. The first class of these drugs is the entry inhibitors, which interfere with the ability of the virus to bind to receptors on the outer surface of the cell, as it attempts to enter. With failed receptor binding, the virus cannot infect the cell. The second group, the fusion inhibitors, interferes with fusion of the virus to the cellular membrane, which keeps the virus from entering the cell. Reverse transcriptase inhibitors prevent reverse transcription – a process in which the HIV enzyme reverse transcriptase (RT) converts single-stranded HIV RNA into double-stranded HIV DNA. Integrase inhibitors block the viral enzyme integrase, which the virus needs to integrate its genetic material into the DNA of the infected cell. Next, protease inhibitors interfere with the HIV-1 enzyme protease, which processes HIV-1 precursors and polyproteins into smaller individual proteins. With the protease inactive, new virus particles cannot be assembled. The last group is the multi-class combination products, which combine HIV-1 drugs from two or more classes into a single product[47].

HIV-1 has the ability to affect the brain by way of passing the Blood-Brain Barrier (BBB)[48]. Early in the course of HIV-1 disease progression, infected macrophages cross the BBB[49] and establish infection within the brain. Microglia, macrophages and astrocytes are major HIV-1 targets in the brain, whereas HIV-1 infected neurons have been rarely observed, suggesting that indirect mechanisms may account for the severe neuronal damage observed in patients with HAND[50-52].

Soluble viral proteins, such as gp120, Vpr, and Tat, are also known to be potent neurotoxins, which contribute to neuronal death and the onset of HAND in patients [53]. The neurocognitive impairment caused by HIV-1 infection is HIV-associated Neurocognitive Disorders (HAND). HAND occurs when the HIV-1 virus enters the central nervous system

(CNS) and compromises the health of the neurons[5]. Virus particularly impairs the brain cells involved in attention, language, memory, decision making, and problem solving – activities collectively known as cognition[48]. There are many symptoms of HAND, such as forgetfulness, headaches, gradual weakening and loss of feeling in extremities, and confusion[5].

HAND has three stages in which an infected individual can progress through. The first of which is the Asymptomatic Neurocognitive Impairment (ANI), in which the infected individual shows cognitive impairment, but the impairment does not hinder everyday function. The second stage is Mild Neurocognitive Disorder (MND), where the affected individual shows impairment and there is a mild interference in everyday function. The third and last stage of HAND is HIV-1 associated Dementia (HAD). Individuals that have progressed to HAD present severe impairment in information processing and new information retention that greatly impact the daily life of the individual[5, 54]. ANI and NMD affect 20 – 40% of HIV-1 infected individuals where HAD has become less common with the advent of Highly active antiretroviral therapy in 1996 (Figure 2)[54, 55].

The prevalence of HAND is significant in patients with well-controlled HIV-1 and this prevalence has been associated with decreased quality of life, increased mortality, and poor adherence to treatment. This poor adherence to treatment causes concern as it could lead to drug resistance[56]. The cause of HAND as well as its prevalence despite the effectiveness of HAART is currently unknown, but there are many theories on the contributors to the development of HAND.

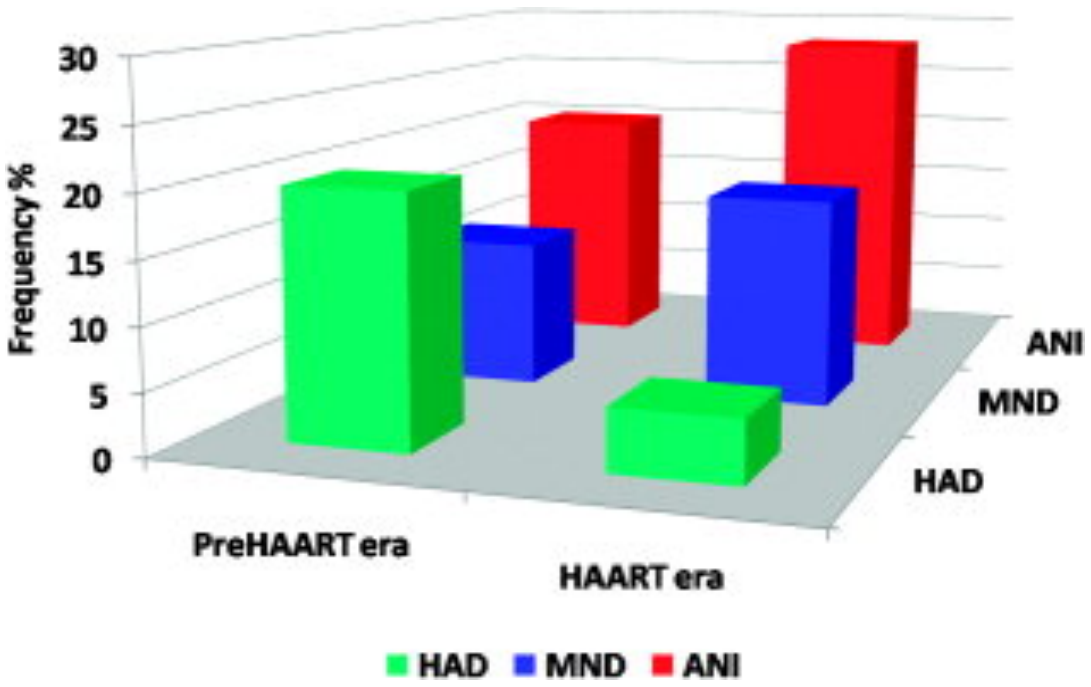


Figure 2. Prevalence of HAND comparing the preV HAART era to the current era of widespread HAART usage.

Reprinted with permission from Wiley Publishers Ltd: ANNALS OF NEUROLOGY, Human immunodeficiency virus-associated neurocognitive disorders: Mind the gap, 2010 [55].

2.4. POTENTIAL CONTRIBUTORS TO HAND

Though the advent of HAND has decreased the incidence of HAD, ANI and MND still continue to affect 20 – 40% of HIV-1 infected individuals on HAART[5]. Although the incidence of HAD has decreased, its prevalence is actually increasing, due to the longer life expectancy for individuals with HIV-1 and to patient resistance to HAART drugs[57]. While neuronal cell damage and death are associated with the development and progression of HAND symptoms, HIV does not directly infect neurons [57]. The cause of neuronal cell death

and dysfunction and HAND is currently not fully defined, both direct and indirect effects are reported. Some of these explanations include that there is accelerated brain aging due to increased β-amyloid and tau depositions facilitated by HIV-1 infection, toxicity of HAART on the CNS, inadequate/partial entry of HAART in the CNS, and depletion of neuronal cells, and the HIV-1 envelope protein, gp120, elevates intracellular free calcium, leading to neuronal damage[56, 58-61].

Numerous studies have provided a strong role for inflammation in triggering events leading to neurodegeneration in HIV infection. The association between the increase of activated macrophages/microglia in the CNS, neuronal damage, and cognitive dysfunction suggests that neuroinflammation resulting from systemic immune activation and/or inflammation triggers the neurodegeneration observed in HAND[62].

Neurons are vulnerable to direct damage by several viral proteins – particularly Tat and gp120. This is mainly mediated through the presence of the neuronal cell surface receptors, particularly the *N-methyl-D-Aspartate* receptors (NMDAR), dopamine transporter, and the chemokine receptors CCR5 and CXCR4. Glutamate is the most abundant neurotransmitter in the brain, and is responsible for spreading excitatory signal transmission by binding to NMDAR and opening cation-specific channels in the cell membrane, allowing the influx of sodium and calcium ions and the efflux of potassium ions[63]. The two major viral proteins that interact with the above receptors to cause neuronal injury are gp120 and Tat. HIV-1 gp120 directly binds NMDAR on human embryonic neurons and can cause a lethal influx of calcium ions (Figure 3)[64].

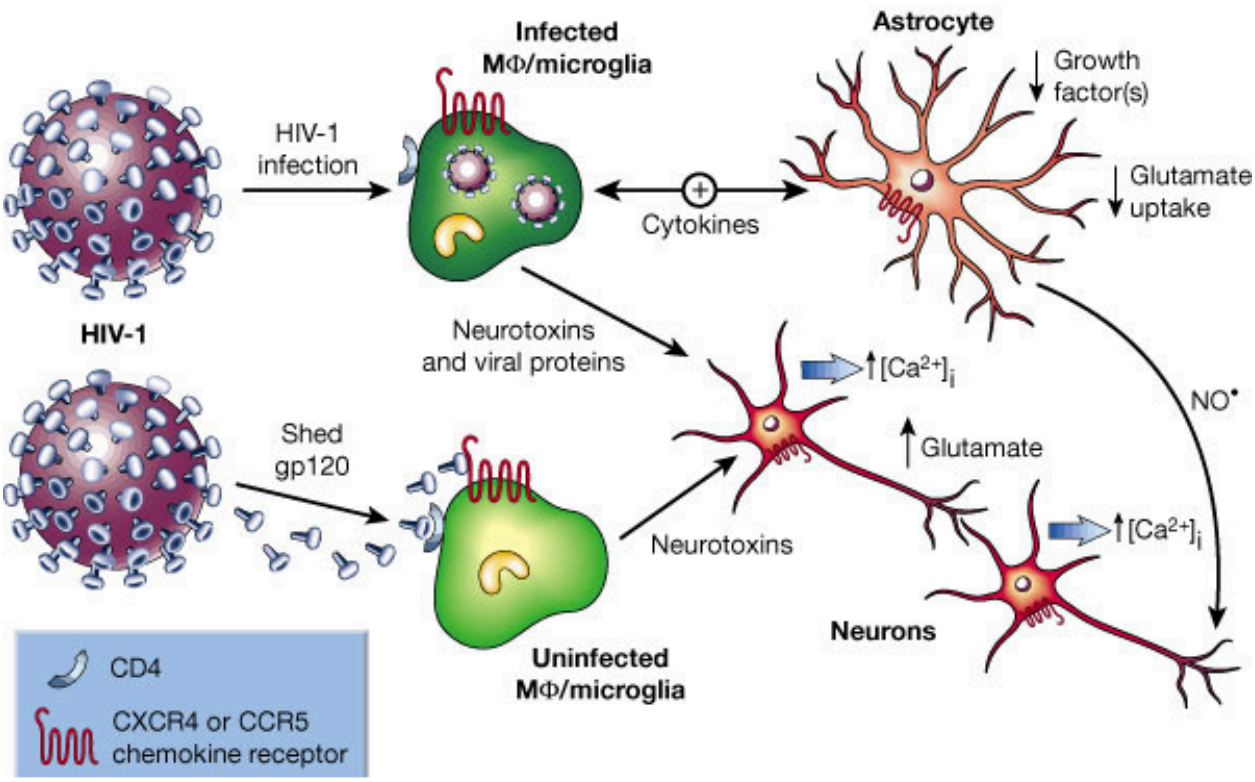


Figure 3. Model of HIV-1 related brain damage.

Reprinted with permission from Macmillan Publishers Ltd: NATURE, Pathways to neuronal injury and apoptosis in HIV-associated dementia, 2001[65].

2.5. NEUROGRANIN

Neurogranin (NRGN) is a synaptic gene that spans 7.3kb of genomic DNA and contains four exons that transcribe a protein of 78 amino acids, which translated to ~17kDa[12, 13]. NRGN is present primarily in neurons and cells of neuronal lineages and accumulates predominantly in the dendritic shafts and spines of neurons and is abundantly expressed in the cerebral cortex, hippocampus, basal ganglia, amygdala, and the hypothalamus[66]. NRGN is a member of the calpacitin protein family, a class of proteins whose role is to control the level and activation state of calmodulin[67]. The calpacitin

proteins are small, abundantly expressed proteins that bind to the Ca^{2+}-free form of calmodulin (CaM) with an affinity equal to or greater than that of the Ca^{2+}-containing form[12, 13, 68, 69]. The most characterized proteins of this class are Purkinje cell protein 4 (PEP-19), neuromodulin (Nm), and neurogranin (NRGN)[69]. NRGN is a cellular factor that plays a role in the calcium-calmodulin signaling pathway and has been suggested to play a role in learning and memory and acts as a substrate for protein kinase C (PKC), specifically the PKC isoform γ (PKCγ)[13, 14, 70, 71].

NRGN has been shown to be involved in synaptic plasticity and long-term potentiation (LTP) and is currently characterized as a synaptic gene [19]. In the process of LTP, NRGN is phosphorylated by PKCγ[72-74]. Once, phosphorylated, NRGN dissociates from Ca^{2+} and localizes to the nucleus where its function is not yet understood[75, 76]. LTP is a very important process involved in learning and memory, which are key parts of cognition that are affected by HIV-1 infection in the brain and synaptic degeneration [19, 20]. LTP can be produced by activating *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, by the synchronized activity of pre- and postsynaptic neurons[18].

Degradation of NRGN has been linked to neurocognitive disorders, such as Alzheimer's Disease[77-81]. Currently, detection of NRGN in cerebral spinal fluid (CSF) is used as a biomarker for Alzheimer's Disease (AD) as degraded NRGN from the neurons are released into the CSF[17, 82]. Individuals infected with HIV-1 and have a high viral load show a downregulation of NRGN[10]. It is currently unclear if these individuals could progress to HAND, and whether degradation of NRGN could be a potential contributor to this disorder.

It would be ideal to study the interactions between HIVA 1 infection in the brain and its effects on the production/degradation of NRGN and how, if at all, it contributes to HAND.

Investigating how NRGN is degraded and/or downregulated in infected individuals could gain insight in possibly predicting the development of HAND in infected individuals as well as developing therapies to curb the degradation or fully combat it.

2.6. microRNAs (miRNAs)

MicroRNAs (miRNAs) are a class of recently discovered 22–24 nucleotide RNA molecules that bind in an imperfect complementarity to their target messenger RNAs (mRNAs) within the 3′A UTR (untranslated region). miRNAs are conserved across species, expressed across cell types, and active against a large proportion of the transcriptome[83]. This binding to target mRNAs can prevent them from being translated into proteins[84, 85]. Since their discovery, much progress has been made in understanding their biogenesis and target site recognition within their target mRNAs[86, 87]. Currently, it is still unclear how miRNAs repress gene expression but what available data suggest that there are interrelated processes involved in miRNAA mediated repression of gene expression. It has been shown that most miRNAA repressed mRNAs are subject to deadenylation and mRNA destabilization[87, 88]. These events appear to be in addition to translational repression, since mRNAs that do not have a poly(A) tail (they are substituted with a histone stem loop) are still subject to miRNA-mediated translational repression without mRNA destabilization[88]. Recently it has been shown that translational repression occurs prior to deadenylation and destabilization[89].

HIV-1 infection mRNA analysis has show that miRNA binding to the 3'UTR regions of mRNAs can either upregulate or downregulate particular protein production[10].

Previous studies in our lab show that there is a potential interaction between mir-564 and the 3'UTR of NRGN that downregulate the production of the protein[10].

3.0 THESIS AIMS

The following are the specific aims designed to investigate how HIV-1 infection impacts the levels of NRGN present in neurons, possibly becoming a contributing factor to HAND.

Aim 1: Characterization of endogenous and over-expressed NRGN in neuronal cell lines.

A. Determine the endogenous expression in neuronal cell lines by performing immunoblot against NRGN and qRT-PCR to determine mRNA transcript levels.

B. Clone and express NRGN-with tag (FLAG) to determine localization of the protein within neuronal cell lines.

C. Localization of endogenous NRGN in neuronal cells using immunofluorescence staining of NRGN.

Aim 2: Examine the effect of HIV-1 infection/exposure on NRGN expression in neuronal cell lines

A. Determine whether neuronal cell lines express HIV-1 receptors and co-receptors by flow cytometry.

B. Infection and exposure of neuronal cell lines with chimeric reporter viruses previously constructed in our lab.

i. NL-YU2-env (CCR5 receptor utilizing virus isolated from the brain)

ii. NL43 (CXCR4 co-receptor utilizing virus)

Aim 3: Determine if NRGN is degraded by HIV-1 infection

A. Using a 3'UTR-NRGN-Luc transfected into HeLa cells to determine if degradation occurs at the 3'UTR of NRGN.

4.0 MATERIALS AND METHODS

4.1. CULTURING OF NEURONAL CELL LINES

The A172 cell line is a brain glioblastoma cell line. This astrocytic tumor cell line that is known to hypoexpress NRGN[90]. NA TTERA 2/D1 (NT2) cell line is a clonally derived pluripotent human embryonal carcinoma cell line isolated from the lung of patient and cultured using retinoic acid to a neuronally committed human teratocarcinoma cell line. [91]. The SH-SY5Y cell line was derived from original cell line, SK-N-SH which was isolated from a four year-old female with neuroblastoma [92]. Neuronal cell lines A172, SH-SY5Y and NT2 cultures were maintained as confluent monolayers at 37°C with 5% CO_2 and 90% humidity in D-10 media (DMEM with 10% fetal calf serum, 20mM HEPES, 2mM L-glutamine).

4.2. CONSTRUCTION OF NRGN WITH FLAG TAG

Forward and reverse primers were determined to PCR amplify NRGN cDNA and obtained from IDT (Coralville, Iowa). The reverse primer was designed to include sequence for the FLAG tag. pcDNA™3.1/V5A His TOPO® TA Expression Kit (Life Technologies) was used to ligate the DNA into pcDNA3.1/V5A HisA TOPO® vector. The ligation was transformed into TOP10 competent cells (Life Technologies) and plated on 100µg/ml ampicillin plates.

4.3. CONFIRMATION OF NRGN-FLAG/NRGN CLONES

Clones were confirmed by performing boiling lysis prep to attain the DNA of transformed TOP 10 cells. The DNA was collected and a restriction digest was performed using EcoRI and XhoI for the pNRGNA FLAG tagged clone and BamHI for the pNRGN

clone. DNA used for restriction digest was yielded using a boiling prep method. Mini preps (Qiagen) were done on the clones that showed a band size of ~330bp and the prepped DNA was sent for sequencing. The clones that had the proper DNA sequence were then grown in E.coli culture and the DNA harvested via maxiprep (Qiagen) and stored.

4.4. WESTERN BLOT ASSESSMENT FOR NEUROGRANIN (NRGN)

Whole cell lysates were prepared using RIPA buffer (containing 50mM Tris (pH 7.5)), 150mM NaCl, 1% Triton XA 100, 1mM sodium orthovanadate, 10mM sodium fluoride, 1mM phenylmethylA sulfonylfluoride, 0.05% deoxycholate, 10% SDS, 0.07 trypsin and protease inhibitors 1μg/ml) and 70 μg equivalents of protein were separated by 12% SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) (BioA Rad). The PVDF membrane was blocked in 0.5% milk in PBSA T for 2 hours and probed with antiA NRGN antibody (1:750, Santa Cruz) for 4°C overnight, and then washed with PBS and 0.1% Tween20 and incubated with goat antirabbit IgG conjugated to horseradish peroxidase (1:3000 Caltag) for 1hr at room temperature. The membrane was developed using an ECL enhanced chemiluminescence kit (Advansta).

4.5. WESTERN BLOT ASSESSMENT FOR P24

Whole cell lysates were prepared using RIPA buffer (containing 50mM Tris (pH 7.5), 150mM NaCl, 1% Triton XA 100, 1mM sodium orthovanadate, 10mM sodium fluoride, 1mM phenylmethylA sulfonylfluoride, 0.05% deoxycholate, 10% SDS, 0.07 trypsin and protease inhibitors 1μg /ml) and 70 μg equivalents of protein were separated by 12% SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) (BioA Rad). The membrane was blocked

membrane was blocked overnight in 0.5% milk in PBSA T for 12 hours. The blocking buffer was poured off and the primary antibody (gagA p24 antibody) was added at a concentration of 1:500 and incubated for 2 hours. The antibody was discarded and the membrane and then washed with PBS and 0.1% Tween20 and incubated with rabbit antimouse IgG conjugated to horseradish peroxidase (1:3000 Caltag) for 2hr at room temperature. The membrane was developed using an ECL enhanced chemiluminescence kit (Advansta).

4.6. TRANSFECTION OF NRGNV FLAG/NRGN PLASMID INTO 293T AND TZM CELLS.

293T cells and TZM cells were seeded in 10cm^2 plates and allowed to reach 85 – 90% confluence. The cells were transfected with FRTA NRGN and FRA NRGN using Polyjet transfection reagent (SignaGen). After 12 – 18 hours, transfection reagent was removed and replaced with complete medium and incubated for an additional 30 hours.

4.7. RNA ISOLATION

TZM, NT2, A172, SHA SY5Y, HeLaA T4 and HeLa 153 cells were used for RNA isolation using the MirVana kit (Applied Biosystems), according to the manufacturer's protocol. Briefly, 5 million cells were lysed with 200µl of lysis buffer. Next, RNA homogenate at a volume equaling 1/10 of lysis buffer was added to the cells, and the cells were incubated on ice for 10 minutes. This was followed by an addition of acid phenol chloroform at a volume equal to that of the lysis buffer. The samples were then thoroughly vortexed for 30 seconds and centrifuged for five minutes at room temperature at 12,000 rpm. The aqueous phase was then removed. Next, 100% ethanol was added. Using a filter cartridge, the samples were spun and washed three times with wash

solutions. Finally, the samples were eluted with 90µl of preA heated elution solution. RNA concentration was determined with NanoDrop2000 spectrophotometer, and the samples were stored at A 80°C.

4.8. GENERATION OF cDNA

cDNA was generated from 100ng of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to Table 1. The PCR was run at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and held indefinitely at 4°C. The cDNA was then either kept at -20°C or immediately used for qRT PCR. The qRT PCR was run in 20µl triplicates (according to Table 2), using the ABI Prism 7000 Sequence detection system, set at 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 sec and 60°C for 1 minute. Relative expression (ddCT) was calculated after normalization to RPLPO.

Table 1. Reverse transcription reagents for mRNA validations

Reagents	Volume (µl)
cDNA	20
Taqman Gene Expression Master Mix	35
mRNA assay	3.5
Nuclease-free water	11.5

Table 2. Real Time PCR reagents for mRNA validations

Reagents	Volume (µl)
RNA	10
10X RT buffer	2
25x dNTP	0.8
Random Primer	2
Multiscribe Reverse Transcriptase	1
Nuclease-free water	4.2
Total	20

4.9. IMMUNOFLUORESCENCE STAINING

In a 6A well plate with coverslips in each well, 500,000 neuronal cells were seeded and allowed to adhere and reach a confluence level of 60 – 75%. The cells were then washed with PBS once and 4% paraformaldehyde was added to the cells and incubated for 15 minutes at room temperature. The cells were then washed with PBS three times and 0.5% Triton XA 100 (with PBS) was added and incubated at room temperature for 15 minutes. The cells were then washed three times with PBS. After washing, the cells were blocked with 2% BSA in PBS and incubated for 1 hour at room temperature. Next, the primary antibody (NRGN or FLAG) was added at a dilution of 1:250 with 2.0% BSA for 1 hour. The cells were then washed three times with PBS. After washing, the secondary antibody (Alexa Fluor 488) was added to the cells at a dilution of 1:500. The cells were then covered with foil and kept in the dark to incubate for 1 hour. After incubation, the cells were washed five times with PBS and DAPI was added (~70uL) and kept on for ~30 seconds. The cells were then washed three times with PBS and mounted onto a slide using Gelvitol then were viewed using a fluorescent microscope.

4.10. FLOW CYTOMETRY TO DETERMINE THE EXPRESSION OF CD4+/CCR5+/CXCR4+ RECEPTORS IN NEURONAL CELLS

Neuronal cells were trypsinized and 100,000 cells were counted then resuspended in 1.0mL FACS buffer (10% FBS in PBS) and kept on ice for 10 minutes. After the incubation on ice, the cells were spun at 2000rpm for 5 minutes and resuspended in 50ul of FACS buffer. 5uL of primary antibody (CD4, CCR5, or CXCR4) was added and the cells were kept in ice for 45 – 60 minutes. After this incubation step, the cells were washed with

PBS 3 times (add 1mL of PBS, spin for 5 minutes at 2000rpm) and then resuspended in 500uL of FACS buffer. Once in the 500uL of FACS buffer, the cells were ready to be loaded on to the LSRFortessa™ Cell Analyzer (BD Biosciences). Flow cytometric analysis was conducted using FACSDiva® software (BD Biosciences).

4.11. CHIMERIC REPORTER VIRUSES

Two reporter viruses used were generated in the laboratory using the primary NL43A EGFP vector. The NL(YUA 2)A EGFP, virus which utilizes the R5A tropic YUA 2 envelope region and the NL4A 3A EGFPA IRES X4A tropic virus were both generated by Jessica K. Sparks and Narasimhan Venkatachari, respectively.

4.12. TRANSFECTION OF HIV-1 CHIMERIC PLASMID TO MAKE VIRUS COMPLEMENTED WITH VSV

In a 10cm^2 plates, 2x10^6 293T cells were seeded and allowed to adhere for 24 hours. Once the cells reach 80 – 90% confluency, cells were coA transfected with 3µg of either NL43A WT or NLA YU2 HIV chimeric viral plasmid and 1µg of VSVA GAG plasmid using Polyjet transfection reagent (SignaGen). The transfection solution was left on the cells for 12 – 18 hours and then replaced with 7mL DA 10 media for 48 hours. After the 48 hours (~72 hours postA transfection) viral particles A was collected and used for infection experiments.

4.13. TZM ASSAY TO DETERMINE INFECTIVITY OF HIVV 1 CHIMERIC VIRUSES

In a 96A well plate, 30,000 TZMA bl cells were seeded per well in a total volume of 100uL and incubated overnight to allow for cell adherence. Once the cells were adherent to the surface of the wells, the HIVA 1 chimeric virus was added to the cells in 1:10 serial

dilutions (1:10; 1:100; 1:1000) in a total volume of 100uL in triplicate. After adding the virus, the cells were incubated for 36 – 40 hours. Post incubation, the virus media was removed from the cells and the cells were fixed using 0.5% gluteraldehyde. Once fixed, the cells were washed twice with PBS and 100ul of XA Gal substrate (Table 3) was added to the cells and incubated at 37°C for 2 – 3 hours. In order to stop the staining reaction, after 2A 3 hours incubation, the cells were washed with PBS once and 50uL of PBS was added to keep the cells for counting.

Table 3. XV Gal Staining Substrate for TZM Assay

PBS	11.10mL
X-Gal (40mg/mL)	240uL
0.5M $MgCl_2$	24uL
K Ferrous CN	300uL
K Ferric CN	300uL

To determine the number of infectious particles (IP) per mL of virus, the number of blue nucleated cells were counted in each well and the triplicate samples were averaged. Once averaged, the following formula was used to determine the number of infectious particles (IP) per mL of virus:

Formula 1. Determine number of infectious particles per mL of virus

(# of blue nucleated cells)(dilution)(10) = #IP/mL

4.14. LUCIFERASE ASSAY

In a 6A well plate, 500,000 HeLa 153 cells were seeded and transfected (using Polyjet) with 1µg of NRGNA 3'UTR plasmid (ActiveMotif). After 18 hours, the transfection media was removed and DA 10 media was added to the cells. After adding the DA 10 media, 0.1 MOI of HIVA 1 chimeric reporter viruses (NLA YU2A env and

NL 4A 3A GFP) were added to the cells and allowed to incubate for 48 hours. Forty-eight hours postA infection, the cells were lysed by adding 100uL of LightSwitch Assay Solution (Switchgear) and incubated for 30 minutes at room temperature. After incubation, the cells were then moved to a black 96A well plate (Nunc) and the plate was read on a luminometer (Veritas, Turner BioSystems) and the luciferase signal was recorded.

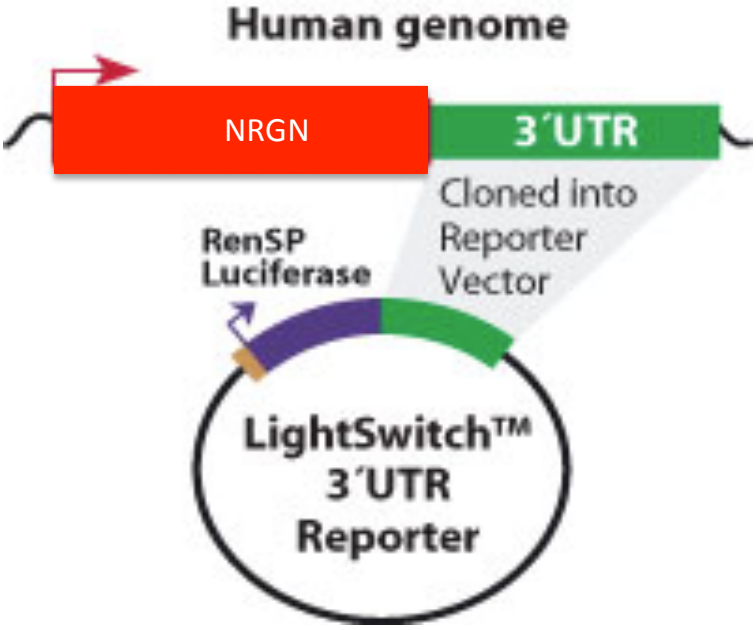


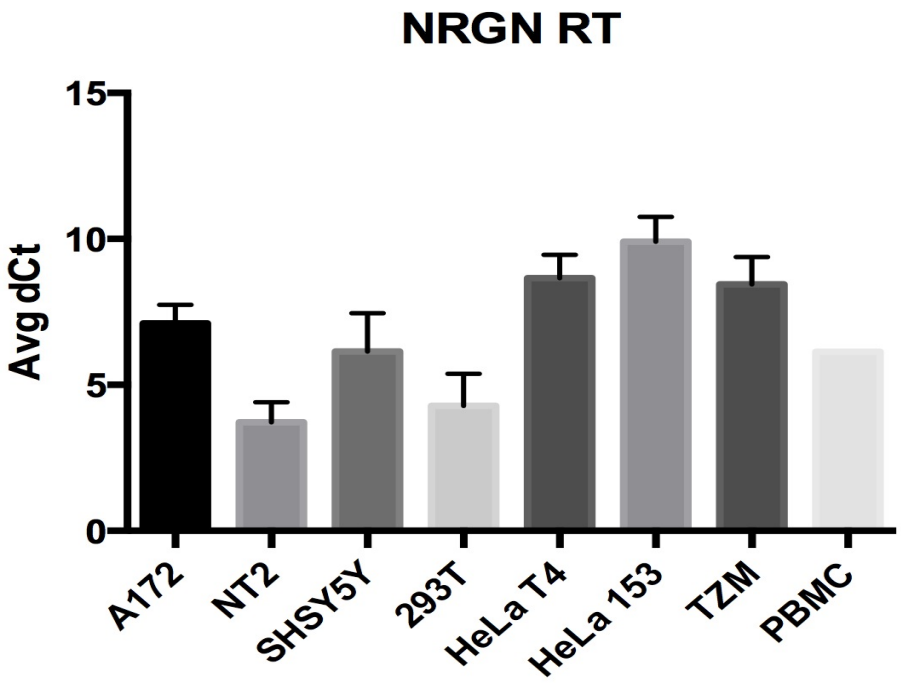
Figure 4. Construct of NRGN-3'UTR Luciferase Reporter Vector[93]

5.0 RESULTS

5.1. AIM #1: CHARACTERIZATION OF ENDOGENOUS AND OVER-EXPRESSED NRGN IN CELL LINES

5.1.1. Determine the endogenous expression of NRGN in neuronal cell lines by immunoblot against NRGN (protein level) and qRT-PCR (RNA level) assays

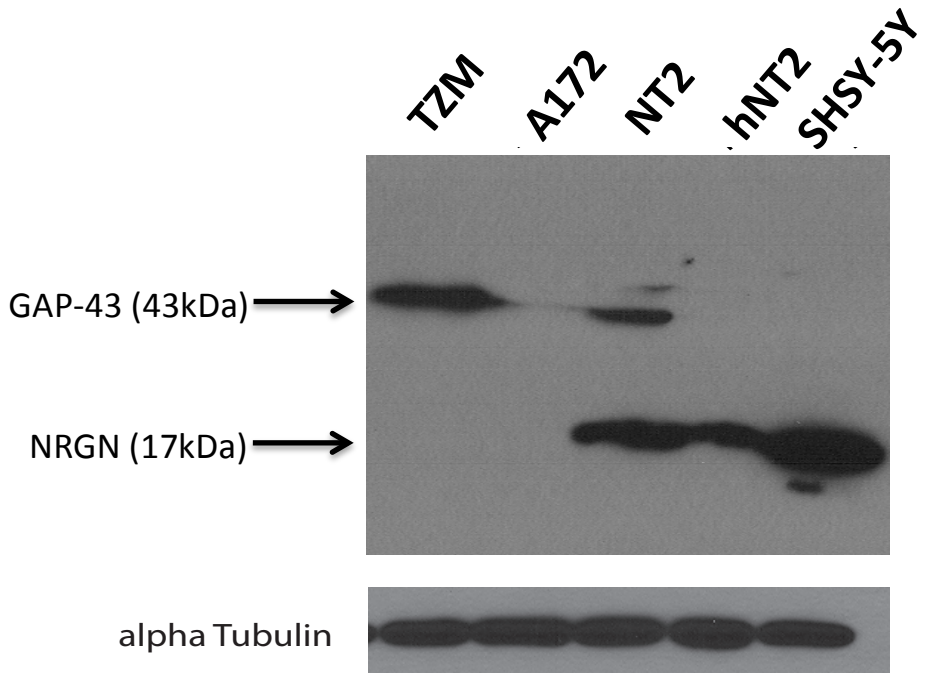
To study the degradation of NRGN within the neuronal cell lines, the level of endogenous expression of NRGN in the cells first I determined the level of NRGN RNA in neuronal cell lineages along with non-neuronal lineage cells. We isolated RNA from A172, NT2, SH-SY5Y, HeLa 153, 293T and PBMCs. The RNA was then transcribed to cDNA and qPCR was performed to measure mRNA NRGN expression (Figure 3). Figure 3 shows that two of the three neuronal cell lines (NT2 and SH-SY5Y) have a high expression of NRGN and the A172 cell lines has a low expression, which coincides with the literature that states this specific neuronal cell line hypo-expresses NRGN[90].



RT-PCR was performed for NRGN expression among the neuronal cell lines as well as control cell lines and the averages of 5 independent experiments (n=5) were collected and graphed. The low number of the average dCt corresponds to a high expression of NRGN.

Figure 5. Endogenous Expression of NRGN in neuronal cell lines by RT-PCR

After concluding that the neuronal cell lines of interest did express NRGN mRNA transcript, immunoblot was performed to determine protein expression using NRGN specific antibody (Figure 4). Figure 4 exhibits that there is no NRGN present in the negative control (TZM) and also that there is very little NRGN expressed in A172 cells, which further confirms the low mRNA expression of that particular neuronal cell line. Importantly, NT2 neuronal cells, the differentiated hNT2 neurons, as well as the SH-SY5Y cells all express NRGN, as expected.

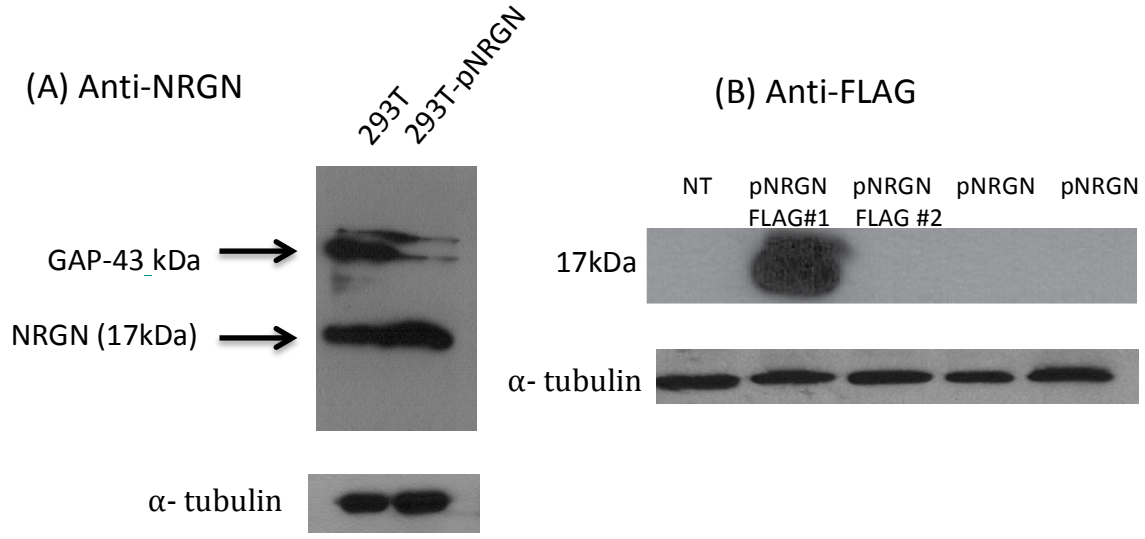


Neuronal cell lines (A172, NT2, hNT2, and SH-SY5Y) and a control cell line (TZM) were lysed and the wholeA cell lysates were run on an SDS-PAGE gel and immunoblotted using anti-NRGN antibody to determine endogenous expression of NRGN within the neuronal cell lines of interest. The blot was then stripped and re-probed using an anti-α-Tubulin antibody to ensure equal loading of lysates.

Figure 6. Immunoblot of endogenous expression of NRGN in neuronal cell lines.

5.1.2. Construction of NRGN-Flag vector to determine the localization of NRGN.

NRGN was cloned into an expression vector that included a FLAG tag in order to perform localization studies. NRGN was also cloned into an expression vector without a FLAG tag. The plasmid DNA containing cloned NRGN with the FLAG tag and the plasmid DNA without the FLAG tag were transfected into 293T. Post transfection cells were lysed and immunoblot was performed to determine if the cloning of the NRGN into the vector was successful using an anti-neurogranin antibody and an anti-FLAG antibody (Figure 7). The confirmed positive plasmids were used for immunofluorescence staining experiments.

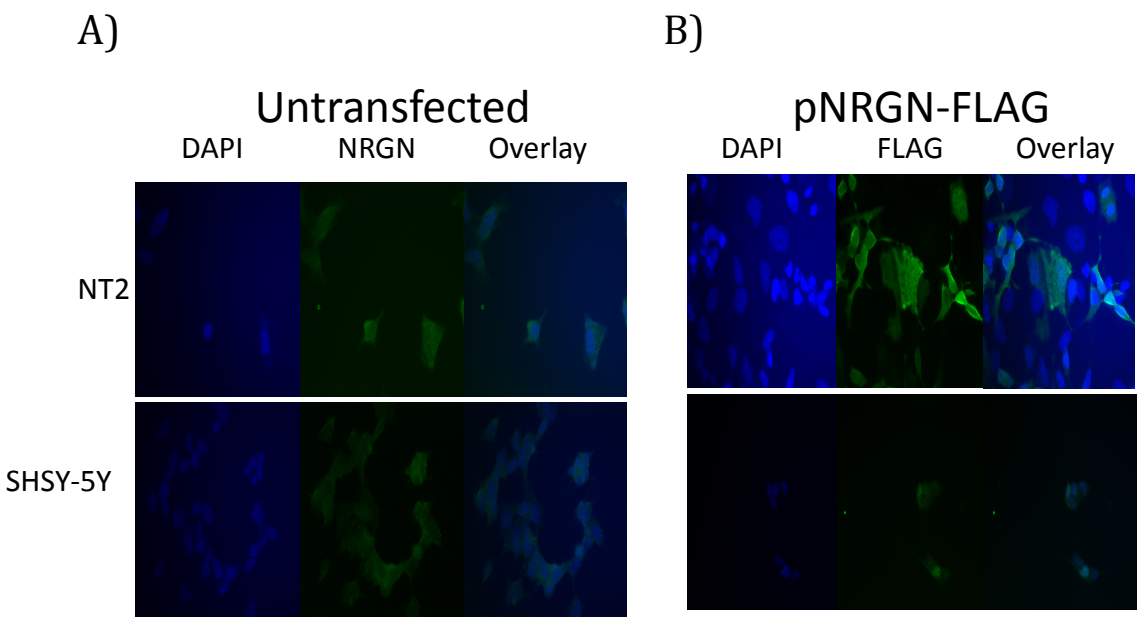


(A) 293T cells were transfected with pNRGN and the lysates were run on a SDS-PAGE gel and immunoblotted using the anti-NRGN antibody. **(B)** Cell lysates from 293T cells transfected with pNRGN and pNRGN-FLAG and run on a SDS-PAGE gel and immunoblotted using an anti-FLAG antibody confirm successful cloning.

Figure 7. Confirmation of pNRGN and pNRGN-FLAG expression in 293T cell lysates.

5.1.3. To determine the localization of endogenous NRGN in neuronal cells using immunofluorescence staining of NRGN.

Immunofluorescence staining was used to visualize the localization of NRGN within the neuronal cell lines (Figure 8A). Using the confirmed clone of pNRGN-FLAG, the neuronal cell lines were transfected and the localization of the transfected pNRGN-FLAG was visualized (Figure 8B). Figure 8 (A, B) shows that the endogenous NRGN and the transfected NRGN-FLAG both localize to the cytoplasm of the neuronal cell lines.



(A) Immunofluoresence staining to determine localization of endogenous NRGN within neuronal cell lines of interest. **(B)** Immunofluoresence staining to determine localization of pNRGN transfected into neuronal cell lines of interest. Green = Antibody; Blue = DAPI; magnification: 60x

Figure 8. Immunofluorescence staining of neuronal cell lines to determine localization of NRGN.

5.1.4. Summary of AIM #1

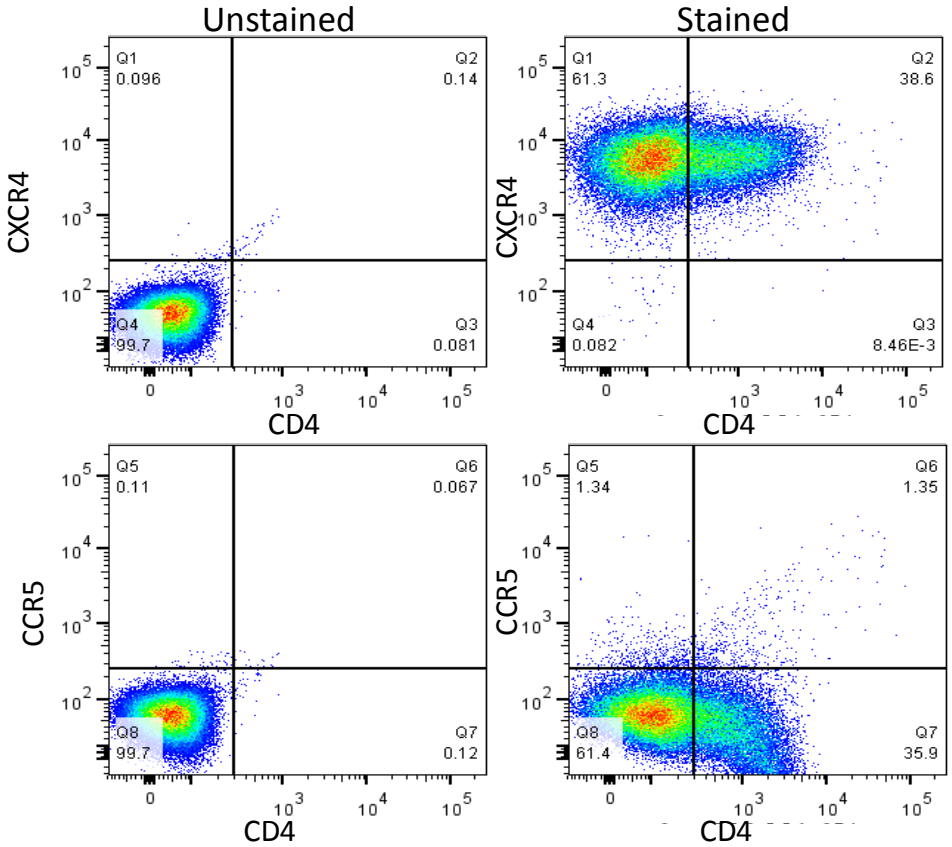
Neurogranin (NRGN) is specific to neuronal cell lines with the exception of 293T cells. The A172 cell line hypoA express NRGN, which can be seen by RTA PCR. A NRGNA FLAG plasmid was successfully cloned for use in immunofluorescence staining experiments for NRGN localization.

5.2 AIM #2: EXAMINE THE EFFECT OF HIV-1 EXPOSURE/INFECTION ON NRGN IN NEURONAL CELL LINES

5.2.1. To test whether neuronal cell lines express HIV-1 receptor and co-receptors using flow cytometry

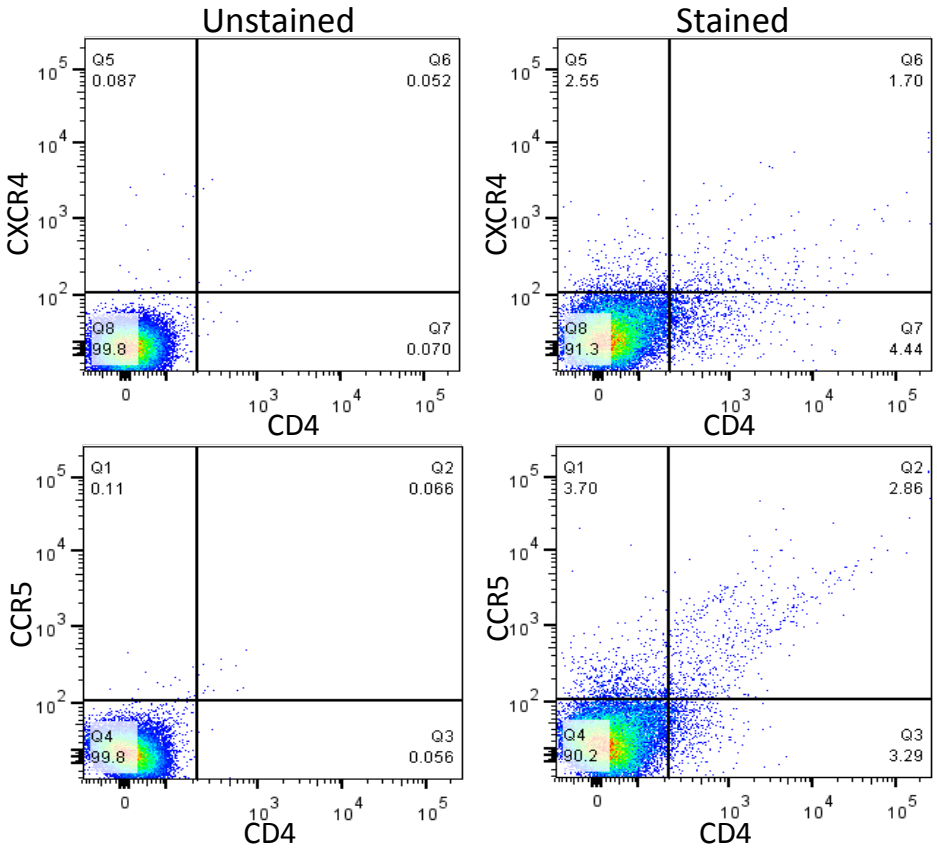
In order to determine if neuronal cell lines were capable of uptaking virus particles, the cells were stained and the expression of surface receptors CD4, CXCR4, and CCR5 were tested via flow cytometry (Figure 9). The surface staining of the neuronal cell lines show that A172 and NT2 cell lines express <3% for CD4+/CXCR4+ and <3% for CD4+/CCR5+. The SH-SY5Y cell line, however, showed ~9% positivity for CD4+/CRC4+ and ~8% for CD4+/CCR5+, which is still low, but much higher than the other neuronal cell lines.

A)



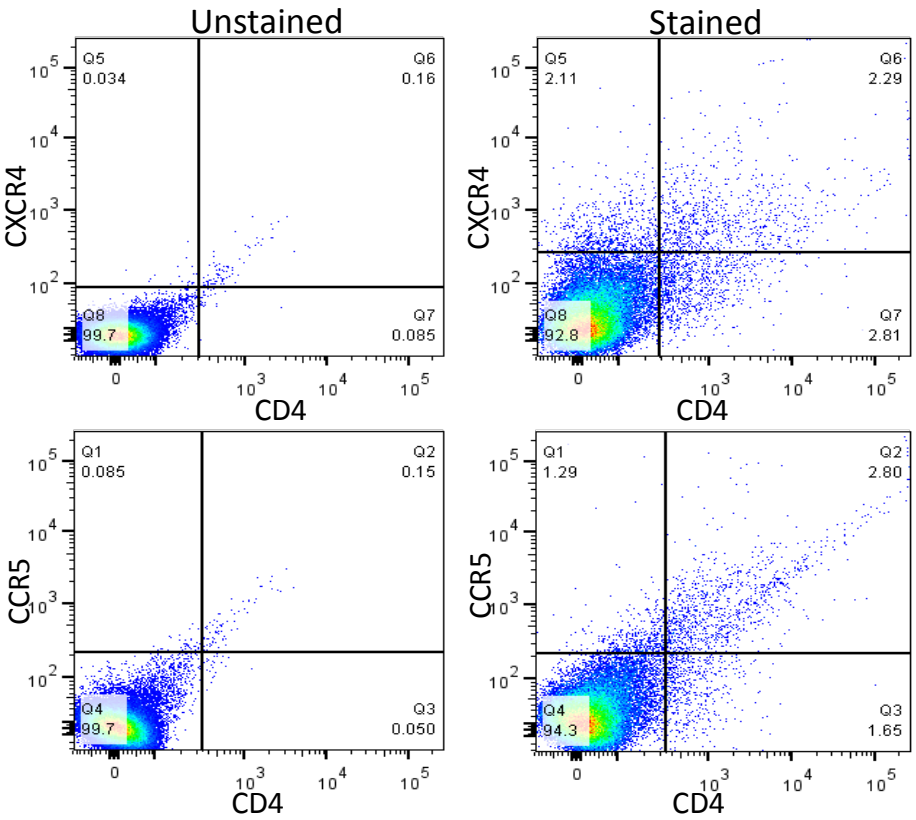
ACH2 (Positive Control)

B)



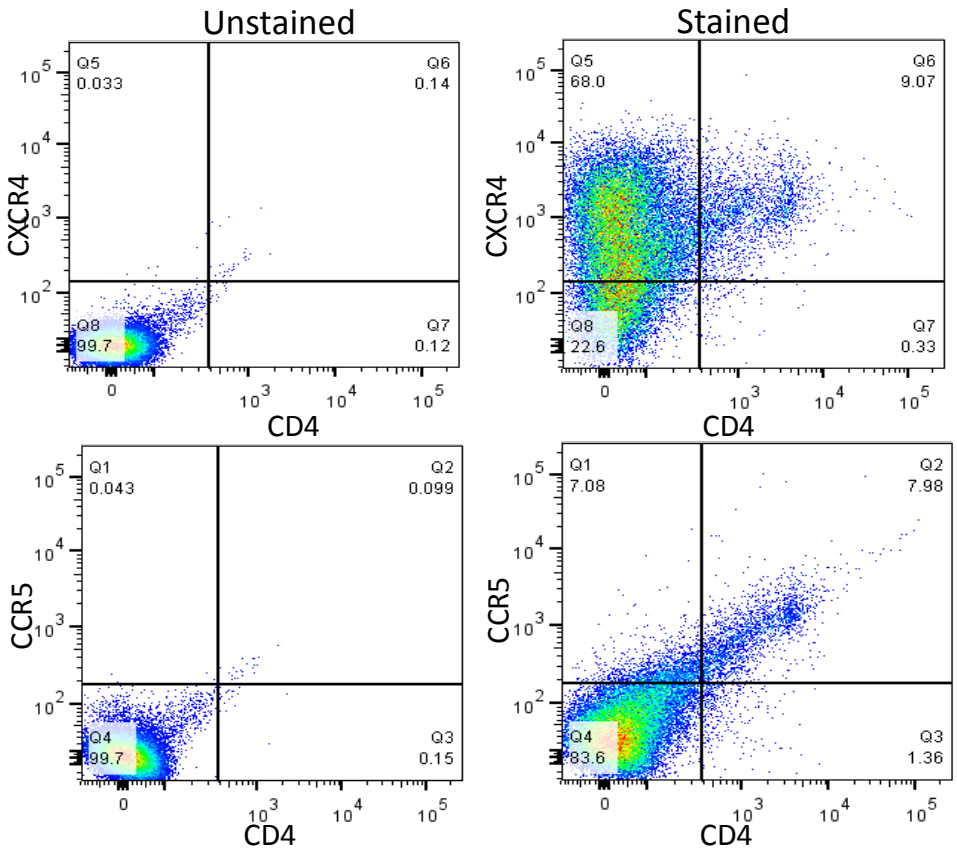
A172

C)



NT2

D)



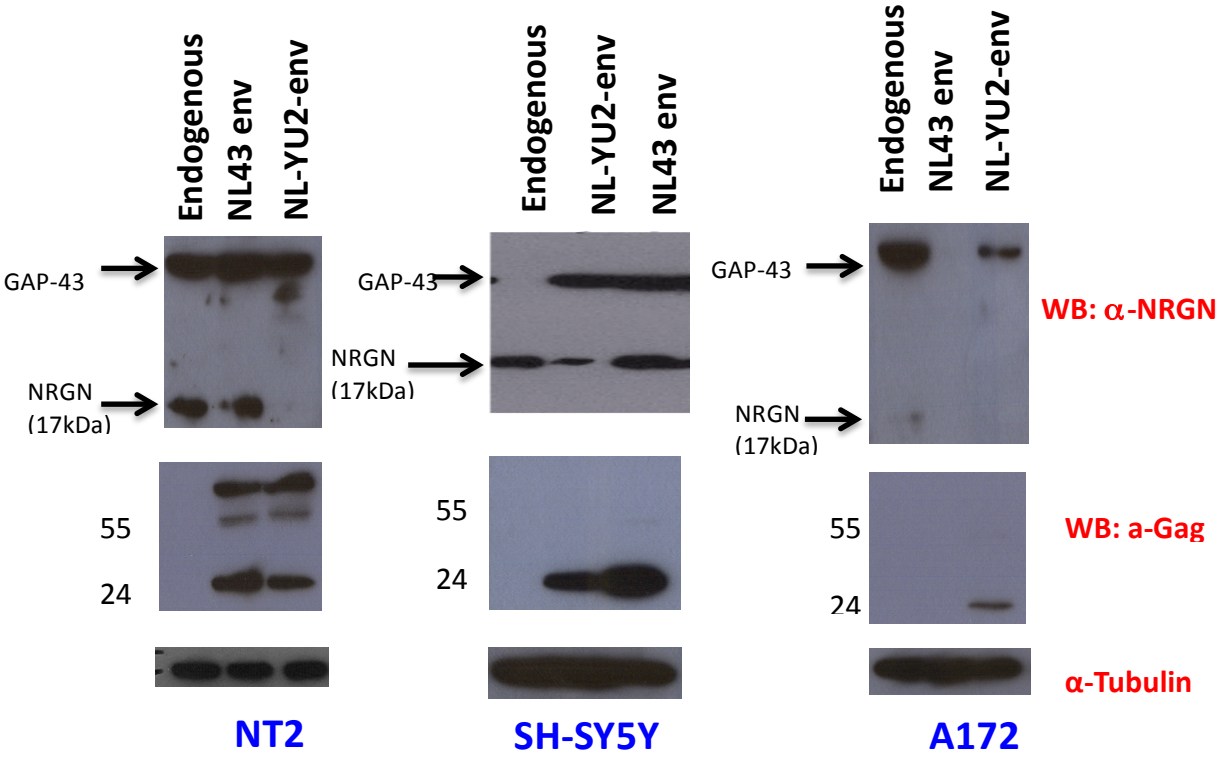
SH-SY5Y

Control cell line ACH2 and neuronal cell lines (A172, NT2, SHA SY5Y) were stained for the presence of the CD4 receptor and the CXCR4 and CCR5 coA receptors necessary for HIV virus entry and HIVA 1 infection.

Figure 9. Surface staining of neuronal cell lines to determine the presence of HIV-1 receptor and co-receptors.

5.2.2. Expose/infect neuronal cell lines with chimeric reporter viruses.

After determining the presence of HIV-1 receptor CD4 and co-receptors CXCR4 and CCR5 using flow cytometry, the neuronal cell lines were then exposed to HIV-1 chimeric viruses, NL-YU2-env and NL43. 48 hours post exposure, the cells were harvested, lysed, and immunoblotted with antibodies specific for HIV-1 capsid protein p24, NRGN, and α-tubulin. Figure 10 shows the effects of HIV-1 exposure on the neuronal cell lines.



Neuronal cell lines were exposed to HIV-1 chimeric viruses complemented with VSV-Env for entry into the cells. 48 hours post-infection, the cells were harvested, lysed and immunblotted using antibodies for HIV capsid protein p24, NRGN, and α-tubulin. In the neuronal cell lines it can be seen that there is a degradation of NRGN with NL-YU2-env (VSV) infection.

Figure 10. Immunoblot of neuronal cell lines after exposure to HIVV 1 Chimeric viruses

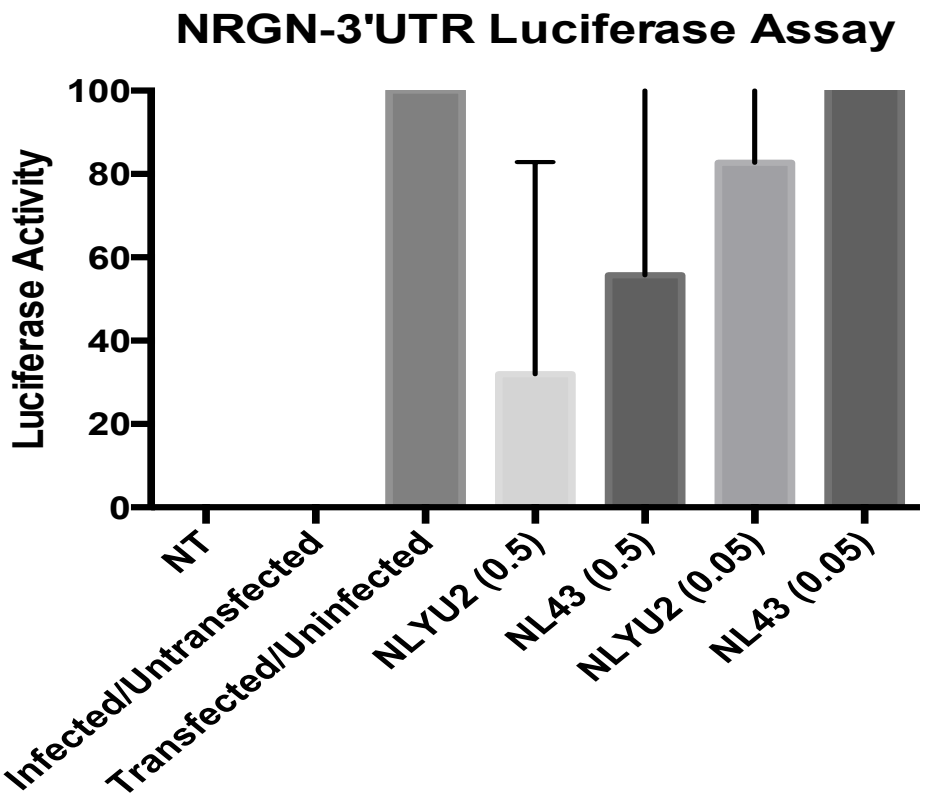
5.2.3. Summary of AIM #2

Based on the flow cytometry data, the neuronal cell lines have a very low percentage of HIV-1 receptor, CD4. There is also a low presence of the HIV-1 co-receptors, CXCR4 and CCR5. With this information, the neuronal cell lines as well as a control cell liines were infected/exposed to HIV-1 chimeric viruses and the potential degradation of NRGN was analyzed via immunoblot. The results show that there is a low level of NRGN degradation in the SH-SY5Y cell line when exposed to the NL-(YU2)-Env chimeric virus.

5.3 AIM #3: DETERMINE HOW NRGN IS DEGRADED BY HIV-1

5.3.1. Using a 3'UTR-NRGN-Luc transfected into HeLa cells to determine if degradation occurs at the 3'UTR of NRGN

Previous microRNA (miRNA) data from our lab indicate that a number of miRNAs could potentially bind at the 3'UTR of NRGN transcript upon HIVA 1 infection and could possibly contribute to the degradation of the RNA transcript. A NRGNA 3'UTRA Luciferace reporter construct was purchased from ActiveMotif and transfected into HeLa 153 cells to test this. PostA transfection (18 hours), the cells were exposed to HIVA 1 chimeric viruses. 48 hours postA infection, the cells were lysed using the luciferase assay buffer and luciferase activity was measured using a luminometer. Based on the luciferase activity data recorded (Figure 11), there is no significant decrease in reporter activity indicating that miRNA binding to the 3'UTR of NRGN is not the pathway. Infection/ exposure of NLA YU2A env virus at a concentration of 0.1 MOI, did show a very high level of luciferase activity (above basal level), at multiple replicate experiments (4 times).



Luminescence activity was recorded from HeLa 153 cells transfected with the NRGN-3'UTR. The cells were infected with HIV-1 chimeric viruses to determine if viral infection would have an effect on the 3'UTR of NRGN. Infection with 0.1μg NL-YU2-Env shows increased luminescence. (n=6)

Figure 11. NRGN 3'UTR Luciferase Reporter Assay

5.3.2. Summary of AIM #3

Based on the luciferase assay data, there is no distinct interaction between HIVA 1 infection and the NRGN degradation via miRNA. This lack of degradation of NRGN transcript by the virus mediated miRNA leads us to conclude that changes in RNA transcript may not be a major contributor to the cause of degradation of NRGN protein in HIVA 1 infection.

6.0. DISCUSSION

Since the first HIV-1/AIDS cases have been reported, it has been about 35 years and the virus is still a great threat to the world population. Even with extensive research, it is not fully clear how the virus interacts with the host and establishes disease state. Additionally, it is unclear why certain individuals progress to HIV-associated neurocognitive disorders (HAND) and others do not. With the advent of HAART, the progression of HIV-1 infected patients to HAD has greatly decreased, but many still progress to either ANI or MND[94-96]. The success of HAART in transforming the lives of HIV-1-infected individuals with access to these drugs by extending lives as well as the quality of life. This extension is overshadowed by the increasing risk of HAND[97].

HIV-1 neuropathogenesis is still not fully understood. It is possible that there is an overlap of other neurodegenerative diseases that are already genetically present in infected individuals that share characteristics with HAND[98-102]. Neuroinflammation is a critical area to investigate as reflected by increased expression of inflammation or immune-activation biomarkers in the brain, cerebrospinal fluid (CSF) and in some cases, plasma in individuals affected by HAND[103-105]. The key role for macrophages in HAND allows for studies of host genetic variation in HAND[101, 106, 107].

Recent studies of HAND have focused on expression of microRNA (miRNA), small non-coding RNA molecules that bind messenger RNA and regulate gene expression at the transcriptional or post-transcriptional levels. miRNA expression studies conducted in cortical neurons exposed to viral proteins such as Tat and Vpr [108, 109] or in tissue from individuals with HIV encephalitis (HIVE) have associated upregulation of the following classes of host miRNAs in HIVE: 1) immune response and inflammation, 2) nucleotide

metabolism, 3) cell cycle, 4) oncogenesis (e.g., miR-21, which targets a neuronal transcription factor), and 5) apoptosis. Downregulated miRNAs were involved in: 1) inflammation, 2) neuronal monoamine oxidase activity (possibly explaining the reduced dopaminergic activity in HAND), 3) apoptosis, 4) modulation of viral replication, 5) mitochondrial function, and 6) axonal guidance[108, 110-112].

Merging short-term memories into long-term memory requires synaptic plasticity, described by altered gene expression and structural changes at the neuronal synapse[113]. A recent study found that many synaptic plasticity genes in HIV-infected astrocytes were downregulated, and there was an increased expression of pro-apoptotic genes, compared to uninfected controls [113, 114]. These findings concluded that there was altered dendritic morphology and reduced dendritic spine density.

Neurogranin (NRGN) is involved in synaptic plasticity and is a key component in the consolidation of short-term memories to long-term memory[15, 115]. Long-term changes in synaptic plasticity depend on protein synthesis and transcription. NRGN is involved in synaptic plasticity through the regulation of CaM (calmodulin)-mediated signaling[116, 117]. NRGN contains an IQ motif that allows for the binding to CaM and PA (phosphatidic acid) and can be phosphorylated by PKC (protein kinase C)[13, 117, 118].

When NRGN is bound to CaM, it is shown to localize to the cytoplasm. When stimulated by PKC and unbound from CaM, NRGN shows a nuclear localization when expressed in cell lines[119]. In our study, we saw that NRGN localized to the cytoplasm in the neuronal cell lines, which correlates with the literature that synaptic activity regulates its translocation to the nucleus[119]. This is very likely due to its being bound to CaM. This could further be studied by immunofluorescence staining against CaM to determine if the

NRGN is indeed bound, hence localizing to the cytoplasm. Another approach would be to stimulate the cells using PKC and performing subcellular fractionation and immunocytochemical techniques or live-cell imaging to investigate the process of phosphorylation of NRGN upon stimulation of PKC and determining localization of the protein.

NRGN is a neuron-specific protein that is concentrated abundantly in the cerebral cortex, hippocampus, amygdala, basal ganglia, and hypothalamus[120]. Because of this information, we chose 293T cells as a negative control for our RT-PCR experiments for NRGN mRNA expression. In our results we observed that 293T cells do express our protein of interest and we found in the literature that 293T cells express an array of neuron-specific proteins[121]. With this information, we used 293T as a positive control and then chose HeLa 153 cells as our negative control, which we knew do not express NRGN. As expected, the results showed low expression of NRGN in the HeLa 153 cells and in the A172 astrocytic cell line, which is known to hypoexpress the protein[90]. As expected, we found high expression of NRGN in our other two neuronal cell lines, NT2 and SH-SY5Y.

Neurons do not have the main surface receptor for HIV-binding and entry into the cell, and therefore cannot directly be infected[122, 123]. In addition to the CD4 receptor, chemokine receptors CXCR4 and CCR5 function as co-receptors for HIV-1 entry into CD4+ T cells. During the early stages of HIV-1 infection, viral isolates use CCR5 for viral entry, and later isolates of the virus utilize CXCR4[124, 125]. To ensure the lack of CD4 surface receptors and to investigate the presence of co-receptors CXCR4 and CCR5, we performed surface staining for the specific receptors and analyzed the cells using flow cytometry. As expected, the A172, NT2, and SH-SY5Y cell lines had very low (almost negligible) presence of the CD4

receptor and co-receptors. The SH-SY5Y cell line, however, did have a higher amount of the receptors than the other cell lines (~7% $CD4^+/CXCR4^+$ and ~9% $CD4^+/CCR5^+$). However, it is not known whether primary neurons or differentiated neurons exhibit similar receptor expression levels in *in vivo* and there are reports supporting both the sides of the observation[126-132].

Which this information, next, we exposed the neuronal cell lines to the chimeric HIV-1 reporter viruses complemented with VSV-Env, which does not need CD4 or its co-receptors for entry into the cell in order to determine if there was a possible degradation of NRGN within the cells. The results show a visible decrease in NRGN in the NT2 and SH-SY5Y cell lines when exposed to the NL-YU2-env chimeric virus complemented with VSV, which shows promise that HIV-1 infection does through some pathway degrade the protein. It is possible that because the YU2-env virus was isolated from HIV-1 that was present in the brain while NL4-3 was not. Further experiments could involve the neuronal cell lines or cultivated neurons being exposed to media of infected macrophages and/or astrocytes to investigate cytokine/chemokine effects on the cells. Exploration of other co-receptors that the virus could utilize that could be present on these cell lines would also be an avenue to pursue, since the viruses used in this experiment were complemented with VSV-G-Env.

Previous miRNA data from Duskova et al[10] indicated a downregulation of NRGN at mRNA level, indicating that it could possibly be due to miRNA inhibition of mRNA translation into the NRGN protein at the 3'UTR of RNA transcript. To explore this hypothesis, the 3'UTR of NRGN in a luciferase reporter vector was transfected into the HeLa 153 cells and the cells were then infected with the chimeric reporter viruses

complemented with VSV-Env. The reporter data indicate that there is no significant effect by HIV-1 via the miRNA-induced degradation and, therefore there must be a different pathway in which the protein is degraded.

6.1. CONCLUSION

This study sought to characterize and elucidate the role of NRGN in HIVA 1 infected/ exposed neuronal cell lines and to find support that there is a correlation between NRGN degradation caused by HIV-1 infection and HAND progression. In order to understand a role of NRGN in HIV-1 infection, neuronal cell lines expressing the protein were exposed to reporter viruses and degradation of the protein was analyzed via immunoblot. Results from this study suggest that NRGN does show degradation upon HIV-1 exposure – particularly in SH-SY5Y neuronal cell lines – which can possibly be one of many contributing factors that can lead to a patient infected with HIV-1 progressing to HAND.

7.0. FUTURE DIRECTIONS

Something of interest in regards to NRGN is what exactly occurs once it dissociates from CaM and migrates towards the nucleus[75]. It has yet to be determined the purpose NRGN serves asides from being bound to CaM and increasing intracellular Ca^{2+}[67]. It is possible that once this is determined, other pathways that NRGN as well as the other proteins in the calpacitin family is involved in can be elucidated.

As a continuation of the current research, it would be interesting to be able to clone NRGN into a mCherry vector to visually investigate the possible degradation of NRGN in the presence of HIV-1 infection. The green fluorescence of the HIV-1 chimeric viruses, in theory, would become more prominent as the red fluorescence of the NRGN-mCherry decreases showing a degradation of the protein in the presence of viral infection.

Another avenue that can be explored are the ties between the hypothalamus-pituitary-thyroid pathway and its link to HIV-1 infection and its effect on the thyroid response element (TRE) present on neurogranin. Recent literature indicates that there is a link between hypothyroidism in HIV-1 infected individuals and a decrease in NRGN [11, 133]. There is no link of the decrease in NRGN and the progression to HAND, but it is something that should be thoroughly investigated.

An area that should be thoroughly investigated is the 3'UTR of NRGN and which, if any, microRNAs (miRNAs) affect the function of NRGN and can potentially lead to its degradation in HIV-1 infection. If there are specific miRNAs involved in the degradation of NRGN, those miRNAs can be targeted and can potentially protect against NRGN degradation.

8.0. PUBLIC HEALTH SIGNIFICANCE

The public health significance of this project is that in determining the underlying contributors to the degradation of cognition in HIV-1 infected individuals that progress to HAND, treatments can be developed to combat the effects on HIV-1 on proteins and cells pertinent to normal brain function. In fully elucidating the complete role of NRGN in the Ca^{2+}/CaM pathway, the PKC pathway and its role in the HPT-axis, it can be determined if NRGN degradation is a key factor contributing to HAND or one of many side effects of a larger dysregulation in the brain caused by HIV-1 infection.

With the advent of HAART, HIV-1 infected individuals are able to live longer, but the effect of HAART on HAND is not enough to removed cognitive degeneration altogether[97]. Though there is a decrease in individuals that progress to HAD, many do experience the earlier stages of HAND (ANI and MND) and there is currently no way to determine which of the HIV-1 infected on HAART will or will not experience cognitive degeneration.

By investigating NRGN, which is currently being used a biomarker for Alzheimer's Disease (AD)[82], it is possible that it or other proteins shown to be degraded in patients with a form of HAND can be used as an early detection tool to determine if an infected individual could potentially progress to HAND and can allow researchers to develop a treatment against such progression. If such a treatment is developed, not only can it be used in conjunction with HAART to extend the lives of individuals living with HIV-1 but to also preserve their cognition and lead to an even small incidence of HAND.

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