UNDERSTANDING THE ROLE OF ASTROCYTES IN HIV-1 NEUROPATHOGENESIS: NOVEL STRATEGIES TO TARGET ASTROCYTE FUNCTION AND HIV-1 ASSOCIATED NEUROCOGNITIVE DISORDER

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BA, University of Pittsburgh, 2017

Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2017

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

This thesis was presented

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December 13, 2017

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ABSTRACT

HIV-1 infection is associated with changes in cognition and neuronal function, known as HIV-associated neurocognitive disorder (HAND), with the most severe form resulting in dementia. Astrocytes in the frontal cortex have a critical role in neuropathogenesis induced by HIV-1 as they undergo "astrocytosis" that is commonly found in HAND subjects. Astrocytes are a type of glial cells located in the central nervous system and are targets for HIV-1 infection, though it is defective in producing replication competent viruses. However, the infected astrocytes are capable of producing viral proteins and are reactivated. These star-shaped cells serve many functions, such as providing structural and metabolic support to nearby neurons, and have a central role in the maintenance of the neuronal synapse and the blood brain barrier. Astrocytes undergo structural and functional transformations in the occurrence of CNS infection, but it is unclear whether this plays a role in HIV-1 pathogenesis. Host cellular factors including microRNAs have been suggested to regulate the balance between neuroprotective and neurotoxic factors in astrocytes. While combination antiretroviral therapy (cART) can be used to treat viral replication, these drugs do not penetrate the central nervous system, and therefore treatment of

infection within the brain is limited. Thus, identifying new therapeutics is important to reduce the burden of HAND, which has great public health significance.

We have previously identified candidate miRNA that are known to have potential neuroprotective effects. In my project, I evaluated whether these miRNA have a role in regulating virus replication and/ or virus reactivation in astrocytes.

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PREFACE

I would like to thank Dr. Jay Venkatachari and Ms. Roberta Reis for their guidance, patience, and advice while teaching me the techniques and procedures used in this project. I would also like to thank Dr. Velpandi Ayyavoo for giving me the opportunity to work in her laboratory.

1.0   INTRODUCTION

Human Immunodeficiency Virus-1 (HIV-1) is a global public health issue, having claimed over 35 million lives to date. Approximately 36.7 million individuals have been infected with HIV-1, as of 2016, with 1.8 million new cases of the virus within 2016 alone. HIV-1 is a virus that targets the immune system and weakens its ability to fight off infections. HIV-1 is spread through sexual behaviors, contact with mucous membranes, and through contact with contaminated needles and syringes [1]. The symptoms of HIV-1 infection can vary depending on the stage of infection. In fact, many individuals are unaware of their status until later stages, as they may not experience any symptoms within the initial weeks of infection. HIV-1 infection is diagnosed through rapid diagnostic tests (RDTs), which measure the levels of HIV-1 antibodies within the blood [1].

There are three stages of HIV-1 infection, with the most advanced and serious stage resulting in Acquired Immunodeficiency Syndrome (AIDS). The first stage of infection is known as acute HIV-1 infection, which develops two to four weeks after infection. Symptoms of the acute stage include fever, headache, and rash. The risk of HIV-1 transmission is very high at this stage of infection, due to high levels of virus within the blood. The second stage of HIV-1 infection is known as chronic infection, where HIV-1 continues to replicate within the body, but at lower levels. Without treatment, chronic infection can lead to AIDS anywhere from 2 to 15 years after initial infection. Finally, the third stage of infection, AIDS, is the most severe form.

At this stage, HIV-1 has greatly damaged the body's immune system, making the individual susceptible to other infections and cancers [2].

HIV-1 in an interesting disease because differences in awareness and treatment practices around the world influence the distribution of HIV-1 cases globally. While HIV-1 does not discriminate against who can be infected with the virus, there are some populations who are at higher risk of being exposed to HIV-1. Those at higher risk of infection include men who have sex with men, those who inject drugs, sex workers, and transgender individuals [1].

There is currently no cure for HIV-1. However, with new technology and advanced research, effective antiretroviral drugs (ART) can help control the replication of the virus and prevent its transmission. ARTs have been successful in reducing the mortality rate of HIV-1 as well as in prolonging the lifespan of individuals infected with the disease [3]. Furthermore, additional treatment such as healthy lifestyle, nutritious diet, and reducing stress can help to alleviate some of the symptoms associated with HIV-1 infection. While there have been significant strides in the understanding and treatment of HIV-1 and its pathogenesis, there is still a great deal of information left to learn about the biology of the virus in order to create a cure for the disease.

1.1   HIV-1 PATHOLOGY

The Human Immunodeficiency Virus is a virus that belongs to the *Lentivirus* genus of the *Retroviridae* family. The virus has two copies of a positive single strand RNA genome that is integrated into the DNA of the host cell, through a seven-step mechanism. HIV-1 infects CD4+ T cells and macrophages through the interaction of surface viral envelope proteins with the CD4

receptor and chemokine co-receptor (CXCR4 or CCR5) on the host cell. These receptors allow for virus entry into the cell by facilitating fusion of the viral and cell membrane [4]. CXCR4 receptors have been shown to facilitate HIV-1 entry into lymphocytes and CCR5 receptors into monocytes, macrophages, and microglia along with the CD4 receptor. For this reason, HIV-1 viral strains that utilize the CXCR4 receptor are known as X4 viruses and those that use the CCR5 receptor are known as R5 viruses [5].

After the virus enters the cell, HIV-1 releases viral reverse transcriptase enzyme, which converts the viral RNA to viral DNA. The viral DNA is then transported into the cell nucleus via an integrase enzyme, which facilitates the integration of the viral DNA into the host cell genome. Once the HIV-1 genome becomes part of the host cell DNA, the virus begins replicating using the host cell machinery. This replication process releases long chains of HIV-1 proteins, which are the building blocks for mature HIV-1 protein [4].

These HIV-1 proteins then move to the surface of the host cell where they assemble into immature HIV-1 virions. In the last step of HIV-1 replication, the immature HIV-1 virions "bud off" from the host cell and the genome releases a protease enzyme, which breaks up the HIV-1 immature virions to form infectious HIV-1 virions [4].

The HIV-1 genome has several structural and regulatory features that help to make it a successful retrovirus within an infected cell. The HIV-1 virion is approximately 120 nm in diameter and contains 2 RNA strands that are each 9 kb long. The genome contains nine genes, which contribute to creating 15 different protein products, which are necessary for the virus to establish an infection within a host cell (Figure 1). These proteins play varying roles within the HIV-1 life cycle, namely structural, regulatory, and accessary functions [5].

5'-LTR

gag

pol

vif

vpr

rev

tat

env

vpu

nef

3'-LTR
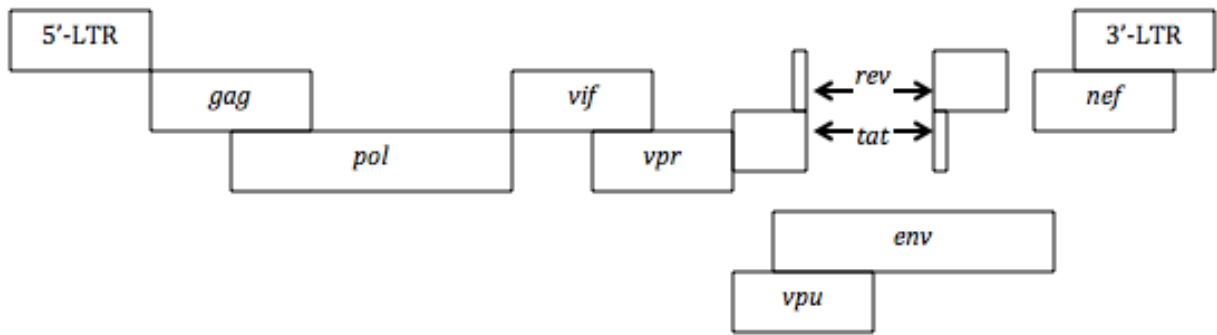
Figure 1. The HIV-1 genome

The HIV-1 provirus has two repeated sequences on either end of the genome, which are known as Long Terminal Repeats (LTRs). The LTR region of the genome contains several binding sites where host transcription factors and viral proteins interact and initiate viral transcription [7]. The genes located within the middle of the genome are responsible for encoding three main classes of proteins: structural, regulatory, and accessory proteins.

The first structural protein on the HIV-1 genome is known as Gag. Gag is a polyprotein that recruits two copies of the viral RNA and other cellular proteins to facilitate the budding of the viral particle from the infected cell. After the infectious particle has budded off from the infected host cell, the Gag protein is broken down into four smaller proteins, MA, CA, NC, and p6, each of which serve various functions in helping the virus establish infection within the host cell [8].

The second structural protein, Pol, encodes four different viral enzymes: Protease (Pro), integrase (IN), RNase H, and reverse transcriptase (RT). The protease enzyme is responsible for preparing the virion for entry into the host cell [9]. Once the virus enters the cell, the reverse transcriptase enzyme makes a cDNA copy of the viral RNA genome. The RNase H then removes the RNA template strand from the DNA copy and allows for the synthesis of a complementary

DNA strand to be made. Finally, the integrase enzyme functions to insert the viral HIV-1 DNA into the DNA of the host cell [8].

Finally, the last structural protein that is encoded by the HIV-1 genome is known as Env. Env is a structural protein that is required to establish infection within the host cell [6]. Env is cleaved into two proteins, gp41 and gp120. Gp41 serves to facilitate the fusion of the viral and cellular membranes, in order to allow the virus to deliver the viral proteins into the cytoplasm of the host cell. Gp120 also mediates viral entry into the host cell, through interactions with the virus receptor CD4. Certain sequences on the gp120 protein interact with the co-receptors CXCR4 or CCR5 and these can affect the infectivity of the virus within a host cell [8].

In addition to structural proteins, the HIV-1 genome contains two regulatory proteins, Tat and Rev, which are responsible for mediating the transcription of the viral RNA. Tat serves several functions, including influencing the splicing of the HIV-1 RNA, modulating the reverse transcription of the RNA genome, and finally, mediating the expression of cellular genes [10]. The second regulatory protein, Rev, is an RNA binding protein and is a necessary for HIV-1 to replicate. Rev facilitates the transport of viral RNAs from the nucleus of the host cell to the cytoplasm and can regulate the levels of splicing that occur with this newly made RNA [8].

Furthermore, the HIV-1 genome contains four accessory proteins, all of which serve various functions within the HIV-1 replication process, particularly to evade the host cell's immune function. The Nef protein, or negative factor protein, is responsible for inducing disease once infection has been established within the host cell. Nef serves to degrade CD4 receptors on the cell surface as well as down-regulate the level of viral replication once the infection has been established, in order to avoid detection by the host cell [11].

Another accessory protein of the HIV-1 genome is viral protein R (Vpr). Vpr, with the aid of some other proteins, facilitates virus entry into the nucleus of the host cell, so that the genome may be incorporated into that of the target cell. In particular, Vpr allows HIV-1 to infect non-dividing cells, which further increases the virus' persistence within the host cell [12].

In a similar way, the viral protein U (Vpu) also plays a role in helping the virus evade the host immune response. Vpu acts in two specific ways. The first is by degrading the CD4 receptor on the cellular surface, in order to allow for virion assembly within the host cell. Secondly, the Vpu protein increases the release of newly formed HIV-1 particles from the surface of the host cell [13].

Finally, the last accessory protein that is present within the HIV-1 genome is the virion infectivity factor (Vif) protein. Vif is the protein that aids in HIV-1 replication within lymphocytes and macrophages, cells that are typically non-permissive. The Vif protein has also been shown to have a role in the assembly of viral proteins. In all, the four accessory proteins of the HIV-1 genome help to establish persistent infection within the target cell and are primarily responsible for evading the host cell immune response [14].

1.2   HIV-1 PATHOGENESIS AND THE CENTRAL NERVOUS SYSTEM

HIV-1 invades the central nervous system (CNS) soon after initial infection via infected macrophages, where it can exist for several years after initial infection. Neurons are not directly infected by HIV-1. However, neurons can be indirectly injured from the presence of viral proteins, such as gp120 and tat, as well as the neurotoxins produced from surrounding infected cells [15].

While ART is used to treat HIV-1 infection and viral replication specifically, these drugs do not penetrate the blood-brain-barrier (BBB) of the CNS, and therefore treatment of infection within the brain is limited. HAND is becoming more prevalent as individuals infected with HIV-1 are living longer [16].

HIV-1 replication within the CNS is regulated by a number of factors, indicating that the surrounding environment influences the ability of the virus to replicate within the brain. When the virus is able to replicate highly actively, the process is known as "productive" infection, whereas a low/undetectable virus production is termed "restricted". Productive infection contributes to the rapid transmission of the virus within host cells, whereas cells that are able to restrict infection are able to survive HIV-1 infection. These cells become virus reservoirs, which are able to produce viral genomes in a stable state. The virus in these reservoirs is able to become re-activated with changes to the surrounding environment, causing infection symptoms much later after initial infection. Both types of infection can occur within the CNS [15].

1.3   HIV-1 PATHOGENESIS AND HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS (HAND)

With modern day advances in treatment and diagnosis of HIV-1 infection, individuals diagnosed with HIV-1 are living longer than those in the past. Many of those who are diagnosed with modern methods are living complete lifespans and experience less complications than before [1]. Infection within the CNS can lead to neurological problems, such as motor and cognition impairments and behavior changes. The infection of the CNS is associated with changes in cognition and neuronal function, known as HIV- associated neurocognitive disorders (HAND).

The least severe form of HAND is called Asymptomatic Neurocognitive Impairment (ANI). Symptoms of ANI include minor neurocognitive impairments, however these have no impact on daily functional performance. A milder form of impairment, known as Mild Neurocognitive Disorder (MND), is associated with neurocognitive impairment that affects activities of daily living. Finally, the most severe form of HAND is known as HIV- associated dementia (HAD). HAD symptoms include impairments in several cognitive domains and have a large impact on daily functioning [17].

Even though treatment with ARTs has reduced the number of HAD cases, neurological problems still persist in those individuals who have prolonged infection. As more HIV-1 positive individuals continue to live longer on ART, the prevalence of HAND has increased. Current treatments do not target virus replication within the brain. Therefore, studying the pathogenesis of HIV-1 within the brain in relation to the development of HAND is an important issue [15].

1.4   HIV-1 AND THE BLOOD BRIAN BARRIER

The blood brain barrier is a layer of endothelial cells that separates toxic substances in the circulating blood and prevents them from entering the brain. The BBB also supplies the brain with essential nutrients. The cells that comprise the BBB are surrounded by astrocyte processes, which serve to provide structural and functional support to the endothelial layer [15].

HIV-1 is unique in that the virus is able to overcome this barrier and enter the brain. There are many proposed mechanisms for this; the most commonly accepted one being the "Trojan horse" hypothesis. This hypothesis suggests that HIV-1 enters the brain through infected monocytes, which cross the BBB. These monocytes serve as carriers of HIV-1 to the brain and

upon entry, release viral proteins that disturb the astrocyte's ability to regulate the permeability of the BBB [15]. It is for this reason that astrocytes are an area of interest for further investigation in regards to HIV-1 pathogenesis within the central nervous system.

When HIV-1 invades the BBB, the infection causes a pro-inflammatory response with the release of numerous cytokines, including TNF-α and IL-1β. Furthermore, endothelial cells that are activated by the virus upregulate the expression of adhesion molecules, which facilitates the leakage of more infected monocytes into the brain [15].

1.5 ASTROCYTES

Astrocytes are star-shaped glial cells that are the most abundant cell type within the CNS. They serve a variety of functions including providing structural support to nearby neurons and maintaining the neuronal synapse and the blood brain barrier. Astrocytes also have a role in regulating brain homeostasis, synaptic transmission, and plasticity. Furthermore, astrocytes can be targeted by the immune system in the event of infection and neuronal injury [18].

Astrocytes actively respond to pathological stress by becoming activated, not only at the site of the injury, but also in the surrounding environment. Several studies have shown that cytokines, growth factors, and cell surface molecules are up regulated within these activated astrocytes. Previous literature has also provided evidence for the role of astrocytes as mediators in the event of HIV-1 neuronal injury. However, the pathogenesis of this process and the specific mechanism by which astrocytes act is still a topic of investigation [18].

1.6   ASTROCYTES AND HIV-1 PATHOGENESIS

Astrocytes are target cells for HIV-1 and upon infection can be activated, a process known as astrocytosis. Though astrocytes are defective in producing replication competent virus, the infected astrocytes are capable of producing viral proteins. These cells undergo structural and functional transformations in the occurrence of CNS infection, but it is unclear what role these cells play in HIV-1 pathogenesis [15]. Host cellular factors including microRNAs have been suggested to regulate the balance between neuroprotective and neurotoxic factors in astrocytes [19].

1.7   ASTROCYTOSIS

Neuronal injury and stress can cause astrocyte activation, or astrocytosis, which is when there is an irregular increase in the size and number of astrocyte cells within the CNS. Astrocyte activation is characterized by hypertrophy of neurons, cell proliferation, alteration in molecular expression, and an upregulation of glial fibrillary acidic protein (GFAP) expression. Furthermore, HIV-1 infection can activate astrocytes and cause them to produce various chemotactic factors [18]. These changes can alter astrocyte activity and affect the surrounding neuronal structures. In it unclear whether these structural and functional transformations play a role within astrocytes in HIV-1 pathogenesis.

Astrocytes, specifically activated astrocytes, are identified by the expression of GFAP, which is an intermediate protein located in the main processes and the body of astrocytes. This protein plays a role in cell communication, cell migration, mitosis, and cytoskeletal changes. The

upregulation of GFAP, an astrocyte specific protein, during activation is caused by neurological injury, and is a hallmark feature of neurological diseases, including Alzheimer's disease, Parkinson's disease, and HAND [20].

In previous studies, HIV-1 infected individuals have shown symptoms of astrocytosis triggered not only by virus infection, but also by the presence of viral proteins. For this reason, astrocytosis is considered to be a hallmark feature of HIV-1 infected brain cells. This leads to the belief that astrocyte dysfunction during HIV-1 infection of the CNS play an important role in HIV-associated neuropathogenesis and the development of HAND [21].

Furthermore, activated astrocytes have been shown to release neurotoxic factors such as excitatory amino acids (ex. Glutamate) and TNF-α. Additionally, gp-120 has been shown to up-regulate Nrf2, a transcription factor that is involved in antioxidant defense mechanism [22]. Gp-120 also induces the expression of several cytokines, such as IL-6, within astrocyte cells, through a nuclear-kappa β-dependent (NF-k β) mechanism. It is hypothesized, therefore, that the neuroinflammation caused by HIV-1 infection is mediated by the NF-k β pathway, however the mechanism of this is still under further research [23].

Another factor that plays a role in astrocytosis and the communication signals between infected astrocytes are gap junctions. Gap junctions are the intracellular space, which allow the cells to communicate with each other, through the transfer of ions, second-messenger molecules, and peptides, between the cytoplasm of neighboring cells [24]. Astrocytes utilize gap junctions to communicate with each other, but also to communicate with neurons and other glial cells [25]. Astrocyte gap junctions specifically regulate ATP release and glutamate homeostasis within the brain. These gap junctions also play a role in filtering out extra K+ ions that become present within the extracellular space after neuronal activity [26]. It is hypothesized that HIV-1 infected

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astrocytes utilize gap junctions to spread toxic signals to uninfected astrocytes. In fact, gap junctions are thought to amplify the toxic signals from infected to uninfected cells, leading to severe HIV-1 associated CNS dysfunction. Evidence for this has been shown with Tat-induced dysfunction of the BBB, which causes an altered expression of specific tight junction proteins [27].

Another important role of astrocytes include regulating the amount of K+ ions that are present within the extracellular space, which affects the ability of neurons to survive and function. During normal neuron function, K+ ions move from the cytoplasm of the cell to the extracellular space. An uncorrected increase in these ions within the extracellular environment would cause the depolarization of the neuronal membrane, which would interrupt synaptic transmission. Astrocytes function by clearing the extracellular K+ ions through a combination of mechanisms, including active uptake, co-transport, and K+ transport channels. It is hypothesized that the function of these K+ channels are interrupted or disturbed during HIV-1 infection, which can potentiate HAND pathogenesis, however the mechanism by which this occurs is still under investigation [28].

In addition to these various functions, astrocytes play another vital role in CNS physiology, by regulating the levels of glutamate within the extracellular space. Glutamate is an excitatory neurotransmitter that is responsible for proper neuron functioning and signaling. However, excess amounts of glutamate present in the synaptic space can lead to neuronal death through excitotoxicity. Astrocytes are the main cell that is responsible for the uptake of excess glutamate, as their membranes contain the necessary transporters [29]. In fact, 90% of glutamate uptake within the brain occurs via astrocytic glutamate transporters [30]. It is believed that excitotoxicity caused by excess glutamate can lead to various neurological disorders such as

Alzheimer's disease. In HIV-1 infected individuals, this excitotoxicity can lead to HAND [18]. The mechanism of this is thought to occur via HIV-1 envelope proteins, gp-120 and gp41, which block glutamate transporters within the astrocyte membrane, which impairs the ability of these cells to uptake and clear the glutamate within the extracellular space [18].

Furthermore, the calcium ion (Ca^{2+}) plays a vital role within astrocyte function, by acting as a second messenger. Ca^{2+} in astrocytes transfers signals between astrocytes and neurons, to further cell signaling. The levels of Ca^{2+} located within the intracellular space greatly affect how well the astrocyte is able to transduce a signal as well as the plasticity of the cells within the CNS. An excess amount of Ca^{2+} within the intracellular space can result in excitability. In astrocytes specifically, Ca^{2+} is responsible for the release of neurotransmitters such as glutamate. In the case of HIV-1 infection, the viral protein Tat can affect the levels of Ca^{2+} within the intracellular space and affect these functions of the astrocyte, which can lead to neuronal excitotoxicity. Ca^{2+} ion is yet another method by which HIV-1 infection induces HIV-1 neurotoxicity and can lead to the pathogenesis of HAND [31].

As mentioned earlier, several glutamate transporters and potassium channels are located within the astrocyte processes, which are constantly in contact with the BBB. In the case of HIV-1 infection, the viral proteins alter the function of these channels within astrocytes, which compromises the integrity of the BBB. Additionally, Tat and gp120 can elevate intracellular levels of Ca^{2+}, which can cause the release of several metabolic factors that regulate the blood flow and dilation of the surrounding capillaries. These proteins also alter the expression of gap junctions in relation to the BBB, which likely increases the permeability of the BBB to harmful toxins. It is believed that it is via this mechanism that HIV-1 infection and further progress within the brain, once already established by the monocytes [30].

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Finally, astrocytes frequently communicate with neurons and vice versa, which contributes to the development of the CNS and the repair and aging process within the brain. It is therefore believed that the impairment of astrocytes via HIV-1 infection can contribute to neurological diseases, especially HAND [18]. For example, Tat released from infected astrocytes has been shown to cause cell death and mitochondrial dysfunction within surround neurons. Additionally, the supernatant of infected astrocytes contains factors that are neurotoxic to neurons. Furthermore, astrocytes that have been infected with virus can greatly affect synaptic transmission. HIV-1 Tat and gp120 activate astrocytes to release cytokines, such as TNF-α, IL-1, and IL-1β, which are known to have proinflammatory affects, and which can lead to neuronal injury within the brain [33].

In conclusion, astrocytes play a critical role in the proper functioning of the brain. These cells regulate brain homeostasis, synaptic transmission, and support the metabolic functions of the surrounds cells. Since astrocytes have so many roles, as stated above, they are of great research interest in order to elucidate the pathogenesis of HIV-1 infection within the brain. Astrocytes could potentially be a point of therapeutic intervention, especially within the realm of HIV-1 pathogenesis and HAND. This serves as the basis for the project of this thesis and why astrocytes were chosen as the cell of interest for this project.

1.8   MICRORNA

MicroRNAs are non coding RNAs generated by host cells that can be targeted as a therapeutic. MicroRNAs are small nucleotide regulatory, noncoding RNA molecules that are encoded by the host genome and are not translated into proteins [19]. MicroRNAs regulate gene expression by

binding to the 3' untranslated region of target messenger RNAs (mRNAs) through complementary base pairing. This binding leads to reduced protein expression, as the miRNA represses the translation of the mRNA or degrades the mRNA so that it is unable to be translated. These miRNA play a central role in cellular processes such as cell proliferation, differentiation, and death [19]. Some recent studies have also shown high levels of miRNA expression within the CNS as well [34].

1.9   MICRORNA AND HIV-1 INFECTION

The virus overcomes several host cellular factors in order to establish active infection within the host cell, which are known as "HIV dependency factors" (HDF's). These dependency factors can be targeted as potential therapeutics to treat HIV-1 infection. The role of miRNA in the pathogenesis of HIV-1 infection is currently an area of interest. In previous studies, some miRNAs have been shown to bind to viral RNA and inhibit the replication of the viral genome [35]. In another hypothesis, some miRNA are believed to affect HDFs in order to reduce HIV-1 virus replication [35]. For this reason, the interaction between miRNA and virus infection is of great interest, in an effort to create therapeutics to target HIV-1 within the CNS [19].

The Ayyavoo lab has shown that specific miRNAs have neuroprotective roles within the CNS in case of HIV-1 infection (Unpublished lab data). These neuroprotective miRNAs are enriched in HAND resistant subjects, which indicates that these miRNAs may play a role in preventing the onset of HAND in cases of chronic infection. Using information from the Multi Center AIDS Cohort Study, the Ayyavoo lab identified 12 HIV-1 positive patients who did not develop HAND for over 15 years after initial infection (HAND resistant group). After comparing

the transcriptomes of these patients with those of HIV-1 negative patients and HIV-1 positive patients with mild neurocognitive disorder (MND) and HIV-1 associated dementia (HAD), unique changes in the transcriptome of the HAND resistant group were found. The analysis found 21 miRNA sequences that target 98 different mRNAs in the HAND resistant group. This is in contrast to the MND and HAD groups, where only 4 miRNAs targeting 10 mRNA and 5 miRNAs targeting 30 mRNAs were found, respectively. By evaluating these miRNA and their gene targets with their biological function, the lab determined that the miRNAs that were upregulated in the HAND resistant group are associated with the gene targets of several cytokines, that have a neuroinflammatory role during neuronal injury (Table 1). This suggests that these miRNAs, if upregulated in HIV-1 positive individuals who do not develop HAND, can neutralize the effects of these cytokines in HAND pathogenesis. This study and the results obtained from the analysis serve as the rationale behind the project that is the focus of this thesis.

Table 1. Candidate miRNA Selected for Study and their Predicted Biological Effect

miRNA	Target mRNAs	Target Cell	Biological Effect
Scramble		Astrocytes	Represses inflammatory response in presence of neuronal injury.
let-7a (miR-141)	IL6R, CCL2	Astrocytes	Regulates inflammatory response by targeting CXCL12.
miR-17-5p (miR-20a and miR-106b)	IL8, STAT3	Astrocytes	Inhibits HIV-1 infection by targeting Tat mRNA and reducing its translation.

2.0   AIMS OF THE PROJECT

Listed below are the specific aims to evaluate the role of microRNAs as therapeutics to block virus replication in astrocytes.

Aim 1: To generate and characterize the astrocyte cell lines expressing candidate miRNAs along with appropriate control.

    A. Transduce normal human astrocytes with candidate miRNA using an miRNA expression vector.

    B. Confirm transduction via microscopy, flow cytometry and RNA Polymerase Chain Reaction (qRT-PCR).

Aim 2: To determine whether the overexpression of these candidate miRNA block virus replication.

    A. Generate 6 different HIV-1 viruses through transfection of 293T cells with viral plasmids.

    B. Infect normal human astrocytes and miRNA expressing astrocytes with each virus type and confirm successful transfection via fluorescent microscope.

    C. Infect TZM-bl cells with the supernatant from the infected astrocytes to determine the infectivity of the virus produced.

    D. Quantify levels of infection using TZM-bl cells based β-Galactosidase assay.

3.0   MATERIALS AND METHODS

3.1   293T CELLS

293T cells were used to initially generate the viruses used in this study. 293T cells are human embryonic kidney cells that are commonly used for virus production for their ability to become easily transfected with viral plasmids. These cells grow rapidly, allowing for the production of a large viral titer for each virus created. The 293T cells were maintained in T-75 flasks, in 10 mL of D10 media.

3.2   TZM-BL CELLS

TZM-bl cells were used in this study to test the titer of the virus created from the 293T cells as well as the infectivity of the viral proteins produced from infected astrocytes. TZM-bl cells are derived from a HeLa cell line that was engineered to express the CD4, CCR5, and CXCR4 receptors. These cells are therefore able to successfully be infected with HIV-1, and can be used to test the viral titer of a given virus. The TZM-bl cells were maintained in T-75 flasks, in 10 mL of D10 media.

3.3   ASTROCYTE CELLS

Normal human astrocytes used in this study were obtained from Gibco. These cells are human brain progenitor-derived astrocytes, which have been tested for the astrocyte-specific marker called glial fibrillary acid protein (GFAP). These cells were maintained in an astrocyte- specific media (obtained from Gibco), which was stored in -20°C. The astrocyte media consisted of DMEM, supplemented with N-2 and FBS (necessary growth factors). The astrocyte cells were maintained in T-75 flasks, in 10 mL of astrocyte-specific media.

3.4   CONSTRUCTION OF MIRNA EXPRESSING ASTROCYTE CELL LINES

This study's first aim was to construct astrocyte cell lines expressing candidate miRNA. In order to accomplish this, normal human astrocytes were transduced with candidate miRNA using a lentivirus expression vector. These miRNAs were previously identified as neuroprotective by profiling and co-expression analysis.

After transduction, puromycin resistant cells were selected for. Finally, microscopy, flow cytometry and RNA PCR were used to confirm the transduction of the candidate miRNA.

Four candidate miRNA were selected for this study:

1. Scramble: Control for miRNA constructs.

2. miR-let-7a: tumor suppressor miRNA that promotes anti-inflammatory factors [36].

3. miR- 141: regulates inflammatory response by targeting CXCL12 [37].

4. miR- 20a: inhibits HIV-1 infection by targeting Tat mRNA and reducing its translation[38].

3.5   VERIFICATION OF MIRNA TRANSDUCTION IN ASTROCYTES BY FLOW CYTOMETRY

Forty-eight hours post transduction, the miRNA transduced astrocytes were washed with PBS and fixed in 3.7% formaldehyde at room temperature for one hour. The cells were then washed and resuspended in 200μL of FACS buffer. Analysis of flow cytometry was performed on the FACSAria™ cell analyzer using the FACSDiva® software. EGFP expression was used to analyze the cells.

3.6   TOTAL RNA EXTRACTION

Total RNA extraction was performed using the *mir*Vana™ miRNA Isolation Kit. MicroRNA expressing astrocytes cell cultures were first washed with PBS and trypsinized to detach them from the T75 flask. After a ten-minute incubation period, the trypsin was deactivated with D10 media. One million cells were then spun down at 1000 rpm for five minutes to obtain the cell pellet. The cells were washed with PBS and lysed in 500 μL of Lysis/Binding Solution. Next, 50 μL of miRNA Homogenate Additive was added to the lysate and mixed for 30 seconds by vortex. The mixture was then left on ice for ten minutes. After ten minutes, 500 μL of Acid-Phenol: Chloroform was added to the solution and vortexed for approximately one minute. The cell solution was then centrifuged at 10,000rpm for 5 minutes in order to separate the organic and the aqueous layers. The aqueous layer (500 μL) was carefully removed from the cell solution and transferred to eppendorf tubes. Next the Elution Solution was heated to 95°C. 625 μL of 100% ethanol was then added to the aqueous phase, and this mixture was then passed through

the RNA binding membrane. Next, 700 μL of miRNA Wash Solution 1 was added to the filter cartridge centrifuged for 15 seconds to allow for the solution to pass through the filter. The flow-through was discarded. 500 μL of miRNA Wash Solution was then added to the filter and centrifuged similarly as in previous steps. This step was repeated a second time with another 500 μL aliquot of the Wash Solution 2/3. After three washes, the flow through was discarded and the filter cartridge was placed in a new collection tube and centrifuged for one minute to remove any residual fluid. Next, the filter cartridge was again placed in a new collection tube and 100 μL of the heated Elution Solution (95°C) was added to the filter. The collection tube was then centrifuged again for 30 seconds and the eluate was collected. Finally, the concentration of the RNA was measured by spectrophotometer. Due to the low concentration of the RNA, it was not converted to cDNA and real time PCR was not performed.

3.7 VIRUS PRODUCTION

In order to produce sufficient virus for this study, 1.0 X 10^6 293T cells were plated in 10 mL of D10 media on a 10 cm^2 tissue culture plate. Cells were grown for 24 hours to achieve 80% confluency before performing the transfection. One hour before performing the transfection, the existing was removed and replaced with 8 mL of fresh media per plate. The transfection was then done using 5 μg of viral DNA, for each of the 6 viruses of interest, in 15 μL of Polyjet transfection reagent, diluted in 250 μL of plain DMEM. This solution was then added drop-wise to each plate of 293 T cells (2 plates/virus). The transfection media was then removed after twenty-four hours, and replaced with 8 mL of fresh D10 media. The plates were the incubated for another 24 hours. Forty-eight hours after transfection, the supernatant of each plate was

collected and centrifuged at 2000 rpm for five min to remove cell debris. The supernatant was then separated into 1 mL aliquots and stored in -80°C for further use. See Table 2 for list of viruses of interest in this study.

Table 2. Viruses of Interest

Virus	Targeted Receptor
NL43	CXCR4
NL43 + VSV-G Env	NA
NLYU25A (brain isolate)	CCR5
NLYU25A + VSV-G Env	NA
NLAD8 (Macrophage Isolate)	CCR5
NLAD8 + VSV-G Env	NA

3.8   QUANTIFYING VIRUS TITER

Virus titer was calculated by using a TZM-bl assay using the supernatants of the transfected 293T cells. 8.0×10^3 TZM-bl cells were plated in triplicate within a 96 well flat bottom plate for 24 hours. Upon reaching over 80% confluency, the cells were infected with various concentrations of virus: 1:10, 1:100, and finally, 1:1000. After 48 hours, the cells were observed under fluorescent microscope. In order to calculate the virus titer, the number of green cells was counted in the wells containing the 1:1000 dilution. The average of the triplicate was calculated and the resulting value was multiplied by 1,000 to obtain the number of infectious particles per μL of media.

3.9   INFECTION OF NORMAL HUMAN ASTROCYTES

In order to transfect normal human astrocytes, 50,000 astrocyte cells were plated in a 24-well culture plate, with 200 µL of astrocyte media, and incubated for 24 hours. Once reaching over 85% confluency, the cells were infected with 1 MOI and 0.1 MOI concentrations of the respective virus. The cells were then incubated. Twenty-four hours post-transfection, the existing astrocyte media was removed, the cells were washed with excess PBS solution, and 200 µL of fresh astrocyte media was added to the wells. Forty-eight hours post transfection, the cells were then observed under fluorescent microscope for evidence of green fluorescence. While the number of green cells were not counted, the appearance of green fluorescence was noted for each virus.

3.10   INFECTION OF MIRNA TRANSDUCED ASTROCYTES

A similar procedure as stated above was performed in order to infect the miRNA expressing astrocytes. However, since these cells already express green fluorescence due to EGFP present in the miRNA vector, the infectivity of the virus was not determined via observation of green fluorescence under the microscope. In order to determine the infectivity of the virus, the supernatant was taken from these cells 48 hours post-transfection and added to plated TZM-bl cells in a 96-well flat bottom culture plate (procedure below).

3.11 INFECTION OF TZM-BL CELLS WITH SUPERNATANT FROM INFECTED ASTROCYTES

In order to test the infectivity of the viral proteins produced by the infected normal and miRNA astrocytes, the supernatant from these cells was added to TZM-bl cells. First, 8,000 TZM-bl cells were plated in a 96-well flat bottom plate with 100 µL of D10 media and incubated overnight until greater than 80% confluency was reached. Viral supernatant, in varying concentrations (100 µL and 10 µL), was added directly to the TZM-bl assay. The cells were then incubated for 48 hours. After 48 hours, the cells were observed under fluorescent microscope for evidence of green fluorescence.

3.12 BETA GALACTOSIDASE ENZYME ASSAY TO TEST THE INFECTIVITY OF VIRUS PRODUCED BY THE INFECTED MIRNA EXPRESSING ASTROCYTES

Seventy- two hours post infection, supernatant from the miRNA expressing astrocytes were collected and the infectivity of the virus released in the supernatant was measured using TZM-bl assay and β- Galactosidase assay as described by the manufacturer protocol. Briefly, supernatants were incubated with the TZM-bl cells in a 96-well plate for forty-eight hours. At this point, the cells were washed and lysed in 50 µL of lysis buffer. After five minutes of lysis on ice, 50 µL of Beta-Glo® reagent (Promega, Madison, WI) was added to the TZM-bl cell monolayers, and the reaction mixture was transferred to a black 96-well plate and chemiluminescence activity was read using a luminometer.

4.0 RESULTS

4.1 GENERATE ASTROCYTE CELLS EXPRESSING CANDIDATE MIRNA

4.1.1 Verification of transduction via fluorescent microscopy

Fluorescent microscopy was used to verify the successful transduction of the miRNA expression vector in the normal human astrocyte cell line. Since the expression vector contained Enhanced Green Fluorescent Protein (EGFP), the cells were observed under green fluorescence light under the microscope. Evidence of green fluorescence indicates successful transduction of all four candidate miRNAs of interest within this study (Figure 2).

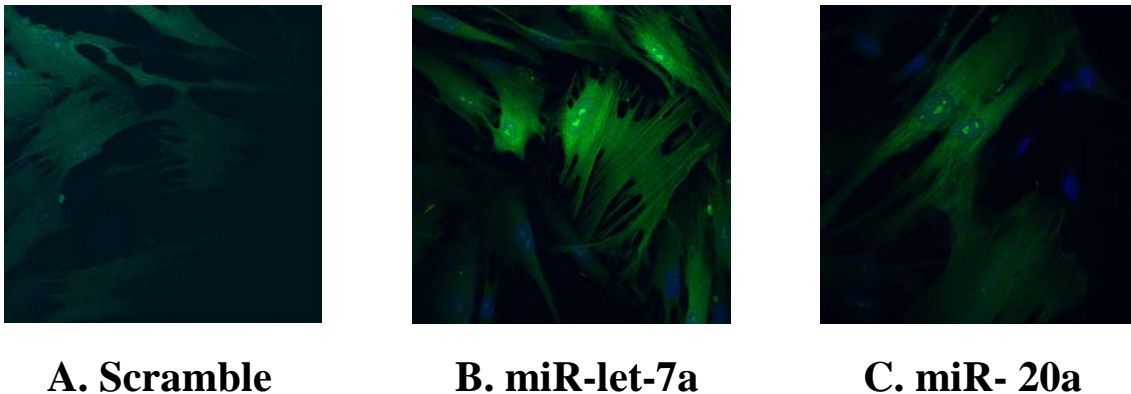## A. Scramble   B. miR-let-7a   C. miR- 20a

Figure 2. Fluorescent Microscope Images of miRNA transduced astrocytes

MicroRNA transduced astrocytes were grown on a cover slip and fixed with 2% paraformaldehyde. Coverslip was viewed under the fluorescence microscope and images were captured at 40X magnification.

4.1.2 Verification of Transduction via Flow Cytometry

Further analysis was performed via flow cytometry to see if the miRNA were transduced into the normal human astrocyte cells. The results from the flow cytometry show that more than 90% of cells are EGFP+, indicating successful transduction of the candidate miRNA (Figure 3).
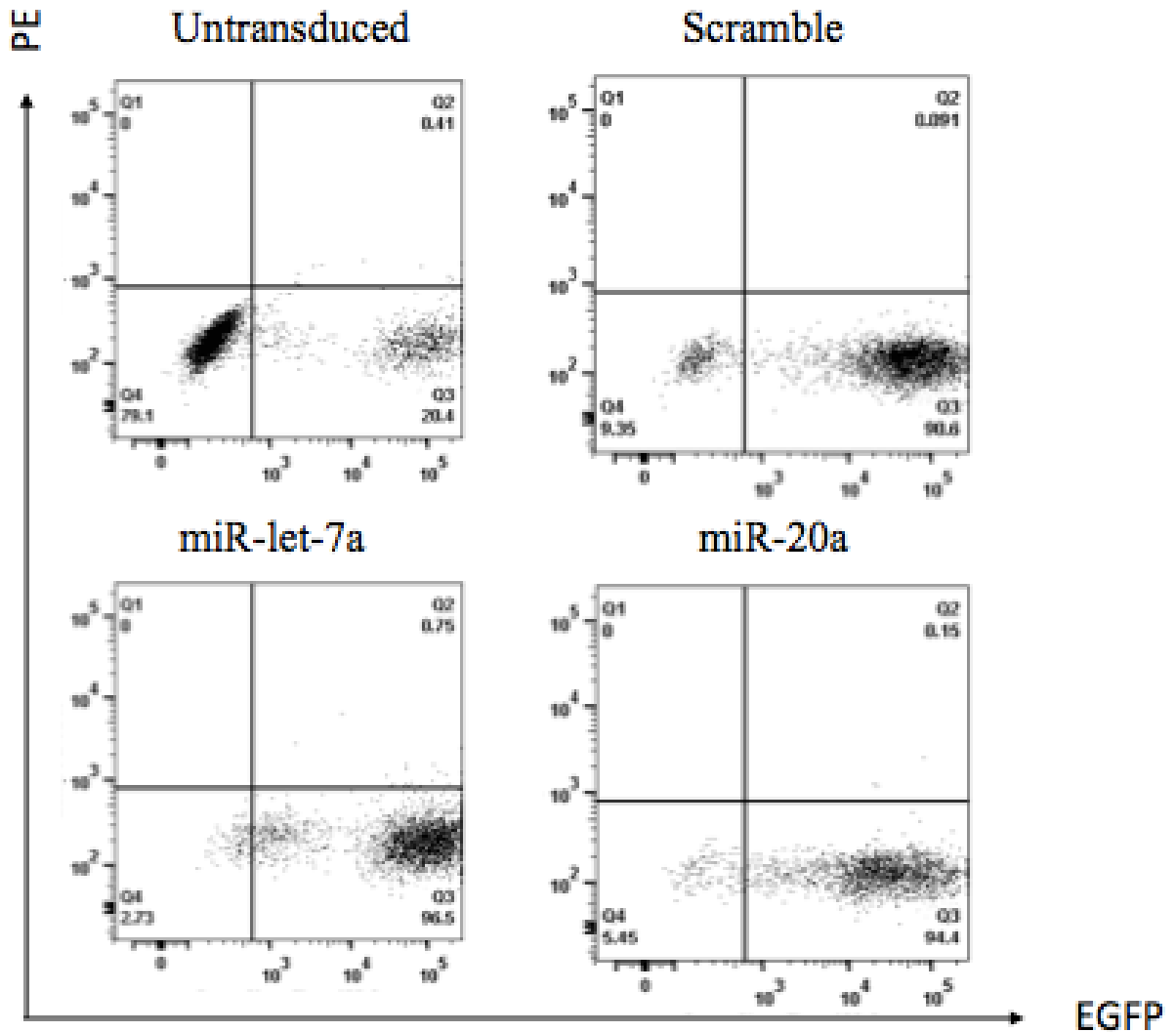

Figure 3. Flow Cytometry Results of miRNA Expressing Astrocytes.

MicroRNA transduced astrocytes were grown and fixed with 2% paraformaldehyde and measured by flow cytometry. The untransduced astrocytes show 20% green fluorescence. Scramble expressing astrocytes show 90% green fluorescence. The astrocytes expressing miR-let-7a and miR-20a are 96% and 94% EGFP+, respectively.

4.1.3   Total RNA Extraction

RNA extraction was done in order to prepare for Real Time PCR to verify the transduction of the miRNA into the astrocyte cell line. Results are summarized in Table 3. Nucleic acid concentrations were low in all four miRNA types, and therefore Real Time PCR was not performed.

Table 3. RNA Extraction Results

miRNA	NA (ng/µL)	A260/A280
miR-20a	63.5	2.11
miR-106b	48.0	2.05
miR-let-7a	30.3	1.84
miR-Scr	42.4	2.09

4.2   INFECTION OF NORMAL AND MIRNA EXPRESSING ASTROCYTES

4.2.1   293T Infectivity Assay

Table 4 shows the results of the infectivity assay conducted for all six viruses of interest for this study. The data are given as the number of infectious particles per mL. The viruses made with VSV-G had the highest titer, with NLAD8 with VSV-G having the highest titer of all six viruses. NLYU25A and NLYU25A + VSV-G had the next highest titer. Finally, the NLAD8, NL43 + VSV-G, and NL43 showed the lowest titer. This information was used to plan the next steps of the experiments, specifically when determining the amount of each virus that is needed to infect the normal human astrocytes and miRNA expressing astrocytes.

Table 4. Estimation of virus titer using TZM-bl cells

Virus	Titer (Infectious particles/ mL)
NL43	0.70×10^5
NL43 + VSV-G	0.98×10^5
NLYU25A	0.10×10^6
NLYU25A + VSV-G	1.5×10^5
NLAD8	0.80×10^5
NLAD8 + VSV-G	8.1×10^5

4.2.2   Verification of Infection of Normal Human Astrocytes via Fluorescent Microscope

Fluorescent microscopy was used to determine if the virus was successful in infecting the normal human astrocytes. The astrocytes plate was read 48 hours post transfection. Results are summarized below in Table 5. The experiment was repeated three times in order to gain a consensus on the ability of each virus to infect the astrocytes.

All viruses, in both concentrations, that utilized the VSV-G envelope protein were able to successfully infect the normal human astrocytes, as observed under the fluorescent microscope. Additionally, the NL43 virus consistently was able to infect the astrocytes, in both concentrations of virus tested. The NLYU25A virus was not able to infect the astrocytes. NLAD8 showed inconsistent results, however from the last two experiments, the virus was not able to infect. Finally, the YU2 and AD8 viruses, used as positive controls, were not able to infect the astrocytes, however their VSV-G counterparts showed evidence of successful infection.

Table 5. Transfection of Normal Human Astrocytes: Evidence of Green Fluorescence as Observed under Microscope

Results of Infection of Normal Human Astrocytes						
	Experiment 1		Experiment 2		Experiment 3	
Virus of Interest	50 µL	5 µL	1 MOI	0.1 MOI	1 MOI	0.1 MOI
NL43	+	+	+	+	+	+
NL43 + VSV-G	+	+	+	+	+	+
NLYU25A	−	−	−	−	−	−
NLYU25A + VSV-G	−	+	+	+	+	+
NLAD8	+	+	−	−	−	−
NLAD8 + VSV-G	+	+	+	+	+	+

Results of Infection of Normal Human Astrocytes- Positive Control						
	Experiment 1		Experiment 2		Experiment 3	
Virus of Interest	50 µL	5 µL	1 MOI	0.1 MOI	1 MOI	0.1 MOI
YU2	NI	NI	−	−	−	−
YU2 + VSV-G	NI	NI	+	+	+	+
AD8	NI	NI	NI	NI	−	−
AD8 + VSV-G	NI	NI	NI	NI	+	+

Table Legend	
+	Green
−	No Green
NI	Not Included

4.2.3 Verification of Infection of miRNA Expressing Astrocytes via TZM-bl Infectivity Assay

Since the miRNA expressing astrocytes already express green fluorescence due to the presence of EGFP within the vector, fluorescent microscopy was not used to determine whether these astrocytes were infected with the viruses of interest. Instead, in order to further determine the infectivity of the miRNA expressing astrocyte supernatant, β-Galactosidase assays were

performed on the infected TZM-bl cells to measure chemiluminescence activity. Forty- eight hours post transfection, the cells were first lysed with lysis buffer for five minutes, after which 50 µL of Beta-Glo® reagent was added to the cells to measure chemiluminescence activity. Results are summarized in the figure below (Figure 4). Figure 4A suggests that miR-20a and miR-141 did not significantly increase NL43 infectivity ($p > 0.05$). miR-106b significantly increases NL43 infectivity ($p < 0.05$). Figure 4B suggests that mi-R20a did not significantly decrease virus infectivity of NL43 + VSVG ($p > 0.05$), and miR-106b and miR-let-7a did not significantly increase infectivity ($p > 0.05$). Furthermore, figure 4C shows that miR-20a, miR-106b, miR-let-7a did not significantly increase virus infectivity of NLYU2 ($p > 0.05$). Figure 4D suggests that mi-R20a and miR-106b did not significantly decrease virus infectivity of NLYU2 + VSVG ($p > 0.05$), and miR-let-7a did not significantly increase infectivity ($p > 0.05$). Figure 4E shows that mi-R20a did not significantly decrease virus infectivity of NLAD8 ($p > 0.05$), and miR106b and miR-let-7a did not significantly increase infectivity ($p > 0.05$). Finally, Figure 4F suggests that miR-20a, miR-106b and miR-let-7a did not significantly increase virus infectivity of NLYU2 + VSVG ($p > 0.05$). Due to the relatively small sample sizes used in these experiments, these results should be confirmed by multiple repeat experiments.
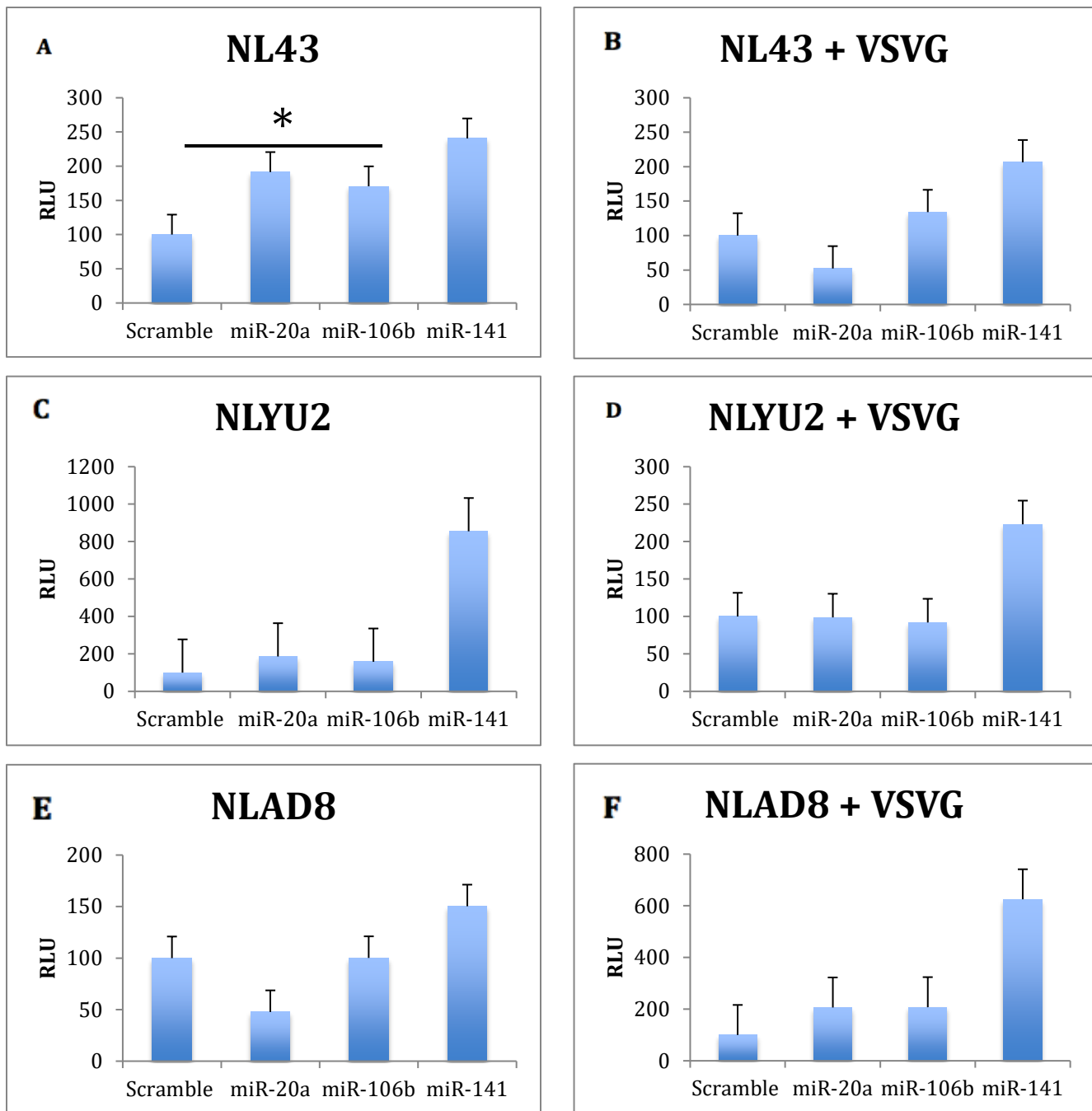
Figure 4. β-Gal Assay to Quantify Virus Infectivity in TZM-bl cells

β-Galactosidase assays were performed on the infected TZM-cells. Forty-eight hours post transfection, the cells were lysed with lysis buffer for five minutes, after which 50 μL of Beta-Glo® reagent was added to the cells and chemiluminescence activity was measured. The assay was done twice. **A.** miR-20a and miR-141 did not significantly increase NL43 infectivity ($p > 0.05$). miR-106b significantly increases NL43 infectivity ($p < 0.05$). **B.** mi-R20a did not significantly decrease virus infectivity of NL43 + VSVG ($p > 0.05$), and miR-106b and miR-let-7a did not significantly increase infectivity ($p > 0.05$). **C.** miR-20a, miR-106b, miR-let-7a did not significantly increase virus infectivity of NLYU2 ($p > 0.05$). **D.** mi-R20a and miR-106b did not significantly decrease virus infectivity of NLYU2 + VSVG ($p > 0.05$), and miR-let-7a did not significantly increase infectivity ($p > 0.05$). **E.** mi-R20a did not significantly decrease virus infectivity of NLAD8 ($p > 0.05$), and miR106b and miR-let-7a did not significantly increase infectivity ($p > 0.05$). **F.** miR-20a, miR-106b and miR-let-7a did not significantly increase virus infectivity of NLYU2 + VSVG ($p > 0.05$).

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5.0   CONCLUSION

The study's first aim was to generate an astrocyte cell line that expresses the selected candidate miRNA. This was successfully accomplished through the use of a miRNA lentivirus expression vector that expresses the Enhanced Green Fluorescence Protein. Through the use of microscopy and flow cytometry, the transduction was shown to be successful, as both procedures produced evidence of green fluorescence. While the initial plan was to perform Real Time PCR on these cells to further confirm the transduction, PCR was not conducted due to low concentration of RNA in all four miRNA expressing astrocytes.

The second aim of this study was to investigate whether the overexpression of the candidate miRNA within astrocytes blocks viral replication. The results from this study first show that HIV-1 is able to infect normal human astrocytes. In particular, NL43 and NL43+VSVG are able to infect. However, NLYU2 and NLAD8 are not able to infect these astrocytes, but are able to infect when trans-complemented with VSV-G Envelope.

Furthermore, the results from this study show that host factors, specifically miRNA, have the potential to play a role in the replication of HIV-1 within astrocytes. From the β-Gal assay, there appears to be some affect of the candidate miRNA on virus infectivity within TZM-bl cells. The NL43 virus infectivity was shown to increase in all three cases of miRNA. This increase was significant in the astrocytes expressing miR-106b ($p < 0.05$). However, miR-20a appears to decrease infectivity of NL43 + VSVG, NLYU2 + VSVG, and NLAD8. miR-106b appears to

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have mixed affects on each individual virus, with no significant differences in any. Finally, miR-let-7a appears to increase the infectivity of all six viruses used in the study.

While these results represent a preliminary investigation into the role of miRNA in virus replication within astrocytes, more research should be done to further elucidate the mechanism(s) of action of these miRNAs. The findings of this study add to existing literature by demonstrating a relationship between miRNA expression and virus infectivity of HIV-1 within astrocytes. The role of miRNA in virus replication is an area of HIV-1 research that should studied in greater detail, in order to potentially create a therapeutic to combat HIV-1 replication within astrocytes and mediate the effects of HAND in HIV-1 positive individuals.

6.0   DISCUSSION

Since 1996, with the availability of combination antiretroviral therapy (cART), the morbidity and mortality rates of HIV-1 infection have decreased significantly, and the life expectancies of those who receive treatment have increased [39]. Along with these improvements, the incidence of HIV-associated Dementia, the most severe neurological disorder associated with HIV-1 infection, has also decreased. However, neurological complications associated with HIV-1 infection still persist in milder forms, which are collectively known as HIV-associated neurocognitive disorder (HAND) [39-40]. Despite the success of cART in treating infection and improving the immune function of those infected, more attention needs to be devoted to understanding the comorbidities associated with HIV-1 infection within the CNS, in order to improve both diagnosis and treatment plans. HAND affects between 30-70% of all HIV-1+ patients, and even those patients who have an undetectable viral load due to effective treatment with cART experience neurological dysfunctions due to HIV-1 infection [40-42]. This indicates that there needs to be a greater focus in studying and developing a therapeutic for the neurological symptoms associated with HIV-1 infection, especially since HIV-1 positive patients are living longer lifespans.

The results of this study provide support for the need of further research into the neuropathogenesis of HIV-1 within the CNS. By demonstrating an affect of candidate miRNA on the infectivity of the viruses studied in this project, it is clear that these miRNA do in fact play

a role in the virus replication process within astrocytes. More research is needed to elucidate the mechanism of action of these miRNAs in order to determine whether they specifically play a helpful or harmful role within the pathogenesis of HIV-1 within the brain. The affect of miRNA on virus infectivity and replication suggests the potential for these candidate miRNA to serve as therapeutics to combat the neurocognitive symptoms that occur over time in HIV-1 positive individuals.

6.1.1 Limitations

While the results of this study offer preliminary insights into the role of candidate miRNA in the pathogenesis of HIV-1 within the CNS, the experiments need to be repeated to test for consistency and confirm the relation between the miRNA and virus infectivity. In order to confirm the successful transduction of the candidate miRNA, real time RNA PCR must be conducted. Since the nucleic acid concentration was found to be too low during RNA extraction, PCR was not conducted. This experiment should be repeated using a greater number of miRNA transduced astrocytes, in order to successfully carry out the PCR and confirm the transduction.

Furthermore, the virus titers of all six viruses used in this project were fairly low, which could affect the levels of infectivity seen within both the normal and miRNA expressing astrocytes. In future studies, viruses with a greater titer should be used in order to increase the efficiency of the virus infection and obtain more consistent results. Additionally, the VSV-G envelope protein was used to increase infection efficiency within the experiments. However, this method of infection is artificial and does not represent the way the HIV-1 virus enters a host cell in nature. Future studies should take this into consideration and could perhaps use more natural

methods of viral entry, as this may affect the ability of the miRNA to regulate the viral replication and infectivity of HIV-1.

6.1.2   Future Directions

The results of this study lend themselves to further research that will be useful in determining the specific role of miRNA in the neuropathogenesis of HIV-1 within the CNS. This study focused on the infection and viral replication of HIV-1 within astrocytes. However, since astrocytes have inflammatory properties, the role of miRNA in regulating neuroinflammation can be a future study that stems from this project. Previous studies have shown that astrocytes become activated and inflammatory in the presence of neuronal injury [18-33]. These activated astrocytes, known as A1 astrocytes, then release toxic factors that can cause cell dysfunction and death of surrounding neurons, which can then contribute to the pathogenesis of neurocognitive disorders within the brain [43]. Furthermore, A2 astrocytes, which are another type of activated astrocyte, have been shown to have neuroprotective properties in the presence of neuronal injury [44]. The affect of miRNA on viral replication of these specific astrocyte types could further elucidate the mechanism of action of these miRNA in the neuropathogenesis of HIV-1 within the CNS.

In addition, there have been several hypotheses for other modes of HIV-1 infection within astrocytes. One of these hypotheses proposes that astrocytes become infected through the cell-to-cell transfer of HIV-1 virus from macrophages to astrocytes. Astrocytes have also been shown to have endocytotic properties, which could be another way the virus enters these cells [45-47]. In future, studies, co-culturing infected macrophages with astrocytes could be another way to induce infection within these cells and study the role of miRNA in the viral replication of virus that is obtained in this way.

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The questions explored in this project can contribute to the existing scientific knowledge regarding the role of host factors on the pathogenesis of HIV-1 within the central nervous system. This research combined with research in the areas mentioned above can significantly help in the development of more specific therapeutics in treating HIV-1 CNS infection and HAND. Since the prevalence of HAND is increasing, research investigating HAND pathogenesis is of great public health significance.

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