Standardization and Optimization of the Detection of Neurogranin (Nrgn), a Synaptic Protein, using the 3D CNS Organoid Model of Human Immunodeficiency Virus (HIV-1) Infection

by

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Standardization and Optimization of the Detection of Neurogranin (Nrgn), a Synaptic Protein, by Human Immunodeficiency Virus (HIV-1) Infection in the CNS using the 3D Organoid Model

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Human Immunodeficiency virus 1 (HIV-1) enters the central nervous system (CNS) and establishes infection during early stages of infection within the brain. Despite advancements in treatment with combination of antiviral therapies (ART), this chronic infection results in HIV-1 associated neurological disorders (HAND) in more than 60% of infected individuals, characterized by memory impairments and executive function deficits in people with HIV-1 (PWH). Although ART successfully controls systemic viral load, low level viral replication still occurs in the brain, creating chronic neuroinflammation due to release of viral protein and other neurotoxic factors which then leads to neuronal damage. Understanding how HIV-1 damages neurons in the brain is needed to develop treatment options and to improve the quality of life in PWH. Neurogranin (Nrgn) is a postsynaptic protein found in the dendritic spines of neurons and plays an important role in synaptic plasticity, memory, and learning. Nrgn has previously been shown to be dysregulated by HIV-1 infection, but the mechanism is less understood. To further understand Nrgn dysregulation within the CNS, our laboratory has recently developed an *in vitro* 3D CNS organoid model. My project aims to standardize and optimize the immunofluorescent staining protocol for the detection of Nrgn along with the identification of multiple cell types found within the 3D HIV-1 CNS organoid in 2D cell culture, human brain tissue samples and ultimately 3D organoid tissue samples.

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1.0 Introduction

It is estimated that more than 84 million individuals have been infected with human immunodeficiency virus (HIV-1) since it first appeared in the 1980s (1). As of 2021, there are estimated to be over 38 million people living with HIV-1 with about 1.5 million new cases and around 650,000 deaths in the year alone (1). With the widespread availability and use of combination antiretroviral therapies (cART), the number of deaths associated with HIV-1 infection has decreased and people with HIV-1 (PWH) are living longer lives (1-3). Despite the advancements made with cART, HIV-1 establishes viral reservoirs within different compartments of PWH, including the CNS. Due to the chronic infection, PWH are at an increased risk for HIV-1-associated comorbidities such as cardiovascular disease, cancer, premature aging, and neurocognitive disorder (4-8). It has been estimated that half or more of PWH develop HIV-1-associated neurocognitive disorders (HAND) even when adhering to cART regimens (9-11). While the mechanisms behind it are not well understood, infection within the CNS causes neurodegeneration that is often accompanied by functional impairments (12, 13). These complications associated with HAND and other comorbidities can greatly impact the quality of life for PWH and taken with the number of deaths, HIV-1 continues to have a significant impact.

1.1 Human Immunodeficiency Virus-1 (HIV-1)

1.1.1 Pathology of HIV-1

HIV-1 primarily spreads from the exchange of body fluid which can occur through multiple ways including sexual intercourse with an individual living with HIV-1, mother to child transfer through vaginal delivery or breastfeeding, or unsafe use of drug paraphernalia including sharing contaminated needles (14). While there is no cure for HIV-1, advancements in combination antiretroviral therapy (cART) have made significant impacts on preventing HIV-1 transmission and spread. This treatment consists of a combination of different antiretroviral drugs that work together to reduce the viral load within the periphery of people with HIV-1 (PWH) and prevent transmission of the virus (15-17). Since 2010, there has been a 52% decrease in the amount of new HIV-1 infection among children and a 30% decrease in new infection among adults globally (1). Improved blood screening along with the recent introduction of prophylactic treatments to high-risk populations has greatly improved the prevention of HIV-1 transmission (18-21).

After initial HIV-1 exposure, it takes about 2 weeks to detect the virus in the infected individual's blood. Symptoms during acute infection are highly variable and can present as asymptomatic but most individuals experience flu-like symptoms, such as fever, headache, or rash (22). HIV-1 targets cells that express CD4, the main HIV-1 receptor, along with other necessary coreceptors, such as CCR5 or CXCR4 (23, 24). This includes monocytes, macrophages, dendritic cells and CD4+ T cells (24-26). Given their importance in the immune response and the high expression of necessary receptors, CD4+ T cells are a primary HIV-1 target. Once infected, initiation of apoptosis via activation of caspase enzymes occurs and results in a significant depletion in the number of CD4+ T cells (27, 28). HIV-1 is able to establish several viral reservoirs

within the periphery and the CNS during this period (25, 26, 29, 30). Due to the high rate of mutation exhibited by HIV-1, the host's immune system is unable to fully eliminate the virus, but it does significantly reduce the viral load until reaching the viral set point (15, 16, 31). Once the viral set point has been established, the number of CD4+ T cells may rebound but they do not fully recover. This indicates the beginning of chronic HIV-1 infection (22).

HIV-1 is able to establish latent infection due to the inability of the host to fully eliminate the viral reservoirs within the body. Within these latent viral reservoirs, HIV-1 is still undergoing low-level replication and continually releasing several viral proteins (32, 33). HIV-1 infection will continue to progress if left untreated and will continue to deplete CD4+ T cells. When the total count of CD4+ T cells drops below 200 cells/mm^3, this is considered to be the final stage of infection known as acquired immunodeficiency syndrome (AIDS) (28). AIDS can be defined by the occurrence of certain opportunistic infections such as pneumonia, cytomegalovirus (CMV), Kaposi's sarcoma (KS), or tuberculosis (TB) due to the significantly weakened immune system (28). Progression to AIDS typically occurs within 8 to 10 years when left untreated (22). However, adherence to a strict cART regimen allows for PWH to maintain asymptomatic, chronic HIV-1 infection for decades and prevents progression to AIDS by controlling systemic viral load (3, 19).

1.2 HIV-1 Neuropathogenesis and HIV-1-Associated Neurological Disorder (HAND)

1.2.1 HIV-1 Neuroinvasion

Several studies have shown that HIV-1 can enter the central nervous system (CNS) within two weeks of initial infection (34, 35). When necessary, circulating monocytes within the

periphery can cross the blood-brain barrier (BBB) and enter the CNS in order to differentiate into macrophages to replace those that may have been injured or damaged. It is thought that HIV-1 is able to enter the CNS by this same mechanism, referred to as the Trojan horse theory. It does so by first infecting a circulating monocyte within the periphery that will then travel to the CNS and differentiate into an infected resident macrophage (36-39). Once these infected cells have entered the brain, they are able to spread HIV-1 infection to surrounding cells of the CNS, including microglia and astrocytes. While astrocytes lack expression of CD4, the primary receptor for HIV-1 entry, there is evidence that supports infection of astrocytes through nonconventional mechanisms, such as cell-to-cell transfer or via endocytosis of virions, viral proteins, and other infectious particles (40-44). However, infection of astrocytes remains controversial as some evidence suggests that while astrocytes in postmortem tissue show integrated HIV-1 DNA, this could be contributed to the astrocyte's ability to engulf infected macrophages while others found no HIV-1 DNA or RNA (45, 46). Macrophages and microglia are major targets for latent HIV-1 infection within the CNS, partially due to their longer life span (47-50). Once infected, the cells of the CNS will begin to secrete cytokines, chemokines and other neurotoxins that ultimately result in neuroinflammation (32, 48, 51).

1.2.2 HIV-1- Associated Neurocognitive Disorder (HAND)

HIV-1-associated neurocognitive disorder (HAND) refers to a wide range of neurocognitive impairments that PWH often experience even when viral suppression within the body is achieved. HAND can be further classified based on severity (52). The least severe form, asymptomatic neurocognitive impairment (ANI), can be determined through psychological tests that indicate a decline in certain neurocognitive abilities without direct impact to daily life, staying

in the asymptomatic stage. Mild neurocognitive disorder (MND) occurs when impairments in the neurocognitive abilities begin to impact daily life and go beyond what is expected with normal aging. HIV-1 associated dementia (HAD) is the most severe form of HAND. HAD is associated with loss of memory, loss of motor skills, and other signs of cognitive decline that significantly impacts daily life and are not contributed to other comorbidities (52). Before the widespread availability of cART, HAD was the prevalent form of HAND and was associated with high mortality (53-55). In the post cART era, HAD prevalence has diminished and is considered to be rare (10, 56). However, possibly due to the poor penetrations of cART drugs across the BBB, the prevalence of HAND still remains around 50% among PWH with cases presenting as milder to moderate impairments (9-11). Further understanding the mechanisms behind HAND can help in identifying biomarkers and other therapeutic targets for treatment and prevention of HAND.

1.2.3 Neuropathogenesis of HAND

While the exact mechanisms behind HIV-1 neurodegeneration are not fully understood and are highly complex, it is known that neurons are not infected by HIV-1. However, they are most affected by the virus due to indirect damage caused by neighboring HIV-1 infected cells (13, 55, 57). Infected microglia and macrophages are continuously releasing neurotoxic factors that result in neuronal damage and neurodegeneration (**Figure 1**). These neurotoxic factors include viral proteins such as gp120, Tat, Nef, or Vpr, chemokines such as CCL2 or CCL3, and cytokines such as TNF-a, IL-1B, IL-6, or IL-10 (48, 49, 57-59). In response to the neurotoxins nearby immune cells undergo activation. These activated microglia and macrophages then begin releasing additional neurotoxic factors such as free radicals, and additional IL-1B and TNF-a (26, 50, 57). The combined release of both host and viral neurotoxic factors promotes (37, 46, 47, 56-58).

Neuroinflammation leads to the hyperactivation of the N-methyl-D-aspartate receptors (NMDAR), a member of the glutamate receptor family involved in synaptic transmission in the CNS (60-63). Known as excitotoxicity, overactivation of NMDARs can cause large influxes of calcium ions within the neurons, leading to synaptic damage and potentially cell death (64-66). To overcome the hyperactivation of NMDARs and attempt to restore homeostasis, neurons will undergo synaptic scalding and significantly decrease the number of excitatory synapses (66-68).

Due to the inability to fully clear HIV-1 from the viral reservoir within the CNS even with strict adherence to cART, infected cells continuously produce low levels of soluble viral proteins (15, 36, 42, 49, 59, 69). Viral proteins such as gp120, Vpr, Nef, and Tat are able to induce neurodegeneration. Some studies using 2D cell culture experiments have shown that exposure to the viral proteins gp120 and Tat can result in synaptic damage (65, 70, 71). The accessory proteins Vpr along with gp120 have been shown to induce neuronal apoptosis (64, 72). Rodent models that have transgenic expression of either gp120 or Tat result in deficits in learning and memory formation (73-76). Gp120 is an envelope protein that is needed for HIV-1 entry into a host cell and is thought to contribute to synaptic degeneration through the hyperactivation NMDARs. This can be either indirectly through inducing more cytokine release or directly by binding to activation sights on NMDARs (60). The viral protein Tat, a regulatory protein for viral transcription, is also able to contribute to the hyperactivation of NMDARs and synaptic degeneration through similar methods such as inducing cytokine and chemokine release (66, 70). However, the mechanisms behind HIV-1 neurodegeneration are very complex and a further understanding of both direct and indirect effects of HIV-1 is needed.

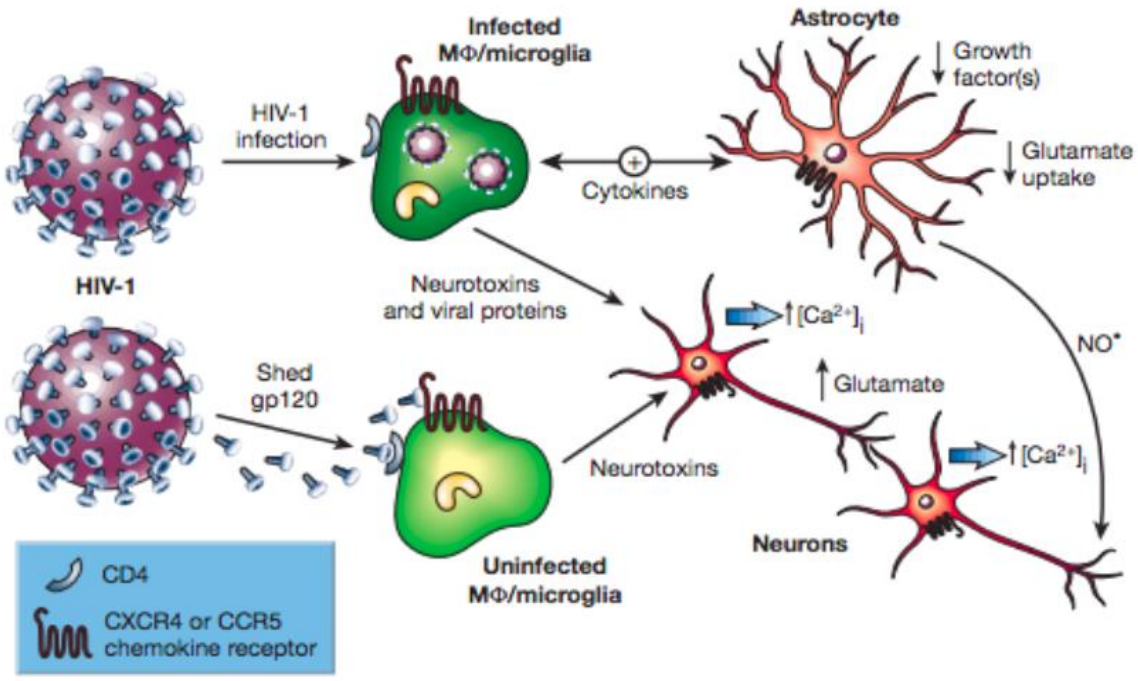

Figure 1. Model of HIV-1 related neuronal injury (77)

HIV-1 infection is able to induce neuronal injury and death, both directly through infected microglia and indirectly through neurotoxins

1.3 Synaptic Plasticity and Neurogranin

1.3.1 Role of Synaptic Plasticity in HIV-1 Neurodegeneration

Synaptic plasticity refers to the ability of neurons to change the strength of synapses over time in response to stimuli and is vital in learning and memory formation within the brain. The two main types of synaptic plasticity include long-term potentiation (LTP) and long-term depression (LTD) (78). Induction of an LTP leads to an increase in synaptic strength and density while an LTD is the opposite, it weakens synapses (78). Both mechanisms require the activation

of the NMDARs, which occurs when a neuron receives a stimulus that results in an influx of calcium ions (Ca2+) (78). However, the difference between the induction of either an LTP or an LTD is dependent on which Ca2+/calmodulin (CaM)- dependent enzyme is activated within the cell based on the calcium ion influx. When there is a large influx of Ca2+, it leads to the activation of the Ca2+/CaM-dependent protein kinase II (CaMKII) and results in an LTP (79). When there is a low-level influx of Ca2+, it leads to the activation of the protein phosphatase calcineurin via CaM, resulting in an LTD due to the high Ca2+ sensitivity of calcineurin (80). Additionally, activation of calcineurin is able to decrease the time that the NMDA receptors are open, contributing to the smaller influx of Ca2+ (81).

1.3.2 Neurogranin, a Postsynaptic Protein

Neurogranin is a post synaptic protein found within neurons, primary in the dendrites and is involved in the synaptic signaling pathway (**Figure 2**) (82). The main function of Nrgn is to bind to and sequester free calmodulin (CaM), normally bound to calcium ions (83). As previously stated, Ca2+ and CaM are important factors in synaptic plasticity and are involved in the production of an LTP. CaM will remain bound to Nrgn until intracellular Ca2+ concentration increases enough to cause Nrgn to release it, resulting in the activation of downstream signaling. Altering Nrgn expression can also alter synaptic strength and density. Overexpression of Nrgn can increase synaptic strength by increasing CaMKII activation resulting in elevated synaptic strength (84, 85). However, the same effect is not achieved when simply increasing CaM levels, demonstrating the importance of Nrgn (86). When Nrgn is depleted, such as in Nrgn-knockout mice, it results in learning and memory deficits, implicating an important role in synaptic plasticity (87, 88).

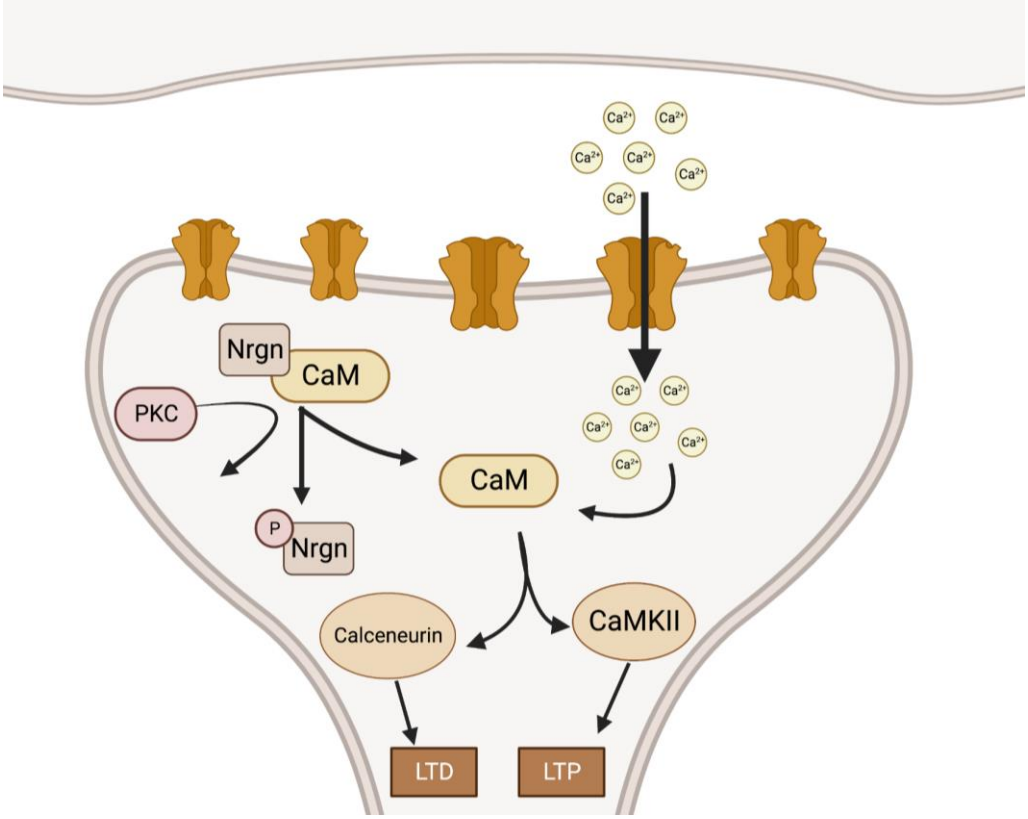

Figure 2. Role of neurogranin in postsynaptic signaling pathway

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1.3.3 Neurogranin as a Potential Biomarker of Neurological Disease

Synaptic plasticity and integrity have been key points of interest within a wide variety of neurological diseases such as Alzheimer's disease, Creutzfeldt-Jakob disease, Parkinson's disease, and HAND (90, 91). In several of these diseases, neurogranin has been identified as a potential biomarker given its important role in synaptic degeneration. In the case of HAND, the expression of Nrgn is significantly reduced in HAND-positive samples compared to uninfected controls (92). Multiple studies support the finding that Alzheimer's patients have higher levels of Nrgn in the CSF in comparison to healthy controls (93-96). Similar results were found in Parkinson's patients

and increasing levels of Nrgn within the CSF was associated with increased cognitive decline and disease progression (97). However, in both Alzheimer's disease and Parkinson's disease, there was no significant correlation found between blood Nrgn levels and disease diagnosis or progression (98, 99). These data support Nrgn as a promising biomarker for different neurodegenerative disorders (95, 96, 99).

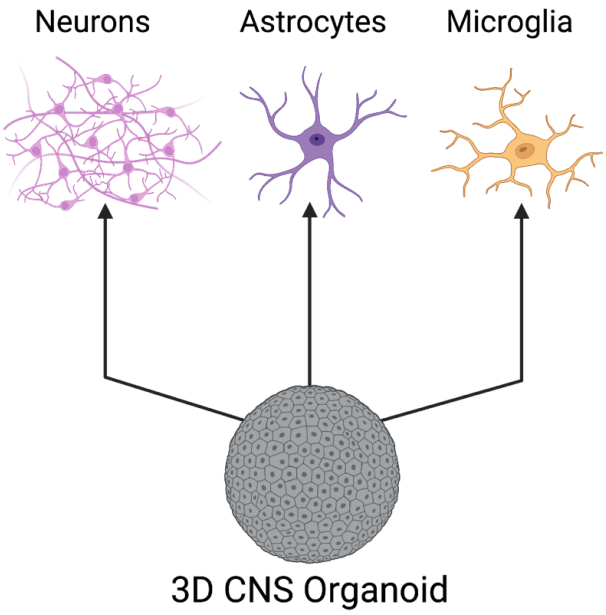

Figure 3. Cells of the 3D CNS prganoid

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1.4 Using 3D Organoids to Study HIV-1 Neuropathogenesis

Studying HIV-1 associated neurocognitive disorders poses unique challenges due to the complexities surrounding the brain. Current models of HIV-1 neuropathogenesis consist of post-mortem HIV-1 infected brain samples, SIV-infected non-human primate studies, rodent models,

and 2D cell cultures. Post-mortem tissues offer limited time points within CNS infection and animal models are unable to fully recapitulate the unique features of the brain. One way to circumvent this issue is the use of a 3D organoid model. Using organoids provides a physiologically relevant model when studying an organ or tissue that under normal circumstances is difficult to access, such as the brain. Our laboratory has recently developed a tri-culture 3D CNS organoid model composed of three cell types, neurons, microglia, and astrocytes (**Figure 3**). We plan to utilize these 3D CNS organoids to study the pathogenesis of HIV-1 in the brain.

2.0 Specific Aims

Among individuals living with HIV-1, it is estimated that at least 50% experience neurocognitive disorders that result in cognitive impairments (9). Neurogranin (Nrgn) is a post synaptic protein that is found in the dendrites of healthy neurons and is involved in synaptic plasticity and cognitive performance. Previous work done using frontal cortex samples of HIV-1 positive individuals has shown a dysregulation of Nrgn expression (92). However, due to unique challenges associated with studying the brain, the mechanisms behind HIV-1 dysregulation of Nrgn are still not well understood. This project aimed to investigate the role of HIV-1 on Nrgn dysregulation using the established *in vitro* 3D CNS organoid model. To accomplish this, I propose the following aims:

Aim 1: Optimize and standardize immunofluorescent staining protocol for neuronal markers in 2D culture, brain tissue, and 3D organoid tissue

A. Optimize antibody concentration for Nrgn, MAP2, IBA-1, GFAP, and p24
B. Standardize protocol in 2D cell cultures
C. Standardize protocol in human brain tissue and 3D organoid derived tissue

Aim 2: Utilize the optimized antibody concentrations to investigate changes in synaptic dendrites in neurons using imaging techniques

A. Quantify changes of Nrgn in presence or absence of HIV-1 infection
B. Measure synaptic density and length

3.0 Materials and Methods

3.1 Cell Culture

Human immortalized embryonic microglial cells (HMC3) (ATCC® CRL-3304) and neuroblastoma SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza Biosciences) supplemented with 10% fetal bovine serum (FBS) (Gemini Biosciences), 1% L-glutamine (Gibco™), and 1% penicillin/streptomycin (Gibco™) (D10 Media). Media was changed every other day. One week prior to immunofluorescent staining, HMC3 cells were transferred to 8-well chamber slides and media was changed every other day. All cells were incubated at 37°C and 5% CO_2.

3.2 Culturing Primary Human Astrocytes

Primary Human Astrocytes were cultured in Gibco complete astrocyte medium composed of DMEM, 0.01% N-2 Supplement, and 0.1% FBS. Cells were grown on PDL-coated plates and media was changed every other day. One week prior to immunofluorescent staining, cells were transferred to PDL-coated coverslips and media was changed every other day.

3.3 Differentiation of SH-SY5Y Cells into Neurons

The neuroblastoma cell line, SH-SY5Y, was differentiated into neurons via a modified protocol described by Shipley et. al. (100) First, SH-SY5Y cells were transferred to a 12-well plate containing coverslips and maintained for 24 hours. The following day, differentiation media containing 10 μM of all-trans-retinoic acid (RA) along with 1% FBS, 1% L-glutamine, and 1% penicillin/streptomycin was added. Differentiation media was replaced every other day for to induce differentiation into neurons. The morphology of the SH-SY5Y can be used to determined differentiation, as best described by Shipley et. al. After 7 days of receiving differentiation media, SH-SY5Y cells begin to display increased numbers of extended processes along with a more pyramidal body shape, indicating successful differentiation into neurons (93).

3.4 Organoid Generation

The organoids used in these experiments have been generously gifted by Dr. Roberta S. dos Reis, University of Pittsburgh Department of Pharmaceutical Sciences. They were previously generated using an established protocol, resulting in a tri-culture organoid (101). Briefly, neural progenitor cells (NPCs) were seeded on top of a microwell device. After allowing the cells to settle within the microwells, NPC media is added, and the devices are incubated at 37°C and 5% CO_2. Once neurospheres begin to form, as confirmed through light microscopy, NPC media is removed from the devices followed by the addition of matrigel (Corning). After allowing the matrigel to solidify, differentiation media is added. To maintain organoids, half of the media was replaced every other day until differentiation is complete.

For the incorporation of microglia, either mock infected or HIV-1 infected microglia were maintained as previously described. 48 hours prior to incorporation into the organoids, HMC3 cells were infected with pNL43-AD8-eGFP-HIV-1 at an MOI of 1.0. Two-week old organoids that have been washed with PBS were incubated with the microglia in a ratio of 1 microglia cell to 20 neural progenitor cells for 24 hours, allowing for the attachment of microglia to the organoids. After 24 hours, the organoids were carefully transferred to a new plate containing fresh differentiation media and were maintained for an additional 15 days. Finally, the organoids containing the microglia are washed with PBS and undergo fixation using 4% paraformaldehyde overnight at 4℃.

3.5 Immunofluorescence Staining of Cultured Cells

Differentiated SH-SY5Y cells, primary human astrocytes, and HMC3 cells were fixed using 4% paraformaldehyde for 15 minutes at room temperature (RT) and then followed by two washes with PBS. Fixed cells were permeabilized using 0.1% Triton-X-100 in PBS for 15 minutes at RT followed by two washes with PBS. After blocking for 1 hour at RT using 3% BSA, cells were then incubated with primary antibodies for 1 hour at RT. Cells are then washed 5 times using a 3% BSA blocking solution and undergo incubation with secondary antibody for 1 hour at RT. DAPI is added followed by the removal of the chambers and the addition of mounting media and a coverslip. Slides are left to dry overnight in a dark location. Imaging was done using confocal microscope as described below.

3.6 Staining of Neuronal, Astrocyte, and Microglial Markers

In order to reliably identify cell types within the 3D CNS organoid, primary antibody markers for each cell type were selected. The first cell type, astrocytes, were identified using glial fibrillary acidic protein (GFAP) antibody. GFAP is highly expressed in the CNS, almost exclusively by astrocytes (102). GFAP is an intermediate filament protein that is responsible for the cytoskeleton of astrocytes, making it a good marker for studying the morphology of astrocytes (103). The next cell type, microglia, were identified using ionized calcium binding adaptor molecule 1 (IBA-1) antibody. The protein is specific to microglia/ macrophages and is involved in the cytoskeleton of the cells (104). Additionally, when microglia undergo activation, expression of IBA-1 is upregulated (105). The final cell type, neurons, were identified using the microtubule accessory protein 2 (MAP2) antibody. Found primarily in the cell bodies and dendrites, MAP2 is an important regulator of the cytoskeleton of neurons (106).

3.7 Immunofluorescence Staining of Organoid and Brain Tissue

Organoids were processed, paraffin embedded, sectioned at 10 μM, and placed on slides by the Pitt Biospecimen Core. Frontal cortex samples from neurocognitive normal individuals were obtained from Neurobiobank (NIH). The slides containing either paraffin embedded frontal cortex tissue or paraffin embedded organoid tissue were stained using a modified immunohistochemistry protocol (R&D Systems®). First, the slides were baked in 60℃ oven overnight to ensure tissue sections adhere to the slides. Following this, the slides undergo deparaffinization via 3 xylene washes for 5 minutes each, followed by a descending concentration

of alcohol starting at 100% followed by 95%, 75%, and 50% for 10 minutes each. Next, the slides are carefully rinsed with water and placed in PBS for 5 minutes. Slides then undergo heat-mediated antigen retrieval using a pressure cooker and a sodium citrate antigen retrieval buffer at pH 6 for 18 minutes. After allowing slides to cool in a bucket of water with ice, they are washed with PBS and tissue sections are circles with Liquid Blocker Mini Pap Pen (Life Technologies). Blocking solution containing 3% BSA and 0.1% Triton-X-100 in PBS was added to sections and incubated for 1 hour at RT. After removing blocking solution, primary antibodies were added and incubated for 1 hour at 37℃. Slides were then washed five times with the blocking solution and following this, secondary antibodies were added and incubated for 1 hour at RT. Slides were then washed for an additional 5 times with blocking solution and DAPI (1:1000) was added and incubated for 1 minute at RT. After washing the slides one final time, Vitogel mounting media was added and coverslips were placed on top. If tissue clearing was performed, following incubation with DAPI, a fructose-glycerol clearing solution is added to slide. The slides were left to incubate for 20 mins at RT then coverslips were placed on top. All slides were left to dry overnight sheltered from light and imaging was done using confocal microscope as described below.

3.8 Microscopy

Imaging was performed using a Z-stacking function on the Olympus FLUOVIEW FV3000 confocal microscope using step size of 0.5-1.5 μM for paraffin sections. Maximum intensity Z-projections were generated and processed using ImageJ (National Institutes of Health, USA).

Table 1. Antibodies Used

Antibody	Host species	Dilution	Manufacturer
Neurogranin	Mouse	1:50	John's Hopkins
GFAP	Rabbit	1:800	Millipore
MAP2	Goat	1:800	Neuromics
IBA-1	Rabbit	1:100	Invitrogen
p24	Mouse	1:200	Invitrogen

4.0 Results

4.1 Aim 1: Optimize and Standardize Immunofluorescent Staining Protocol for Neuronal Markers in 2D Culture, Brain Tissue, and 3D-Organoid Tissue

4.1.1 Optimized Antibody Concentrations for GFAP, IBA-1, MAP2, Nrgn, and p24 in 2D Cell Culture and Brain Tissue Sample

In order to reliably identify the three cell types within our 3D CNS organoid model, astrocytes (GFAP), microglia (IBA-1), and neurons (MAP2 and Nrgn), I first performed immunofluorescent staining of 2D cell cultures to optimize antibody concentrations, followed by optimization in paraffin-embedded brain tissue samples. The expression of the astrocyte marker GFAP was tested in primary human astrocytes (PHA) (**Fig. 4A**) and in human brain tissue (**Fig. 4B**). Within the CNS, GFAP is almost exclusively found within astrocytes and is responsible for the cytoskeleton structure of the cells (107, 108). Next, expression of the microglia marker IBA-1 was tested on human microglia clone 3 (HMC3) cells (**Fig. 5A**) and in human brain tissue (**Fig. 5B**). The IBA-1 antibody stains the ionized calcium binding adaptor molecule 1 (IBA-1) which is expressed only in microglia/macrophages and localizes within the cytoskeleton (104, 109). The expression of the neuronal marker MAP2, a vital protein in the cytoskeleton of neurons, was tested in differentiated SH-SY5Y cells (**Fig. 6A**) and in human brain tissue (**Fig. 6B**). Finally, the expression of neurogranin (Nrgn) was also tested in differentiated SH-SY5Y cells (**Fig. 7A**) and in human brain tissue (**Fig. 7B**).

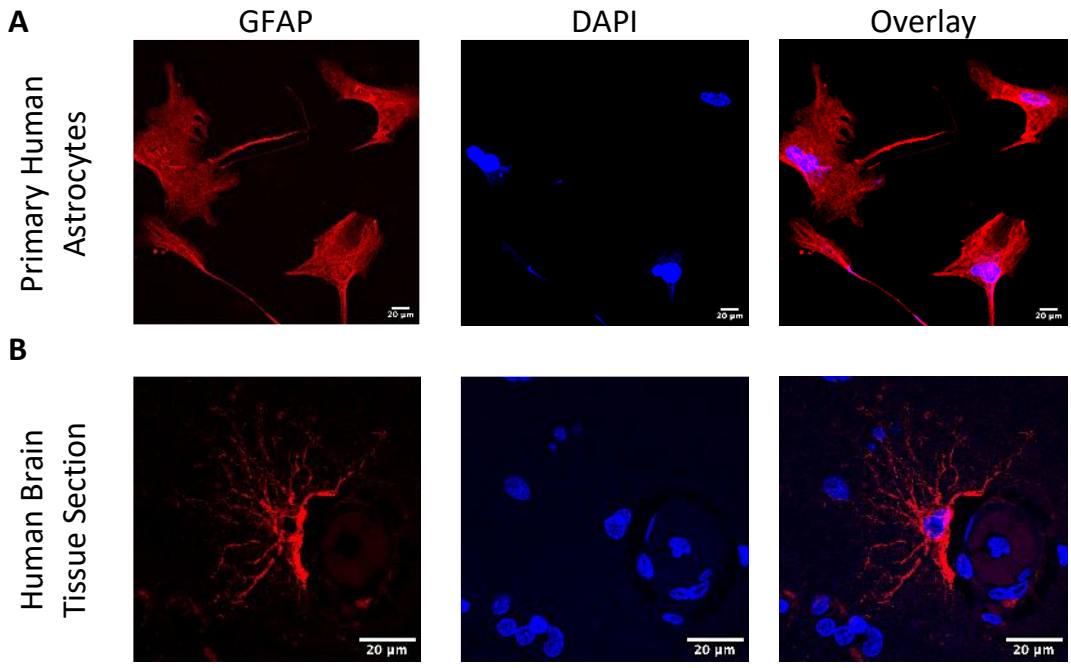

Figure 4. GFAP staining in (A) primary human astrocytes and (B) human brain tissue sections

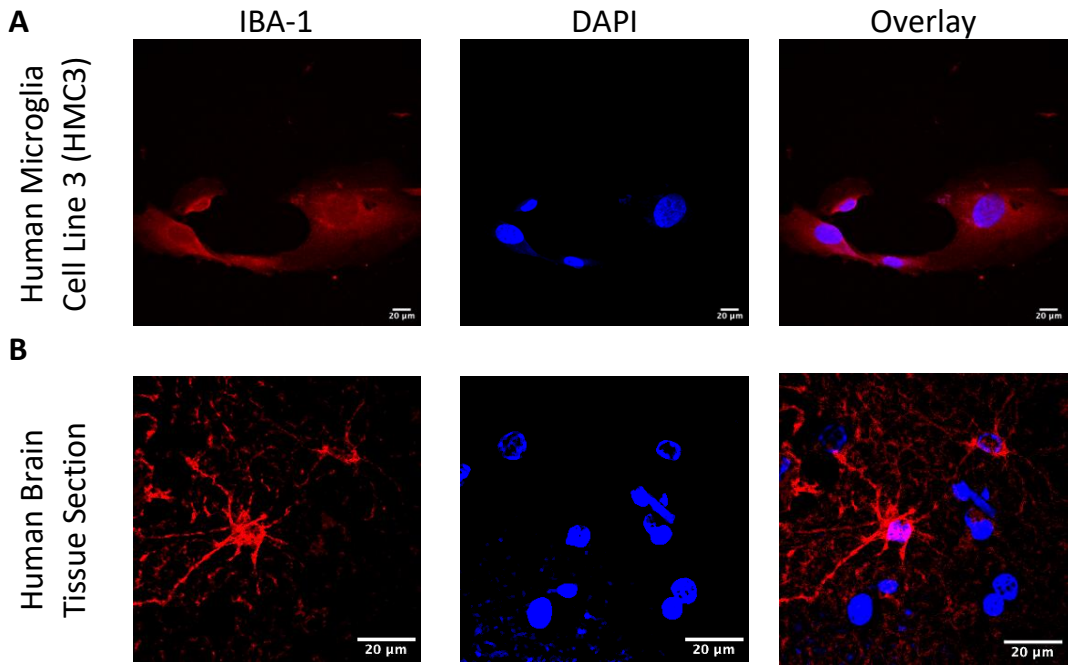

Figure 5. IBA-1 staining in (A) human microlia cell line 3 (HMC3) and (B) human brain tissue sections

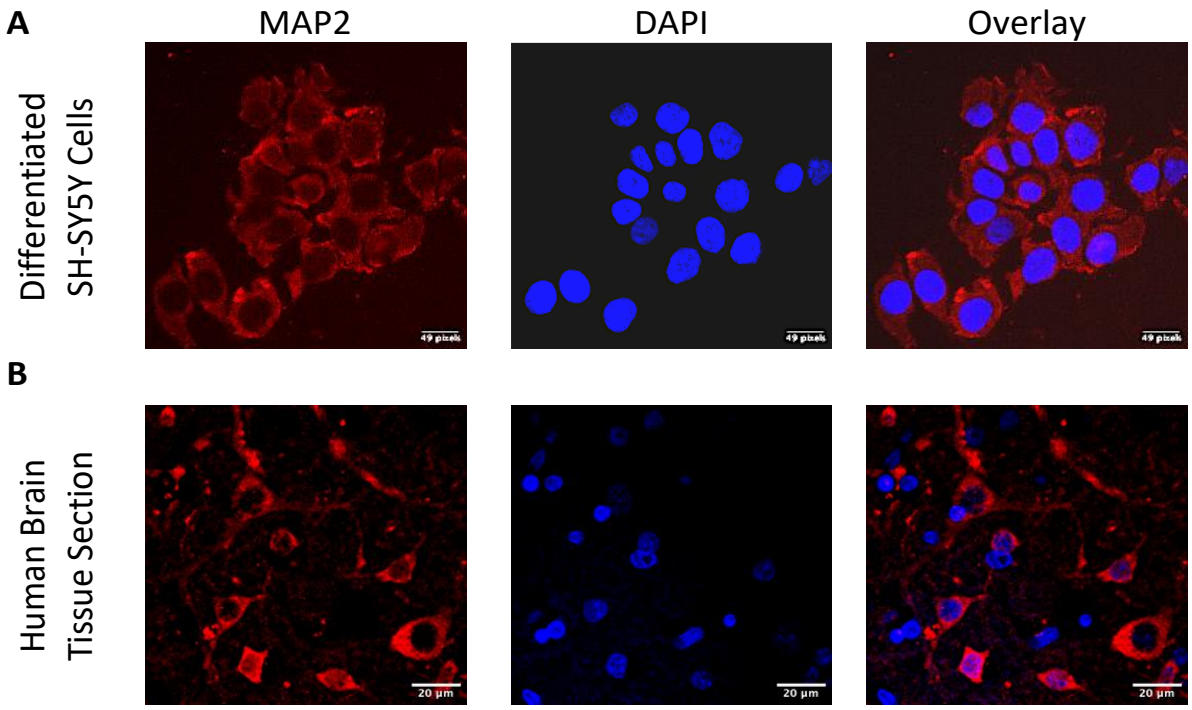

Figure 6. MAP2 staining in (A) differentiated SH-SY5Y and (B) human brain tissue sections

In addition to the cellular markers, the HIV-1 viral capsid protein p24 antibody was used as a marker of successful HIV-1 infection. To test the expression of p24 antibody, HMC3 cells were infected with the HIV-1 YU2 (env)-EGFP reporter virus at an MOI of 1 for 72 hours followed by immunofluorescent staining protocol (**Fig. 8**). Optimization in HIV-1 positive brain tissue sample was attempted however, the staining resulted in high autofluorescence indicated by overlapping, non-specific staining (**Fig 9, arrows**). The optimized concentration for each primary antibody in 2D cell culture and brain tissue sections is summarized in **Table 1**.

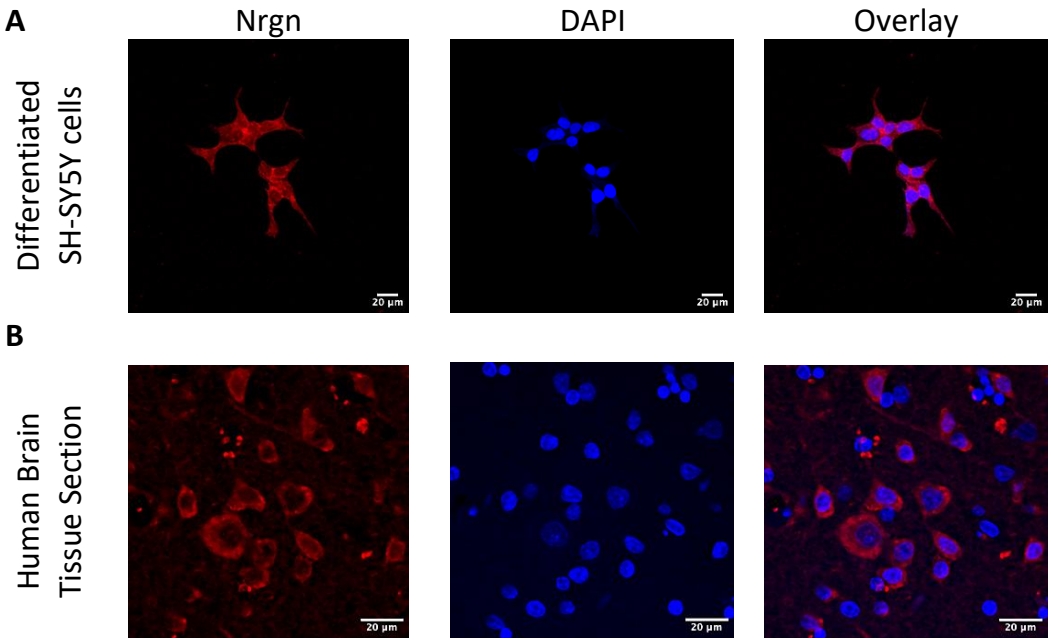

Figure 7. Nrgn staining in (A) differentiated SH-SY5Y cells and (B) human brain tissue sections

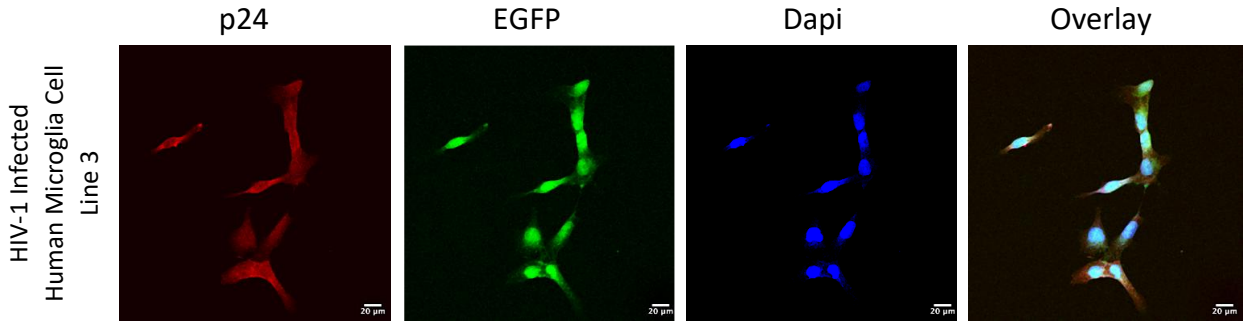

Figure 8. p24 (red) staining in HIV-1 infected HMC3 cells

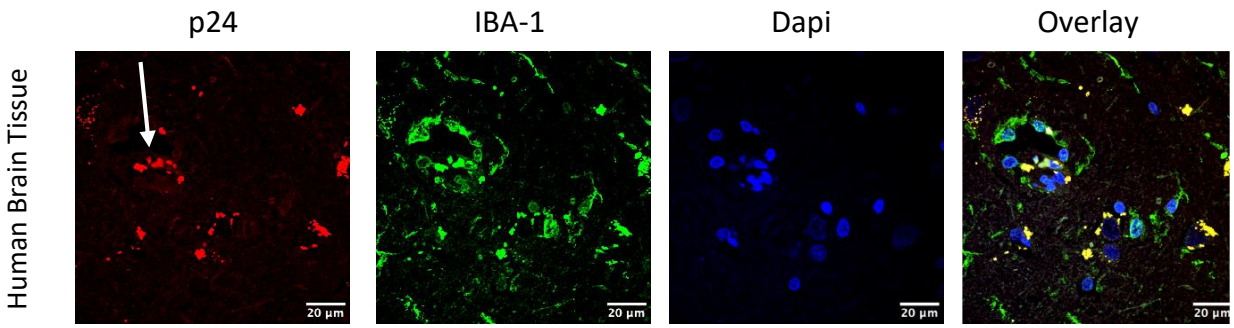

Figure 9. p24 (red) and IBA-1 (green) staining in HIV-1 infected human brain tissue sample. Arrows point to areas of high autoflourescence (yellow)

4.1.2 Standardized Protocol in 2D Cell Cultures and Tissue Samples Derived From Human Brain or 3D Organoid Model

Before staining the 3D organoid tissue samples, I first tested a modified fluorescent immunohistochemistry protocol (R&D Systems®) in formalin-fixed paraffin-embedded human derived brain tissue samples. Slides containing brain tissue first underwent deparaffinization using xylene followed by a rehydration of the tissue using descending concentrations of alcohol. Following this with a wash step, the slides then undergo heat-mediated antigen retrieval. After cooling, the tissue sample on the slides is outlined with Liquid Blocker Mini Pap Pen (Life Technologies) and a blocking solution is added. Following incubation with blocking solution, primary antibody is added at concentrations previously determined for one hour at 37℃ (**Table 1**). Slides are washed 5 times with blocking solution and secondary antibodies are added and incubated for 1 hour at RT. Following an additional 5 washes with blocking solution, DAPI is added at a concentration of 1:1000 for 1 minute at RT. After washing the slides one final time, Vitogel mounting media was added and coverslips were placed on top.

With the R&D protocol as a starting point, I sought out to continue to optimize this protocol to best work for our organoid samples first using paraffin embedded brain tissue samples. The first point of optimization was the antigen retrieval step. While there are a few different methods for antigen retrieval, I tested two different heat induced methods. The first used a citrate buffer antigen retrieval solution at pH 6.0 followed by heating via microwave (**Fig. 10A**). The second method used a citrate buffer with 1% Tween at pH 6.0 followed by heating via a pressure cooker (**Fig. 10B**). The pressure cooker method allows for a more even distribution of heat, allowing for higher sensitivity and improved staining (**Fig. 10**).

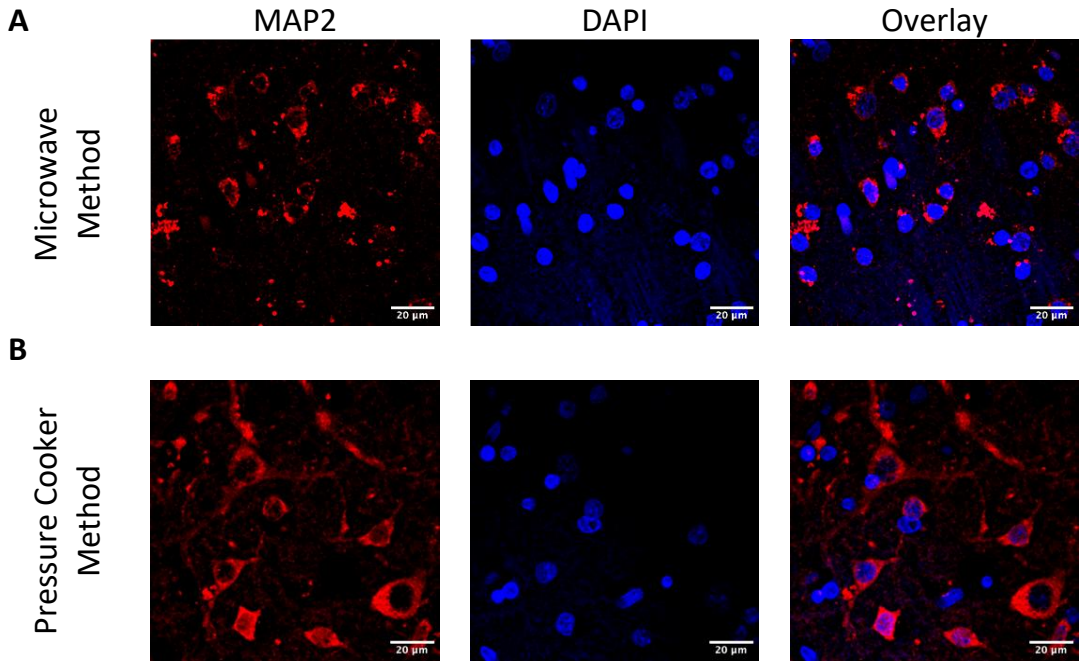

Figure 10. Immunoflourescent staining of MAP2 on human brain tissue comparing two antigen retrieval heating methods, (A) a microwave and (B) a pressure cooker

In order to reduce high background staining, different concentrations of BSA within the blocking solution were tested. Blocking solution containing with either 1%, 3%, or 5% BSA in addition to 0.1% Tween was tested on slides containing brain tissue sections (**Fig. 11**). Based on the results, blocking solution containing 3% BSA was selected for future use.

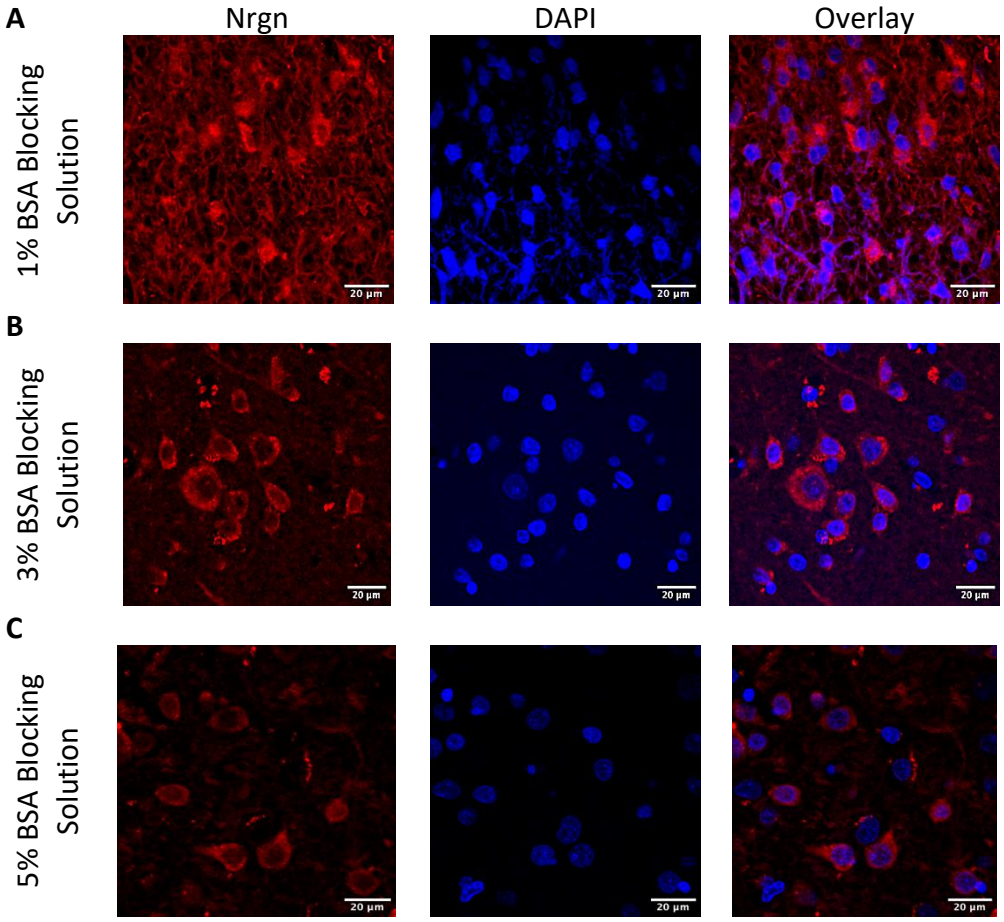

Figure 11. Neurogranin staining on human brain tissue sections comparing blocking solutions containing different concentrations of BSA, with (A) 1% BSA, (B) 3% BSA, and (C) 5% BSA

Finally, I wanted to continue to improve our staining and reduce excessive background, so a tissue clearing step was added. Using a fructose-glycerol clearing agent, optical clearing allows for better visualization of thicker tissues and decreased autofluorescence. Results of the use of a clearing step on organoid tissue sections supported the idea that performing the tissue clearing step does decrease non-specific staining (**Fig. 12**).

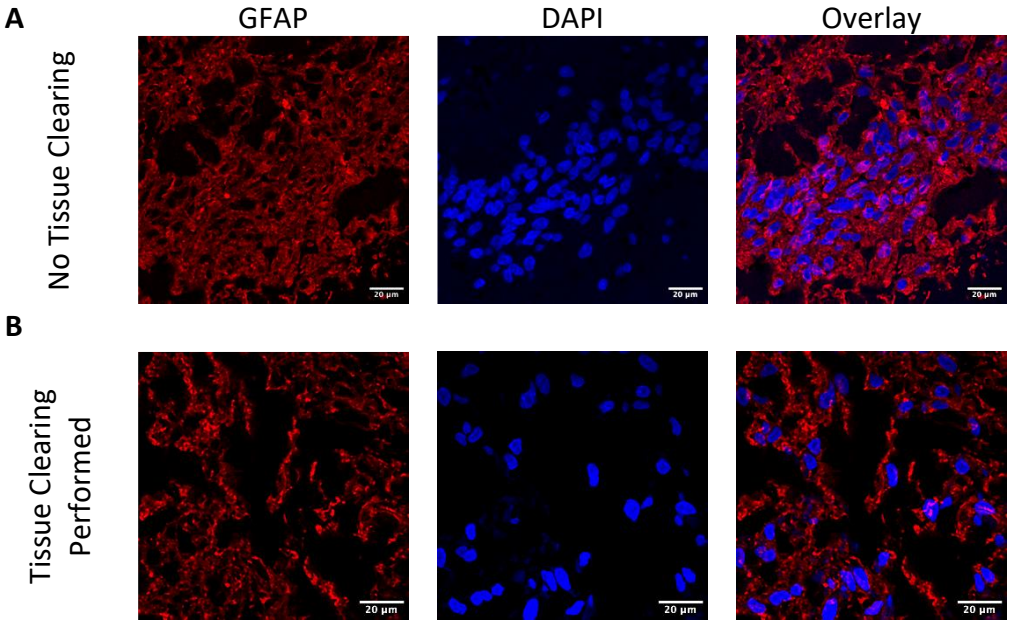

Figure 12. Immunoflourescent staining of GFAP on organoid tissue without tissue clearing (A) and with tissue clearing (B)

4.2 Aim 2: Utilize Optimized Antibody Concentrations to Investigate Changes in Synaptic Dendrites in Neurons Using Imaging Techniques

4.2.1 Quantify Changes of Nrgn in Presence of and in Absence of HIV-1 Infection

After optimizing antibody concentrations in 2D cultures and optimizing the protocol for immunofluorescent (IF) staining in human brain tissue samples, the next step was to apply these to the organoid tissue samples. Immunofluorescent staining using Nrgn antibody was performed on organoid tissue samples, either containing HIV-1 infected microglia or mock infected microglia. However, images obtained displayed high non-specific staining, despite obtaining clear images of the IF-stained human brain tissue sample (**Fig. 13**). Due to this, I was unable to quantify changes of Nrgn within the 3D organoid model.

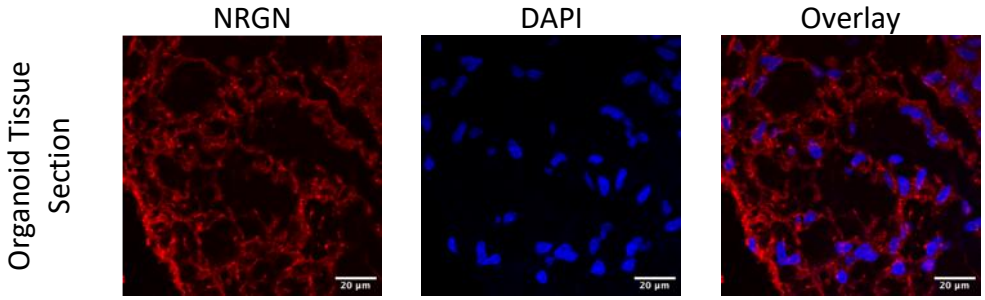

Figure 13. Immunoflourescent staining of Nrgn in organoid tissue section

4.2.2 Measure Synaptic Density and Length

The final step was to utilize the optimized antibody concentrations and IF staining protocol to measure synaptic density and length of neurons to look for differences between HIV-1 infected and mock infected organoids. Images obtained were to be analyzed using ImageJ software. However, staining of the organoids with the neuronal marker MAP2 again resulted in high nonspecific staining, preventing the ability to quantify the images (**Fig. 14**).

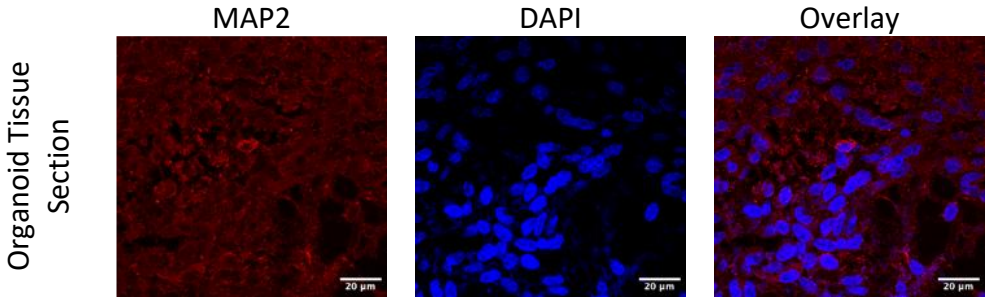

Figure 14. Immunoflourescent staining of MAP2 on organoid tissue section

5.0 Conclusions and Discussion

Neurogranin (Nrgn) is a post synaptic protein that is found in the dendrites of healthy neurons and is involved in synaptic plasticity and cognitive performance. Previous work done using frontal cortex samples of HIV-1 positive individuals has shown a dysregulation of Nrgn expression (92). However, due to unique challenges associated with studying the brain, the underlying mechanisms behind HIV-1 dysregulation of Nrgn are still not well understood. This project aimed to investigate the role of HIV-1 on Nrgn dysregulation using the established *in vitro* 3D CNS organoid model by first standardizing and optimizing the immunofluorescent staining protocol in 2D culture, brain tissue, and 3D CNS organoid tissue followed by utilizing the optimized protocol to understand changes in Nrgn within synaptic dendrites using imaging techniques.

The 3D CNS organoid model used contains the 3 main cell types of the CNS, astrocytes, microglia, and neurons. Antibody concentrations were optimized for a cellular marker corresponding to either astrocyte (GFAP), microglia (IBA-1) and neurons (MAP2) first in 2D cell culture then in paraffin embedded human brain tissue sample before ultimately staining organoid tissue.

The first cell type found within the 3D CNS organoid model are astrocytes, the most abundant cell type in the brain. Astrocytes are often a point of controversy in HIV-1 infection, as they lack CD4, the primary receptor for HIV-1 entry into the cell but are thought to be an important reservoir within CNS infection (36, 41, 45, 46). Despite this, it is well supported that HIV-1 causes dysregulation of astrocytes, often resulting in the production of proinflammatory signaling (110-113). In response to HIV-1 infection, astrocytes may undergo activation, also known as

astrogliosis, resulting in a change of the morphology of the cell. Exclusively expressed by astrocytes within the CNS, glial fibrillary acidic protein (GFAP) plays an important role in the cytoskeleton structure and morphology of astrocytes (107, 108). Using primary human astrocytes and human brain tissue samples, antibody concentrations were optimized for the GFAP antibody (**Fig. 4).** GFAP staining in the brain samples was able to visualize the long projections of astrocytes, known as the astrocytic end feet. Additionally, we are able to see the end feet of the astrocytes surround what appears to be a blood vessel within the brain. Astrocytes play an important role in the regulation of blood flow in the brain and are often found surrounding blood vessels within the CNS (114).

It is well established that HIV-1 enters the brain via infected monocytes early in infection and established infection in resident microglia in the brain, making microglia a vital cell type in an HIV-1 organoid model (36-39). The cellular protein IBA-1 is an established cellular marker that is specific to microglia and macrophages within the CNS. IBA-1 is found in the cytoskeleton of microglia and is involved in membrane ruffling and phagocytosis (104, 109, 115). When microglia undergo activation or differentiate into macrophages, the morphology of the cell changes from a ramified cell to a more ameboid cell which can result in an increase in IBA-1 expression (115-117). This makes IBA-1 a reliable marker for all both resting and activated microglia within the CNS. Using first HMC3 cells followed by brain tissue samples, the antibody concentration for IBA-1 was optimized (**Fig. 5**). When looking at the positive stained cells in the brain tissue sample, we can see a ramified morphology with many long processes, indicating healthy microglia.

The last cell type found within the organoids is the neurons. To first optimize antibody in 2D cell culture, the neuroblastoma cell line SH-SY5Y was used. In their undifferentiated form, SH-SY5Y cells express markers associated with an immature neuron (118, 119). After inducing

differentiation of cells for just 7 days with the use of retinoic acid (RA) and serum deprivation, SH-SY5Y cells begin to express markers associated with a mature neuron, including expression of microtubule associated protein 2 (MAP2) (100). MAP2 is expressed primarily by mature neurons, and it is involved in the bundling and stabilization of microtubules of the cytoskeleton. In neurons, the bundling effects of MAP2 are essential for the formation of the dendritic structure (120, 121). Using the MAP2 antibody lets us see the morphology of the neurons both in differentiated SH-SY5Y and in the brain tissue sample (**Fig. 6**). Staining with MAP2 resulted in clear visualization of the neuronal bodies and some staining within the dendrites.

In addition to MAP2, differentiated SH-SY5Y cells were used to optimize the antibody concentration for Nrgn, along with brain tissue samples (**Fig. 7**). Similar to MAP2, Nrgn is primarily expressed in neurons within the CNS as it is a post-synaptic protein. Staining using Nrgn allows for visualization of the dendrites of neurons along with their cell bodies.

In addition to IBA-1, HMC3 cells were used for the optimization of the viral protein p24. Prior to staining, HMC3 cells were first infected with HIV-1 at an MOI of 1 and 72 post infection, cells underwent immunofluorescent staining. The virus used to infect the HMC3 cells expressed EGFP, so we were able to track infection within the cells. Often used as a marker of successful infection, the viral protein p24 is found in the capsid of HIV-1 and is the most abundant HIV-1 protein. Successful staining of p24 was achieved in infected HMC3s, with p24 expression co-expressed with EGFP, indicating successful infection (**Fig. 8**). However, when attempted in brain tissue sample from an HIV-1 positive brain, the tissue displayed a high amount of non-specific staining, which was consistent between each color channel even those not used in the staining, indicated by the yellow (**Fig. 9**)

Following the optimization of antibody concentrations in 2D (**Table 1**), the next step was to optimize and standardize the immunofluorescent protocol for the paraffin embedded organoid tissues. This was first done using slides containing human brain tissue samples, also paraffin embedded. While formalin-fixation and paraffin-embedding allow for a well-preserved tissue architecture, they also can cause masking of important epitopes, resulting in reduced antigen-antibody binding (122). To overcome this, an antigen retrieval step is included and optimized in this protocol. There are two main forms of antigen retrieval, proteolytic-induced epitope retrieval (PIER) and heat induced epitope retrieval (HIER). PIER uses peptidases to unmask the epitopes, which can cause damage to the tissue and is a harsher method and compared to HIER which uses specific buffers and heat to reveal hidden epitopes (123). Given this, I tested two different heating devices for HIER, a microwave and a pressure cooker, both using a citrate buffer with pH 6.0 (**Fig. 10**). The use of a pressure cooker allows for more even heat distribution and eliminates the worry of drying out slides in the microwave due to boiling over and produced clearer images.

In addition to the antigen retrieval step, I also sought to optimize the blocking step of the protocol. The initial protocol called for a 1% BSA and 0.1% Tween blocking solution. When initial staining was performed on brain tissue sections with this concentration, we experienced high background staining (**Fig. 11A**). To combat this, I tested different concentrations of BSA within the blocking solution (**Fig. 11**). While an increase to 3% BSA did improve the quality of staining in 2D and brain tissue samples, when applied to the organoid samples there was still a significant amount of non-specific staining (**Fig. 12A**). Because of this, we decided to add an addition tissue clearing step.

Tissue clearing is used to take thick tissues sections that may be difficult to see and cause them to become transparent, creating clearer, easier to analyze images (124). They do this by

minimizing the refractive index, resulting in increased passage of light through the sample (125, 126). While there are a few different approaches, we used a fructose-glycerol solution. Tissue clearing did have a positive impact on the staining of GFAP on organoid tissue samples however, a high amount of non-specific staining remained (**Fig. 12**).

Nrgn has been identified as a biomarker for several neurodegenerative diseases, including Alzheimer's (93, 94, 99). In addition, previous work has demonstrated Nrgn dysregulation in the frontal cortex of HIV-1 positive individuals (92). After optimizing in 2D and brain tissue samples, the next step was to stain organoid tissue sections with the goal to investigate changes in Nrgn expression between HIV-1 infected and mock infected organoids along with measuring synaptic length and density. However, when applied to the organoid tissue, Nrgn staining, along with other cellular markers, resulted in high non-specific staining. As seen in these figures, almost every cell is staining positive for Nrgn (**Fig. 14**) or MAP2 (**Fig. 13**), indicating non-specific staining. The addition of the tissue clearing step did help decrease some background staining seen in the organoids, however it was not sufficient. This non-specific staining could be contributed to the preparation of the organoids. Prior to undergoing sectioning, organoids are embedded in Matrigel, which could cause an increase in non-specific staining.

6.0 Future Directions

The use of the 3D CNS organoid model to study HIV-1 neuropathogenesis allows for a more biologically relevant model. Utilizing 3D CNS organoids can allow for a deeper investigation on the mechanisms behind HIV-1 associated neuropathogenesis. The standardization and optimization of an immunofluorescence staining protocol for the identification different cell types found in this organoid model is a necessary step in this process. The goal of this project was to create a protocol that could be used in future experiments using the 3D CNS organoid model. Utilization of an optimized protocol can allow for investigations into the changes caused by HIV-1 infection in multiple cell types including microglia and astrocytes. Due to being unable to fully optimize this protocol, future studies will be needed to finalize the protocol in the 3D CNS organoid model.

Additionally, we focused on the postsynaptic protein Nrgn and its role in HIV-1 infection. Previous studies have demonstrated that Nrgn becomes dysregulated upon HIV-1 infection. Due to the important role of Nrgn in cognitive function and synaptic plasticity (75, 85, 88), we speculate that dysregulation caused by HIV-1 neuropathogenesis may contribute to HAND. Future experiments are needed to quantify changes within the 3D-CNS organoid model.

7.0 Public Health Significance

Since being first declared an epidemic in the 1980's, HIV-1 still remains a significant public health concern, claiming the lives of over 40 million (1). Advancements in the treatment of HIV-1 have allowed for slower disease progression and reduced mortality however, PWH are at an increased risk of developing comorbidities compared to HIV-1 negative individuals (1, 4, 7, 8). Common comorbidities such as cardiovascular disease, cancers and others can have a significant impact on the quality of life of people with HIV-1 (4, 6). Despite the significant decrease in the prevalence of HIV-1 associated dementia (HAD), up to half of PWH continue to experience HIV-1 associated neurological disorder (HAND) even when following a strict cART regimen (10, 11, 56).

Due to difficulties in studying the brain, current models of HIV-1 neuropathogenesis come with many limitations. The use of an *in vitro* 3D-CNS organoid model to study HIV-1 neuropathogenesis allows for a more physiologically relevant model. Understanding the mechanisms behind neuropathogenesis is the first step in the identification and development of therapeutics to improve HAND diagnosis, treatment, and prevention. By using a previously developed 3D-CNS organoid, we sought to contribute to the understanding of this mechanism by optimizing and standardizing the immunofluorescent staining protocol for the organoids in order to investigate HIV-1 induced dysregulation within the CNS.

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