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Profiling the neurovascular cell interactions in alcohol exposure and HIV-1 infection

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ABSTRACT

PROFILING THE NEUROVASCULAR CELL INTERACTIONS IN ALCOHOL EXPOSURE AND HIV-1 INFECTION

by
Agnieszka Agas

Alcohol use is known to exacerbate the progression of human immunodeficiency virus associated acquired immunodeficiency syndrome or HIV/AIDS in the brain, known as the NeuroAIDS. The mechanisms of this accelerated progression are still poorly understood. The purpose of my thesis is to review the parameters contributing to the co-morbid effects of alcohol in the progression of NeuroAIDS. The ***first aim*** will evaluate the exacerbating effects of alcohol on HIV-1 transmission, infection, and the role of metabolic energy imbalance during NeuroAIDS progression, which will enable me to formulate the possible mechanism for NeuroAIDS progression. The ***second aim*** will help me establish the technique of rat embryonic neuronal isolation, which will be used for testing the neurotoxicity of HIV proteins (Tat, gp120) in the setting of interactive neuroimmune cell culture (brain endothelial cells, astrocytes, microglia, and neurons) with or without the presence of alcohol. The synergistic effect of alcohol on HIV associated neurotoxicity will pave the future research path to examine the unique mechanism for HIV/AIDS progression and a possible cure for HIV/AIDS with active antiretroviral drug(s).

PROFILING THE NEUROVASCULAR CELL INTERACTIONS IN ALCOHOL EXPOSURE AND HIV-1 INFECTION

by
Agnieszka Agas

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APPROVAL PAGE

PROFILING THE NEUROVASCULAR CELL INTERACTIONS IN ALCOHOL EXPOSURE AND HIV-1 INFECTION

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To mom and dad. Thank you for your constant love and support.

Dla mamy i taty. Dziękuję wam za ciągłą miłość i wsparcie.

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CHAPTER 1

INTRODUCTION

1.1 Objective

The objective of this thesis is to conduct a literary review on the exacerbating effects of alcohol for HIV-1 transmission, infection, and the role of metabolic energy imbalance during NeuroAIDS progression. This knowledge will be used to formulate a possible mechanism for accelerated NeuroAIDS progression (first aim). However, for the scope of this thesis, the mechanism will not be experimentally studied. Instead a technique of rat embryonic neuronal isolation will be established (second aim) and used for testing the neurotoxicity of HIV proteins (TAT) in the setting of interactive neuroimmune cell culture (brain endothelial cells, astrocytes, microglia, and neurons) with or without the presence of alcohol. It is expected that there is a synergistic effect of alcohol on HIV associated neurotoxicity.

1.2 Importance for Continued HIV Research

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that infects immune cells to suppress the immune response, which ultimately causes human immunodeficiency virus associated acquired immunodeficiency syndrome (HIV/AIDS). There are about 36.7 million people are living with HIV globally, which can be controlled by the combination of active antiretroviral therapy (ART) drugs (Figure 1.1a). This steady increase signifies that the ART drugs are becoming more effective and durable at inhibiting viral replication and progression. Advancement of ART makes it more

convenient, easy to administer, and more accessible worldwide. In this regard, the number of AIDS-related deaths is progressively declining with about 1.1 million people globally (Figure 1.1a). However, when this decline is compared to a graph of people receiving ART (Figure 1.1b) the slope of people receiving ART is profoundly steeper than the slope of AIDS-related deaths. This high mortality rate can mean that ART is less effective towards certain types of people. One of the objectives of this thesis is to review the exacerbating effects of alcohol on HIV-1 progression and offer an explanation for this poor inverse correlation.

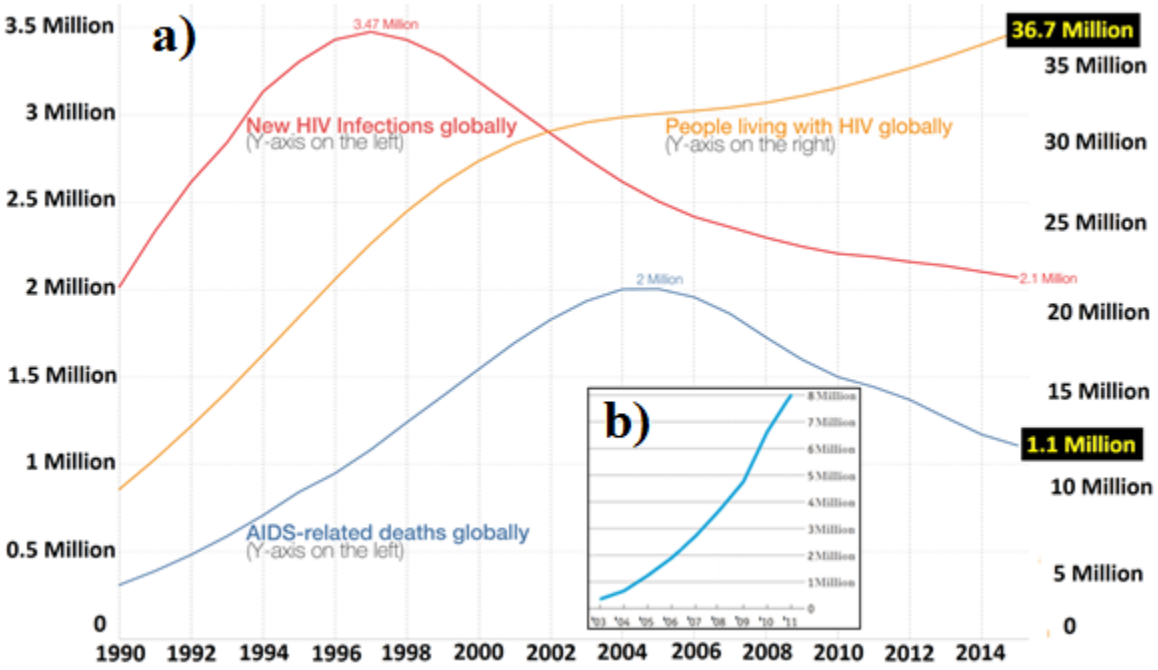

Figure 1.1 Empirical views of size and response to the HIV epidemic. a) Rate of people living with HIV and rate of AIDS-related deaths globally (1990-2014). b) Rate of people receiving antiretroviral therapy globally (2003-2011).

Sources: Max Roser (2016) – 'HIV / AIDS'. Published online at OurWorldInData.org. Retrieved from: https://ourworldindata.org/hiv-aids/ [Online Resource] and Joint United Nations Program on HIV and AIDS (UNAIDS).

Despite being an industrially advanced country with one of the world's highest standards of living, the United States does not have the lowest number of infected people

(0.37% of its total population; China has an upwards of 0.11% of its total population) (Figure 1.2). Also, countries with once low socioeconomic prospects that have been developing and can now supply their people with the most advanced knowledge and treatment are still seeing high infection numbers (such as India Figure 1.2). The reasons for HIV/AIDS persistence and influence in the era of ART are still not fully understood. Meanwhile, for the still remaining low-income economies, HIV/AIDS is their fifth leading cause of death, above malaria and tuberculosis (World Health Organization, 2015).

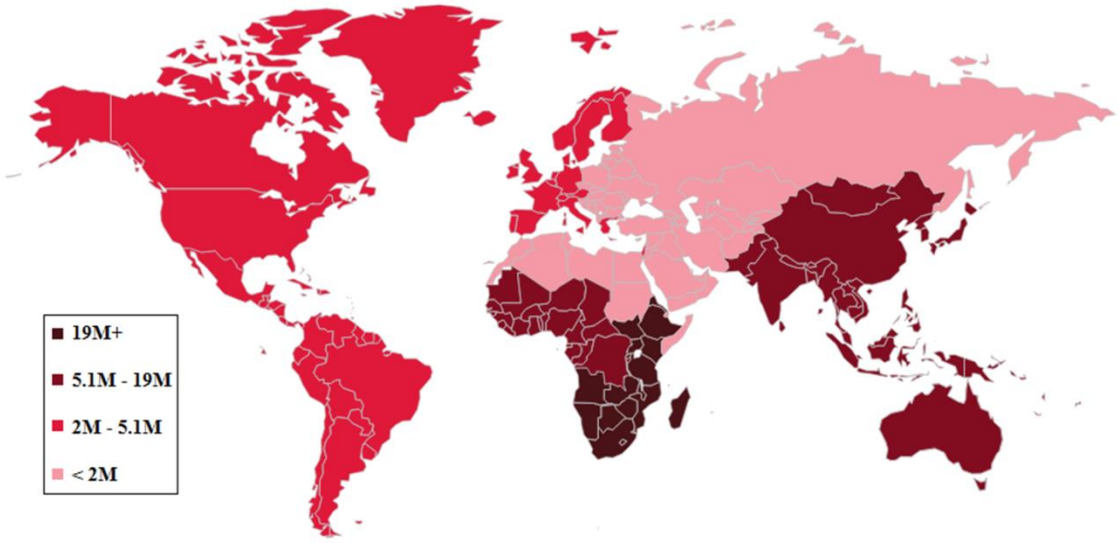

Figure 1.2 Regional mapping in number of people infected with HIV/AIDS (2015). Less than 2 million people represented with < 2M. More than 19 million people represented with 19M+.

Source: Centers for Disease Control and Prevention; UNAIDS (2015) http://aidsinfo.unaids.org/.

New Jersey and New York represent about 15% of all infected persons in the United States, however almost 50% of all AIDS-related mortalities are occurring in these two states. Of all infected people in New York State, more than 80% are coming from New York City (i.e. the Bronx, Queens, Manhattan, etc.) and they constitute almost 35%

of all AIDS-related mortalities across the country (Table 1.1 and Figure 1.3a). This means that significant transmission than progression of HIV/AIDS is happening in urban, densely populated areas. The two most common routes for HIV transmission are risky sexual behavior and needle sharing with someone already infected (Center for Disease Control and Prevention – HIV/AIDS). In these regards, New Jersey and New York are consuming some of largest quantities of ethanol (Figure 1.3b). Also both states show predominant numbers of heroin users (Figure 1.3c), a drug administered by needle injection.

Table 1.1 Prevalence of HIV/AIDS infection and AIDS-related mortality in New Jersey and New York. (± 0 people)

State	City	Prevalence	Percentage of all infected in US (%)	Percentage of all infected in state (%)	Mortality	Percentage of all mortality in US (%)	Percentage of all mortality in state (%)
NJ	ALL	37,832	~3.15	N/A	828	~12.32	N/A
	Newark	5,718	~0.48	~15.11	142	~2.11	~17.15
NY	ALL	130,691	~10.89	N/A	2,335	**~34.7**	N/A
	New York City	105,487	~8.79	**~80.71**	1,499	~22.30	~64.20

Source: Multiple AIDSVu (aidsvu.org) webpages on New York and New Jersey. Emory University, Rollins School of Public Health.

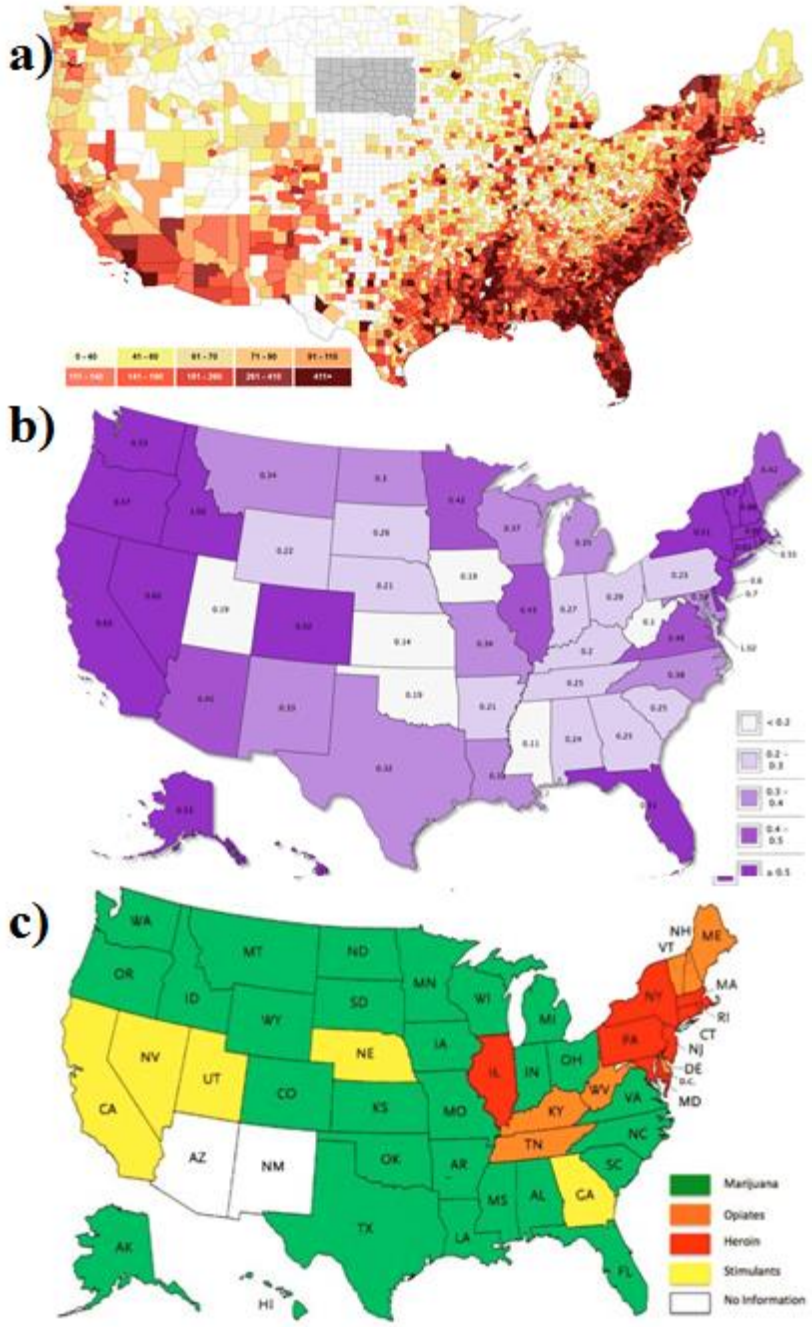

Figure 1.3 Maps on prevalence of HIV/AIDS infection, alcohol and drug use by state. a) Prevalence of HIV/AIDS infection by rate, across the country (2015). b) Wine consumption in gallons of ethanol based on the adult population per state; from a NIAAA surveillance report 2012 (the darker the purple the more ethanol consumed). c) Most prevalent drug treatment admissions per state (red color signifies treatment for heroin).

Source: AIDSVu (https://aidsvu.org/map/); Short, Kevin. "He State Of Drug Use In America, In 9 Maps." Huffington Post 22 Oct. 2014: n. pag. Web. <http://www.huffingtonpost.com/2014/10/22/america-drug-use-maps_n_5974592.html>.

1.3 Overview of HIV Progression in the Brain

The blood-brain barrier (BBB) is composed of closely packed non-fenestrated brain microvascular endothelial cells. Pericytes have a close physical association with this endothelium while astrocyte projections surround the endothelial cells, providing biochemical support. Neurons and immune cell like microglia and oligodendrocytes contribute to the neurovascular unit [1]. Circulating monocytes/macrophage and B-lymphocytes play an important role in the innate and adaptive immune response. They are also important target cells for human immunodeficiency virus type 1 (HIV-1) infection [2]. The selective permeability of the BBB prevents HIV-1 virions from freely entering the central nervous system [3].

Infection increases expression of key proteins involved in chemokine recognition, enhancing invasion. Through the Trojan horse mechanism HIV-1 virions are transported to the central nervous system via the infected macrophage for releasing HIV-1 virions in the brain for microglial cell infection [4, 5, 6]. Efflux of HIV-1 virions into to circulation from infected microglia brain reservoir causes reinfection/resurgent of HIV-1 and complicates the barging of HIV-1 and ART effectiveness [7, 8, 2]. Infected microglia subsequently infects more microglia. Macrophage are viral hideouts during the chronic phase of infection [9].

HIV-infected microglia produce chemokines that through paracrine type signaling from immune to endothelial cells, recruit more immune cells from the circulation. These would include more monocyte/macrophage, T-cells, and other leukocytes. This influx of immune cells over time results in enhanced inflammation and tissue damage. Consequently, all these cells secrete neurotoxic viral shedded proteins, such as TAT and

gp120, which interact with neurons triggering cellular death. Activated astrocytes can also die subsequently compromising the BBB [4, 5].

Antiretroviral therapy drugs cannot readily pass the BBB because of efflux system such as the multi-drug resistant proteins and metabolizing enzymes of the brain endothelial cells. The BBB is a bidirectional barrier. Accumulated virions in the brain during NeuroAIDA progression can efflux into the blood circulation and re-infect the immune cells [10]. The inability of drugs to freely pass the BBB promote opportunistic re-infection from the brain side, as such NeuroAIDS can no longer be overlooked area in HIV/AIDS progression.

1.4 Summary

Chapter 1 outlined discrepancies in HIV/AIDS statistics, i.e. high mortality in an era of superior treatment effectiveness and availability. It described highest AIDS-related mortality rates occurring in urban populations, linking large alcohol consumption and heroin use to prevalence in HIV/AIDS transmission and accelerated progression. Finally, Chapter 1 demonstrated the importance for NeuroAIDS research, i.e. the blood-brain barrier prevents entry of antiretroviral therapy drugs to the brain region and an efflux system can purge HIV virions in the brain-to-blood direction to re-infect the body.

CHAPTER 2

AIM 1: MECHANISMS FOR NEUROAIDS PROGRESSION IN ALCOHOL ABUSE

2.1 Hypothesis Approach

The first reported cases of HIV/AIDS in the United States occurred in the early 1980s. By the mid-1990s the mortality rate caused by HIV/AIDS peaked and antiretroviral therapy (ART) drugs were being developed to decrease viral load and sustain those infected from further disease progression and death. The development of ART led to the discovery of latent reservoirs, hideouts containing inactive forms of the virus. It was found that ART drugs were not able to penetrate these reservoirs therefore the virus could never be completely eliminated from the body or, since HIV embeds itself in the host's genome, in the very least, silenced. Subsequent research was disillusioned of any cure instead focusing on developing better ART and more durable and convenient ways of taking it (e.g. cART, HAART). However, today there is new hope; if these latent reservoirs can be eradicated and eliminated, HIV/AIDS can be cured (AIDS.gov timeline).

There are two foreseeable problems with this approach to a cure: 1) the eradication method calls for reactivating latent reservoirs; therefore would this reactivation re-start viral replication that could replace or even create more latent reservoirs? 2) Are latent reservoirs the only obstacle needed to be overcome to succeed in a cure for HIV/AIDS? Many HIV/AIDS positive patients are also abusing substances such alcohol (National Institutes of Health – National Institute on Drug Abuse), as a result, can alcohol have some interplay in progression? Alcohol has been understood to exacerbate HIV progression through inflammation and oxidative stress. Alcohol has also

been shown to interrupt energy uptake to the brain (Chapter 2.2.4). Therefore, can alcohol help disrupt the energy metabolism to favor infected cells and, in so doing, progress NeuroAIDS? This thesis will demonstrate how HIV/AIDS with alcohol abuse can have a synergistic effect. That is, HIV/AIDS under alcohol abuse can awaken a novel mechanism for faster progression of NeuroAIDS.

2.2 Literary Review

2.2.1 Alcohol as a Risk Factor for HIV/AIDS Transmission

Alcohol use is admitted of provoking depression [11], reckless sexual behavior [12], and needle sharing by drug-users [13], all of which can lead to opportunistic human immunodeficiency virus type 1 (HIV-1) infection [14-16]. Mentally, alcohol interrupts regular functions of the brain involved in intelligence and rational thinking [17] thereby perverting the abuser towards such harmful behaviors.

Physiologically, the effects of alcohol use on immune defenses can further encourage HIV-1 acquisition. Inebriating doses suppress the immune system increasing risk of infection and replication of virus while chronic doses activate it through inflammation and oxidative injury [18-20] promoting HIV-infected cell entry into the brain. Chronic activation has also been shown to create an anergic effect by changing immune cells' abilities, increasing surreptitious viral entry into the brain and viral persistence in the central nervous system (CNS). These changes in immune cells ability are caused by alcohol-triggered interferences in B-cell and T-cell development (especially under utero exposure) [21] and deletions of anti-viral factors [22-24]. Therefore, alcohol-impaired immune defenses combined with HIV's ability to evade

immune attack through quick antigenic variation [25] make cells very susceptible to infection. In effect, alcohol use augments and accelerates HIV-1 transmission (Figure 2.1).

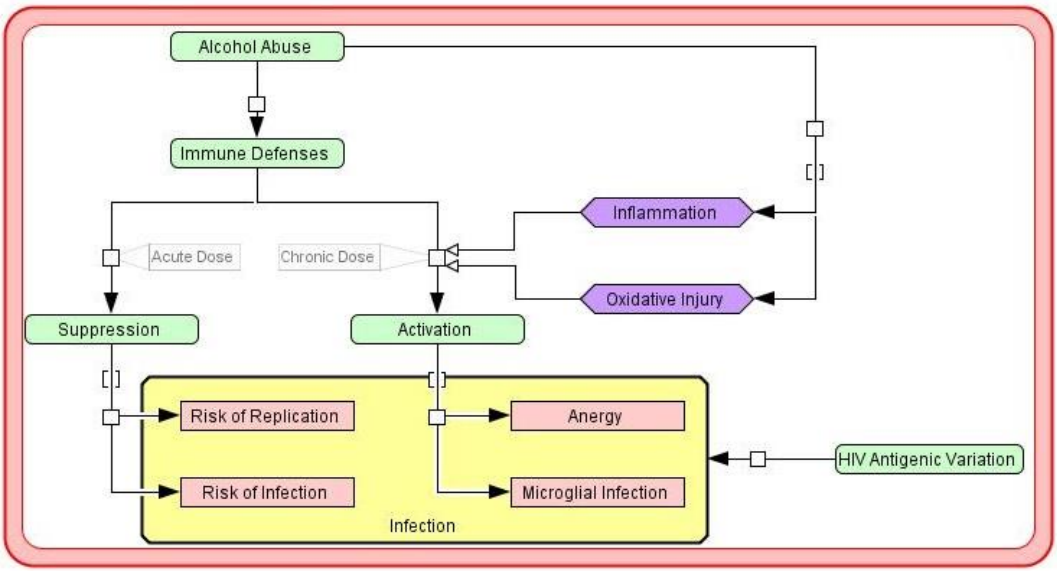

Figure 2.1 Process diagram for alcohol abuse impairing immune defenses and causing opportunistic infection. Alcohol has been shown to have a biphasic effect on the immune system. However, either effect can progress infection; a suppressed immune system can increase transmission and replication while an active immune system, with increase inflammation and oxidative stress, can cause anergy making immune cells less effective towards HIV-1 as well as allow more infected cell to infiltrate and infect the brain.

2.2.2 Alcohol on Vascular Injury and HIV Infected Cell Entry into the Brain

A unique result of ethanol metabolism in the brain is the generation of free radicals ROS/RNS. Their accumulation leads to an imbalance of redox and causes oxidative stress [26]. Further oxidative stress is caused by alcohol-induced activation of NF-κB, TLR4- and IL-1R signaling, interference of antioxidant mechanisms, increased CYP2E1 activity, and mitochondrial injury [27-29]. One consequence of oxidative stress is the stimulation of inositol 1,4,5-triphosphate receptor and release of Ca^{2+} leading to the activation of myosin light chain kinase (MLCK) and subsequent phosphorylation of myosin light chain

(MLC) and tight junction (TJ) proteins of the specialized blood brain barrier (BBB) [30]. The degree of permeability across the BBB is dependent on the integrity of TJ proteins, especially occludin and claudin-5. Therefore, their decrease injures the microvasculature allowing passage to otherwise restricted substances such as HIV-infected macrophage. Also a decrease in occludin has been shown to increase HIV transcription [31] (Figure 2.2).

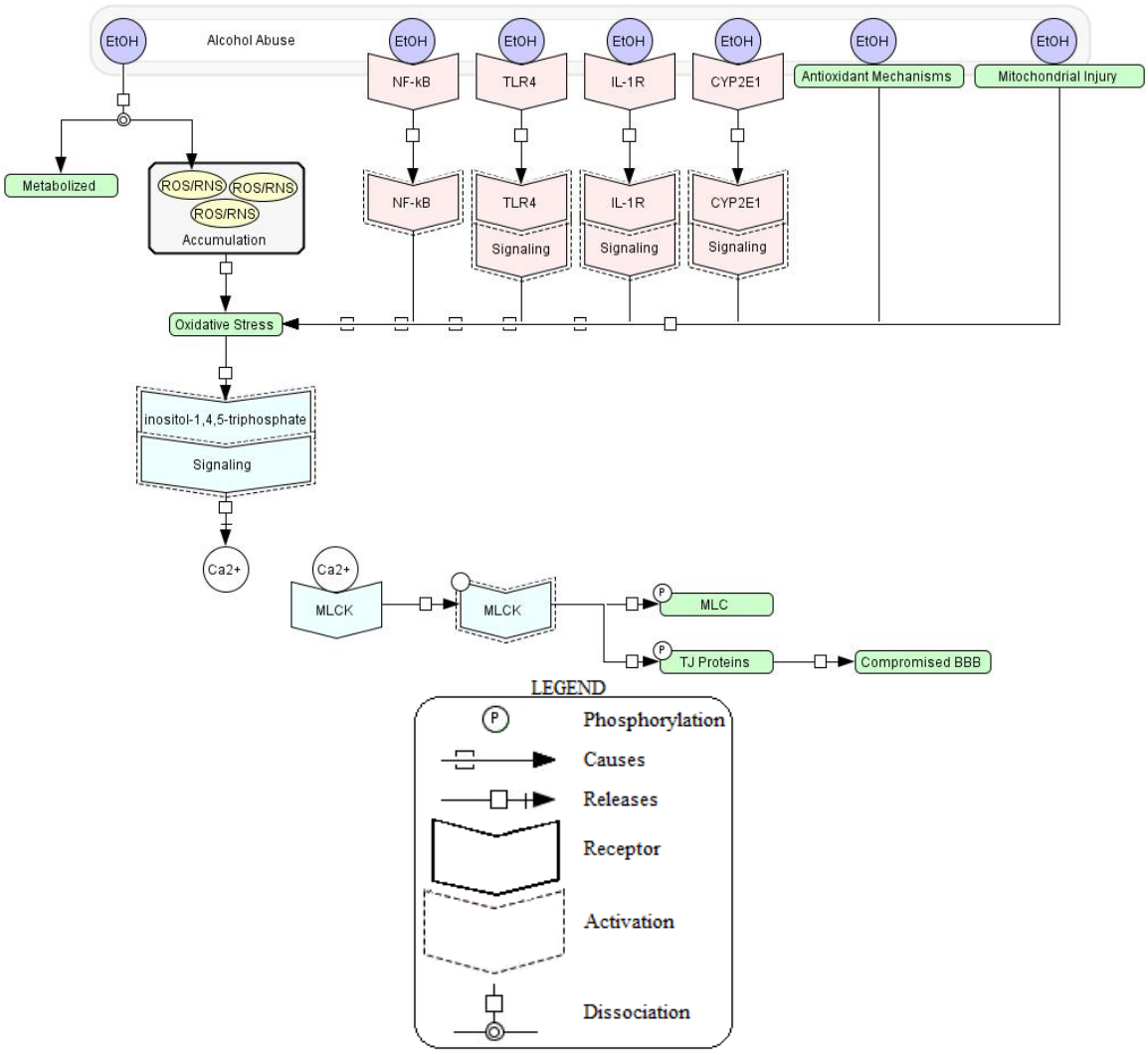

Figure 2.2 Process diagram for alcohol abuse exacerbating oxidative stress and compromising the blood-brain barrier. Ethanol metabolism results the activation of several inflammatory pathways, suppression of antioxidant mechanisms, mitochondrial injury and the prolonged production and accumulation of free radicals, all of which can

elevate oxidative stress in the brain. Oxidative stress can activate a signaling cascade that results in a blood-brain barrier that is more permeable.

2.2.3 Alcohol Interrupts the Energy Supply to the Brain

Alcohol use has been established to inhibit glycolysis in the brain [32].One such way is by down-regulating the uptake of glucose to the brain by reducing glucose transporter 1 (GLUT1), a uniporter protein involved in the transport of glucose [33-37]. In disturbing the translocation of mRNA for its biosynthesis, ethanol decreases the levels of glycosylated GLUT1 protein and its isoforms [38] (Figure 2.3). However, other ethanol-induced maladies can also lead to deficiencies in glucose uptake. For example, a prevalence of inhibitory neurotransmitters [39, 40], ethanol-damaged insulin signaling [41], and heightened levels of acetate in the blood stream [42], a precursor of ethanol consumption, have all been linked to an impaired uptake of glucose. To this effect, upregulation of acetate to the brain has been found to be a means of an alternative energy source [43-45]. Current research has been steered towards defining and understanding this shift in energy sources as an adaptive response to alcohol abuse (for example, [46]). Regardless, all related studies agree that alcohol can interrupt the energy supply by preventing entry of glucose into the brain; therefore there is an overall reduction of available energy in the brain following alcohol consumption.

Energy wasting in infected cells and malnutrition in non-infected cells accelerate Neuro-AIDS progression in alcohol abuse. Alcohol-mediated deprivation of glucose to the brain parenchyma reduces energy for astrocytes and neurons [47]. Additional total energy reductions are caused by alcohol's disruption in nutrient metabolic pathways [48]. This resulting state of malnutrition has been suggested as a mechanism for accelerated HIV progression [49].

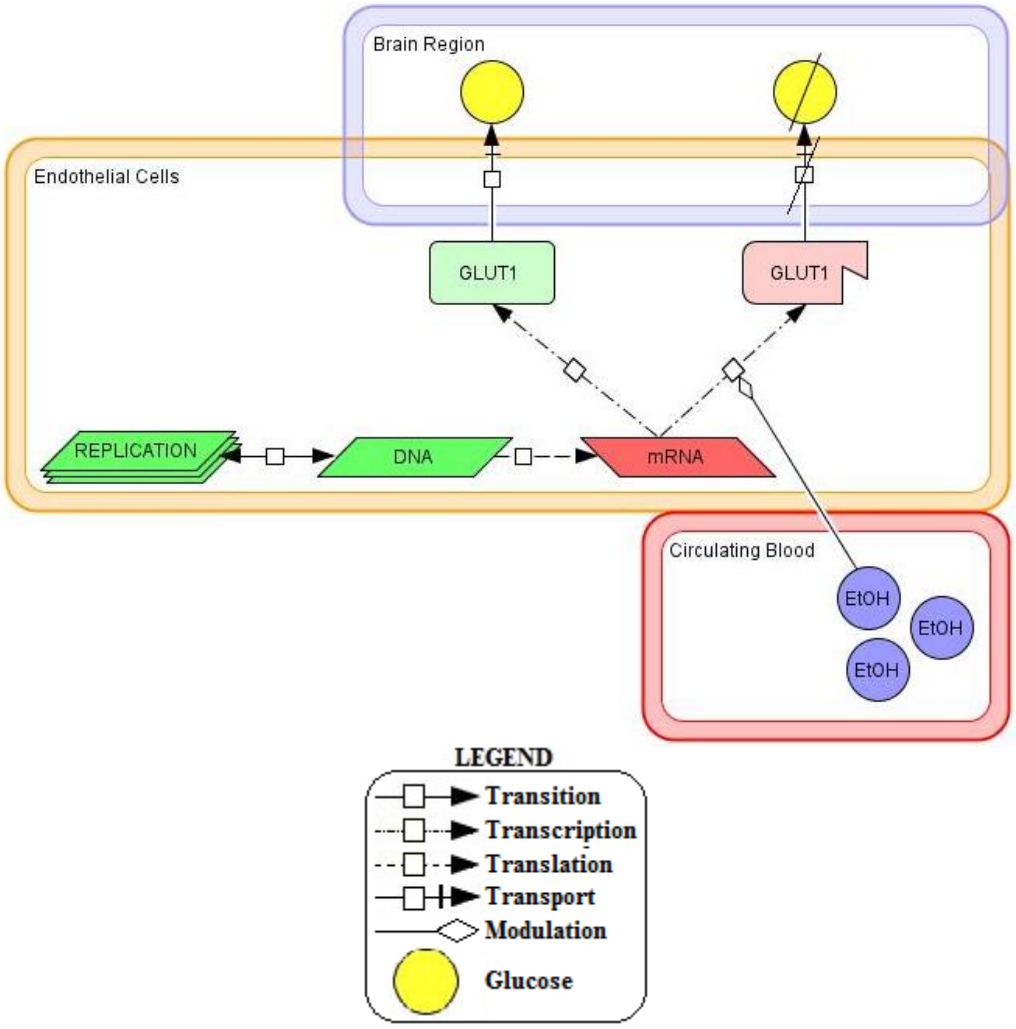

Figure 2.3 Process diagram for alcohol abuse causing malnutrition in the brain. Ethanol impairs transcription of mRNA for GLUT1 transporter protein. This transfigured GLUT1 is incapable of transporting glucose from circulating blood into the brain region. This decreases the amount of glucose in the brain.

2.2.4 Infected Microglia Scavenge

Neurons and astrocytes damaged by malnutrition and HIV shedded proteins/cytotoxins leak cell necrosis factors [50] into the surrounding extracellular space and signal P2Y12 receptors for microglia chemotaxis. These molecules subsequently stimulate the membrane of infected microglia and provoke ATP mediated activation of P2Y4 purinergic receptors [51, 52]. This causes a morphological ruffling-type behavior (actin

and tubulin rearranging) in the membrane issuing macropinocytosis ingestion [53] (Figure 2.4).

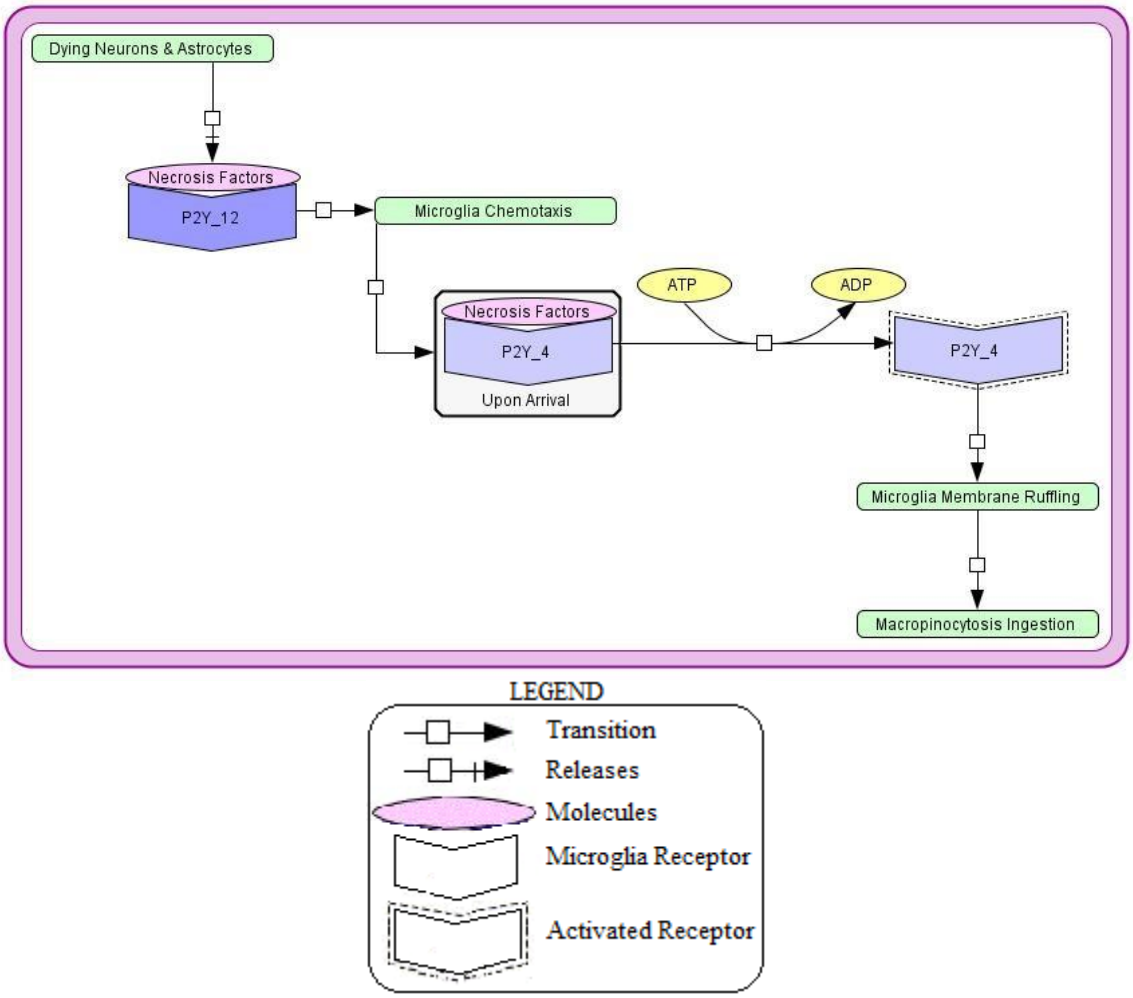

Figure 2.4 Process diagram for a mechanism for microglial pinocytosis. Necrosis factors released from dying cells stimulate microglial chemotaxis. Upon arrival these necrosis factors further stimulate energy dependent pinocytosis ingestion.

2.2.5 Adverse Effects of ART in Alcohol Abusers

Antiretroviral therapy (ART), although able to suppress HIV-1, fails to eradicate viral DNA compartmentalized in latent reservoirs of the CNS [54, 55]. This incapability prevents the complete removal of the virus and, with inconstant adherence to the therapy, can result in reactivation of the HIV-1 infection [56]. While emerging research has

suggested that forced reactivation of latent HIV-1with ART leads to its removal, results have not been definitive of this effect because research has been predominantly focused on identifying stimulant compounds, [57-61] of the ensuing degeneration caused by the toxicity of these stimulants [62], and purging may re-start replication and re-place viral DNA back into reservoirs. The side effects of ART are also controversial with reports of neurotoxicity [63], contraction of immune reconstitution inflammatory syndrome (IRIS) [64], cognitive impairment [65], and other adverse, unwanted reactions [66] especially within indigenous people [67]. Finally, even with successful ART, HIV-associated neurocognitive disorders (HAND) still persist, especially neuropsychological deficits [68, 69].

Alcohol abuse further exacerbates the consequences of HIV infection in the brain and undermines ART [4, 70, 71]. Alcohol abuse causes poor adherence to ART [72] but, moreover, can interrupt its treatment [73-76]. Overlapping pathways in ART and alcohol metabolism may explain these limiting and/or disrupted effects of ART and aggravation of HAND [49, 77].

2.3 A Matter of Energy Imbalance

One hallmark of viral infection of the central nervous system (CNS) is the immediate immune activation and enhanced energy metabolism of brain phagocytes, specifically macrophages and microglia [78-81]. Furthermore, HIV-1 infection of these phagocytes promotes their sustained survival [82]. With acute ethanol use, microglia is additionally reactive in order to help alleviate ethanol-induced neurodegeneration [83]. Reasonably, such prolonged activation needs a steady supply of energy; however, as above mentioned,

alcohol use deters the uptake of glucose causing a significant depletion of this energy source in the brain. There has been much evidence to suggest an abnormal or redistributed energy metabolism in the brain following infection [31, 79, 84-87]; resulting pathologic deficits provide further evidence. Metabolic shifts are expected in reaction to infection, however their gradation is still not fully understood. This thesis postulates that when metabolic reprogramming of infected microglia renders them reactive and subsequently insatiably hungry for biosynthetic precursors. Under this nutrient deprived and malnourished environment these infected microglia resort to a macropinocytosis-like ingestion [88] and amassing of available energy for infected cells survival, thereby causing malnourishment of uninfected surrounding cells. In this biological interaction, the highly reactive infected microglia predator feeds on its undernourished neuron and astrocyte prey (Figure 2.5).

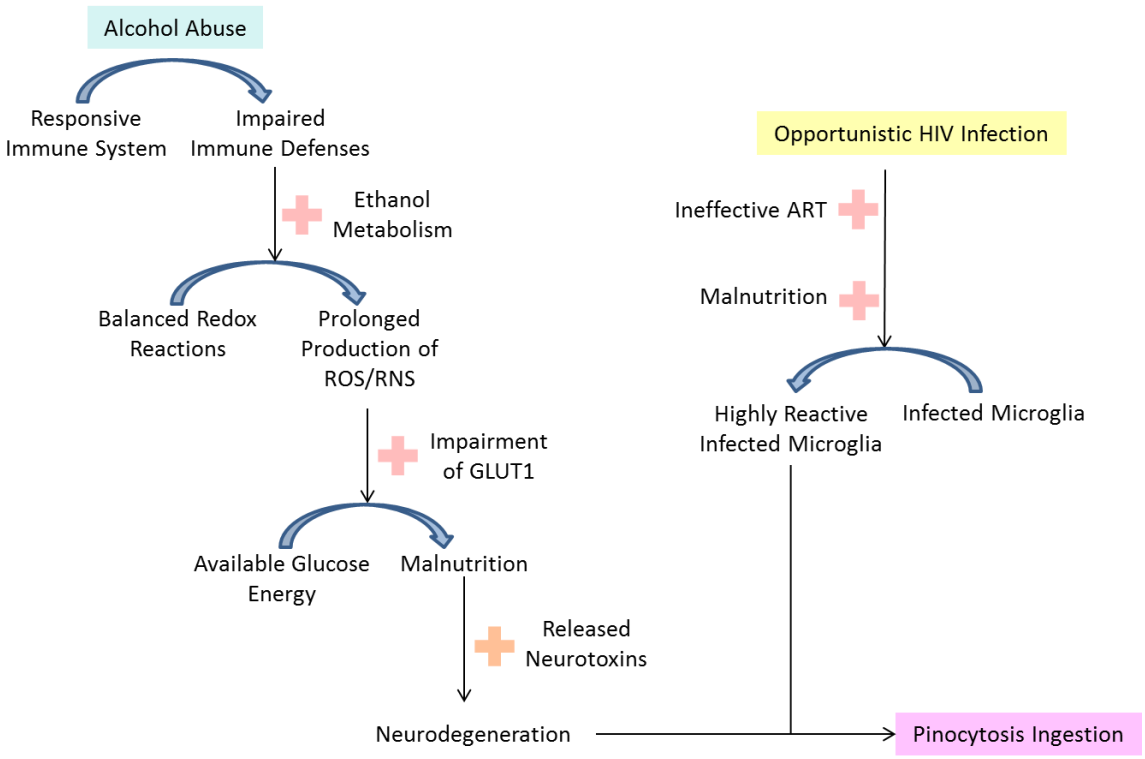

Figure 2.5 Comprehensive presentation of a mechanism for NeuroAIDS progression in alcohol abuse. Alcohol abuse impairs immune defenses and can lead to opportunistic transmission of the HIV-1 virus. Alcohol metabolism prolong the production of free radicals and impair GLUT1 subsequently causing a state of malnutrition in the brain. Alcohol abuse also causes poor adherence to treatment drugs. Ineffective treatment coupled with this state of malnutrition in the brain can cause infected microglia to become highly reactive. Infected microglia release HIV proteins/cytotoxins, that can further neurodegeneration. The theory is that infected microglia would ingest dying neurons and astrocytes for energy thereby progressing NeuroAIDS.

2.4 Comprehensive Experimental Approach

In order test the validity of this mechanism several pertinent questions must be answered.

They are as follows:

1. Is there truly a state of malnutrition in the brain under HIV + alcohol condition?
    a. How does the use of alternative energy sources such as lactate and acetate alter malnutrition? Are these sources enough for malnutrition to no longer be a problem? (Exploring metabolism in the brain)

b. For neuronal malnutrition, is there any energy compartmentalized in myelin sheaths? Can they access this energy?
c. Can HIV impair the utilization of these alternative energy sources? If so, can this further exacerbate malnutrition?
d. What is malnutrition's effect on neurodegeneration and astrocyte death?

2. Can shedded cytotoxins/HIV proteins cause sufficient neuron and astrocyte death?
    a. What is the concentration needed to achieve this? Do multiple cellular interactions affect this concentration?
    b. Does death occur by necrosis or apoptosis? Does it matter on the process? Can concentration affect which process?
    c. Can alcohol aggravate cellular death? What are the effects of alcohol concentrations on cellular death?
    d. Can alcohol increase the release and/or production of these proteins by infected cells?
3. Can alcohol increase infected macrophage blood-brain barrier transmigration or does this only depend on chemotactic stimulants?
    a. Does alcohol damage or impair the blood-brain barrier? What are the effects of alcohol concentrations on degree of injury?
4. Will malnutrition get infected cells more active towards finding energy? Will it awaken a survival instinct? Would this be the cell or virus's need to survive?
    a. With little to no available energy can they feed on surrounding cells? Can infected microglia use the debris for energy?

b. Would the brain cells experience a survival of the fittest scenario, a consequence of predator versus prey?

c. Can infected cells become greedy, mutate from constant hunger to consume all available energy? Can they overeat grow? Can microglia become multi-nucleated giant cells?

5. Would ingestion occur through a pinocytosis-like mechanism?

The possibility and extent of the proposed novel mechanism for NeuroAIDS progression in alcohol abuse will be uncovered by their answers. However, for this thesis only question 2a, 2b, and 2c will be examined because they do not require the use of the live HIV-1 virus. The results will determine if any synergistic effect of alcohol on HIV associated neurotoxicity is possible and whether further examination of this unique mechanism for HIV/AIDS progression would be meaningful.

2.5 TAT Neurotoxicity

TAT is the trans-activating protein of HIV-1 involved in viral replication. It is actively shedded by infected cells and can be found in high concentrations in circulating blood and cerebrospinal fluid [89]. TAT has a toxic effect on neuronal cells; however the nuances of TAT-induced neurotoxicity have not been fully investigated.

Experiment 1 will explore TAT concentrations and determine which concentration initiates single cell neuronal death. 2ng to 40ng/mL is the concentration found in the serum of many HIV-1 infected patients [90]. Also Eugenin et al. 2003 found significant neuronal death (>50%) at 10ng/mL after 24hrs exposure in mixed culture [91]. Therefore Experiment 1 will test neurotoxicity on pure neuronal culture at 2ng/mL, 5ng/mL, 10ng/mL, and 20ng/mL concentrations of TAT for 24hrs.

Experiment 2 will explore whether the addition of 2.5mM alcohol has an effect on neurotoxicity progression. 2.5mM is about 115mg/L blood alcohol concentration; for a 90kg person this would be the equivalent of having one drink (clinlabnavigator.com). With 2.5mM alcohol the personal would appear normal and experience no obvious impairments (http://flightphysical.com).

Finally, neuronal cultures after exposure to experiments 1 and 2 will be stained with Caspase-3 to determine if the cells are entering apoptosis.

2.5.1 Method and Materials

Neuronal cells were isolated from rat embryos (see Chapter 3) and plated on 12mm poly-D-lysine coverslips (Corning; Teterboro, NJ, USA) at a density of 50,000cells/cm2 in cortical media. The cortical media was comprised of Neurobasal medium (Thermo Fisher Scientific; Fair Lawn, NJ, USA) with 1% Penicillin/Streptomycin (Thermo Fisher Scientific), 2% 50X B-27 Supplement (Gibco, under Thermo Fisher Scientific), and 0.2% L-Glutamine (Sigma-Aldrich; MO, USA). 24hrs after plating, half of the media was changed; subsequent culture involved changing all media every 48hrs. These cells cannot be passaged. After 14daysin culture they lose quality and are no longer appropriate for experimentation.

TAT recombinant protein was generously provided by Dr. Eliseo Eugenin at the Public Health Research Institute (PHRI) of New Jersey Medical School (NJMS) Newark, NJ, USA at a concentration of 25ng/µL. In experiment 1 rat neuronal cells were cultured for 7 days then exposed to 2ng/mL, 5ng/mL, 10ng/mL, and 20ng/mL concentrations of TAT for 24hrs. In experiment 2 rat neuronal cells were cultured for 7 days then exposed to these same with the addition of 2.5mM stabilizer-free EtOH for 24hrs. After which,

cells were fixed onto the coverslips with 4% paraformaldehyde (Sigma-Aldrich) and stained for Neu-N, Neurofilamets, and Caspase-3 (Abcam; MA, USA).

2.5.2 Results

At 2ng/mL of TAT exposure no neuronal death was observed (Figure 2.6b). At 5ng/mL fragmentation was seen for some axon extensions (Figure 2.6c). Also smaller aggregations of neurons demonstrated loss of synapses and axonal shrinkage at this concentration (Figure 2.7b). However at concentrations greater than 7ng/mL all axonal and dendritic projections were gone (Figure 2.6d). For densely aggregated neurons, complete axon shrinkage was observed at concentrations greater than 9ng/mL (Figure 2.6d and 2.7c). At 10ng/mL of TAT exposure no neuro-filaments were observed for all single cell and aggregated neuron populations instead much cellular debris was seen (Figure 2.6d). However within these aggregations neuro-filament projects were still visible (Figure 2.7c). At 20ng/mL all aggregations appeared dislodged (Figure 2.6e).

With the addition of 2.5mM EtOH, at 2ng/mL neuronal cultures were projecting more axons and dendrites (Figure 2.8b). At 5ng/mL most neuronal extensions were lost (Figure 2.8c). At 10ng/mL all neuronal extensions were lost and aggregations appeared dislodged (Figure 2.8d). Again neurons of these dislodged aggregations still showed neuro-filament connections among themselves (Figure 2.8d).

Caspase-3 staining was seen in most neurons at 2ng/mL of TAT exposure (Figure 2.9a). At 5ng/mL, staining was observed at aggregated neurons however little to no staining was seen in surrounding single cells (Figure 2.9b). With the addition of EtOH, staining was observed in single cell neurons; however most neuro-filaments were lost

(Figure 2.9c). Caspase-3 staining was increased in 5ng/mL of TAT exposure with the addition of EtOH (Figure 2.10).

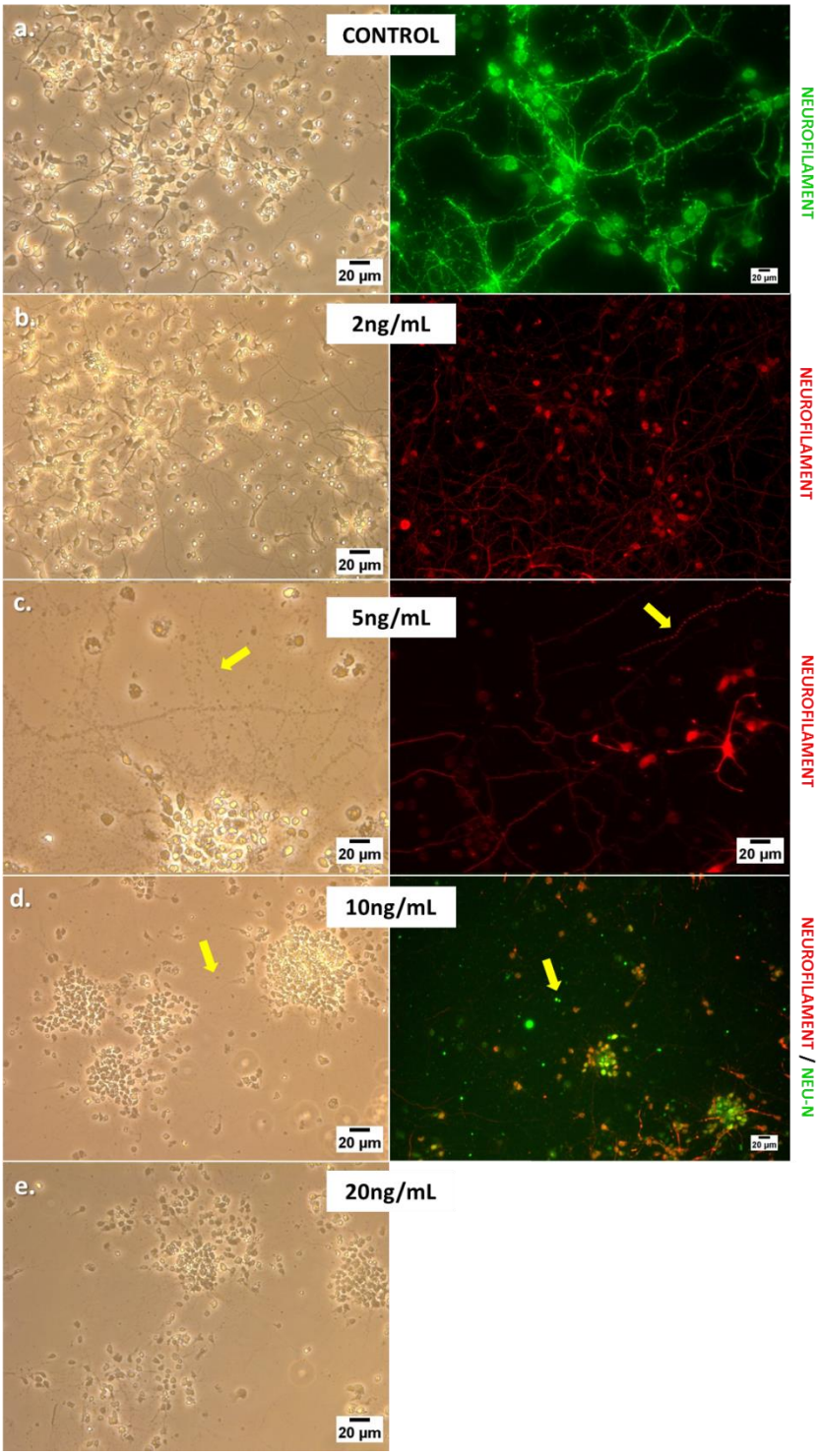

Figure 2.6 Results of TAT neurotoxicity. a) no TAT, b) 2ng/mL, c) 5ng/mL (arrow point to axon fragmentation), d) 10ng/mL (arrow points to cell debris), and e) 20ng/mL exposure. Cells were exposed to respective TAT concentrations for 24hrs. Left paneled are phase contrast images and right paneled are florescent images. Not shown is a florescent image at 20ng/mL TAT exposure.

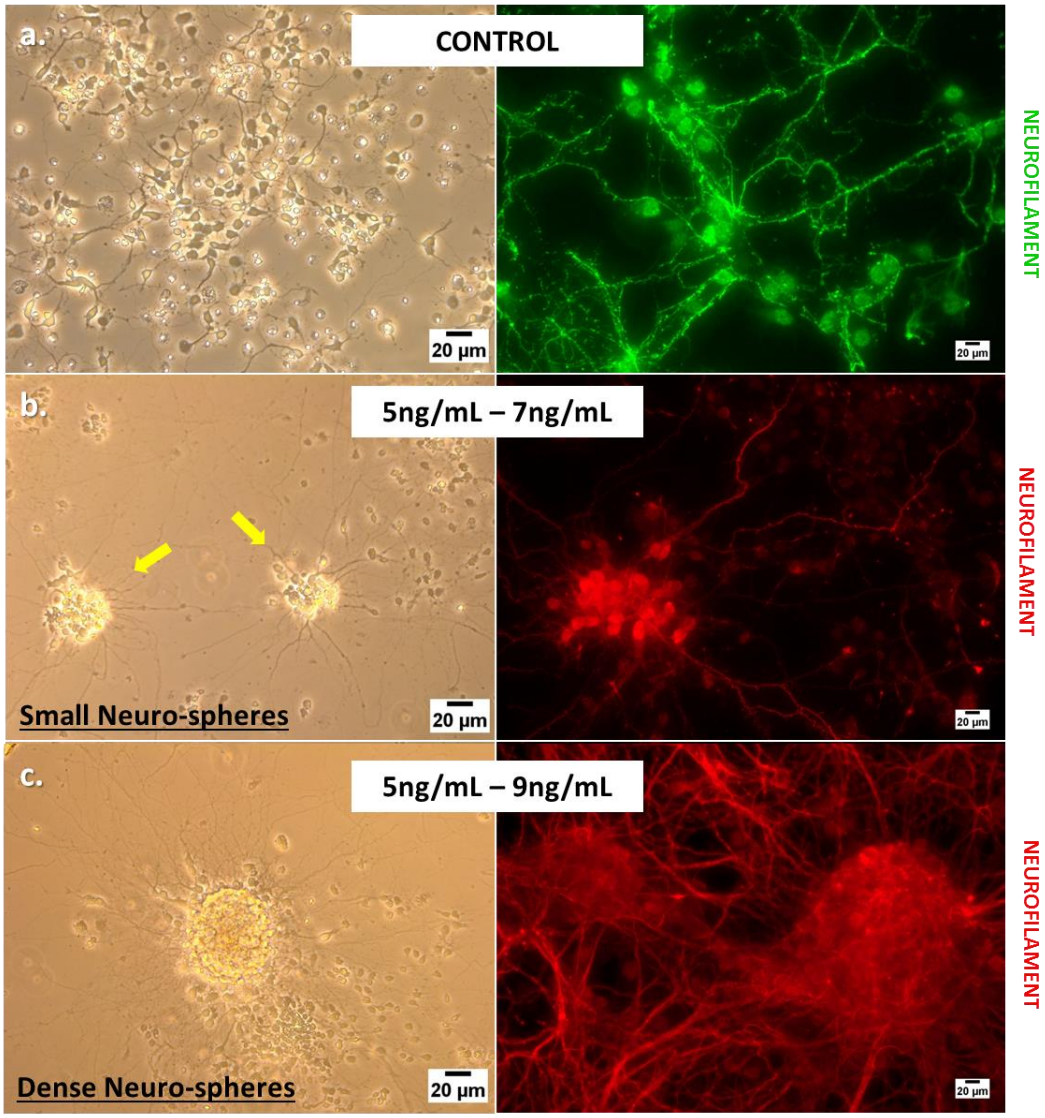

Figure 2.7 Results of TAT neurotoxicity on aggregated neurons. a) control, b) small neuronal aggregates, and c) dense neuronal aggregates. Cells were exposed to respective TAT concentrations for 24hrs. Arrows point to loss of synapse and axon shrinkage. Left paneled are phase contrast images and right paneled are florescent images.

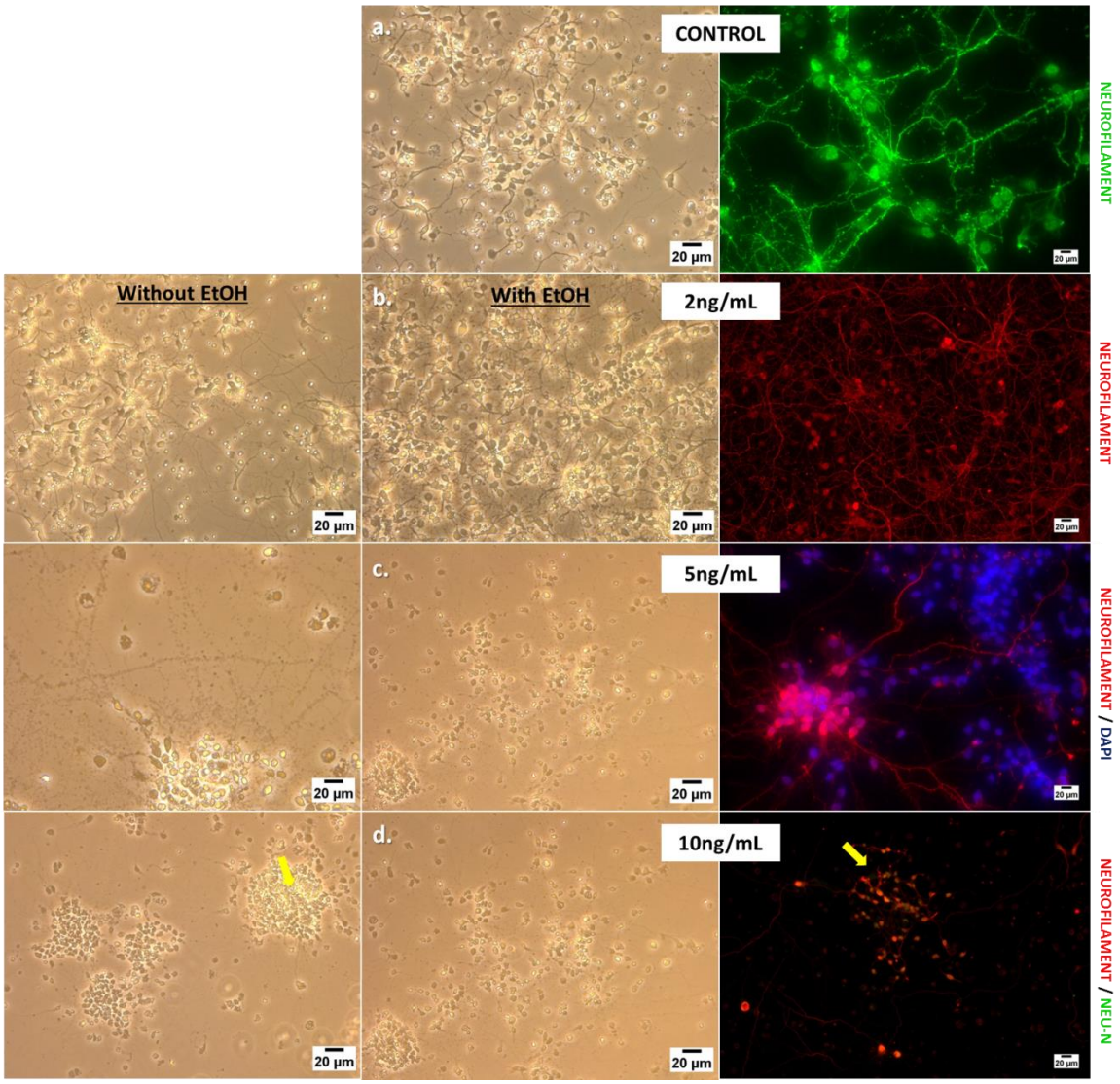

Figure 2.8 Results of TAT neurotoxicity with 2.5mM EtOH. a) control, b) 2ng/mL, c) 5ng/mL, and d) 10ng/mL TAT with 2.5mM EtOH (arrow points to remaining neuro-filaments). Cells were exposed to respective TAT with EtOH concentrations for 24hrs. Left paneled are phase contrast images of cells not exposed to EtOH, middle paneled are phase contrast images of cells exposed to EtOH, and right paneled are florescent images of cells exposed to EtOH.

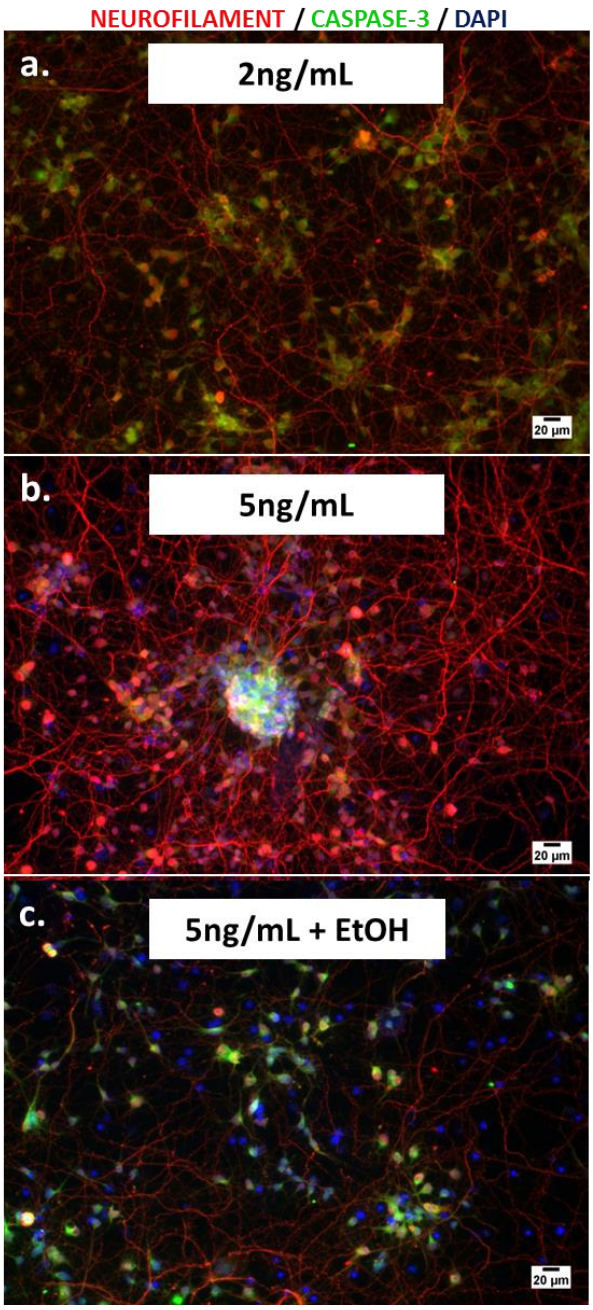

Figure 2.9 Results of Caspase-3 staining. a) 2ng/mL, b) 5ng/mL, and c) 5ng/mL with EtOH. Cells were exposed to respective concentrations for 24hrs.

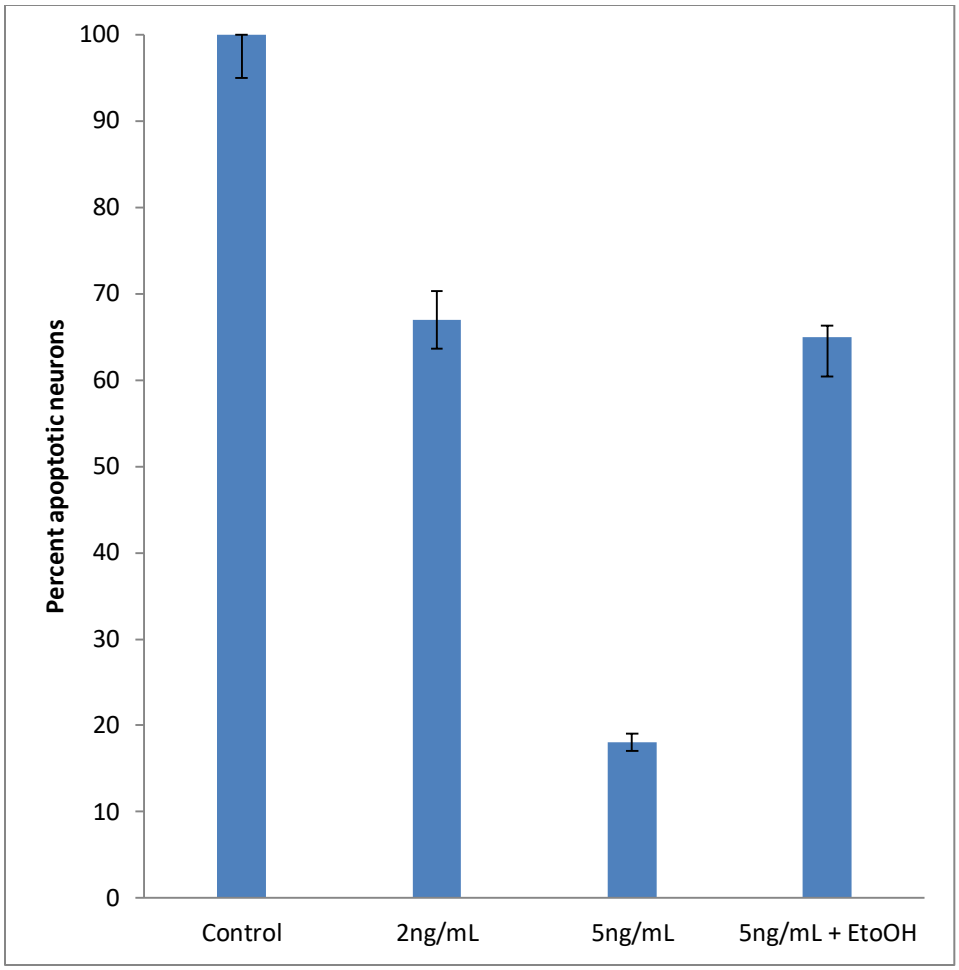

Figure 2.10 Percent of Caspase-3 staining. Total number of nuclei was found by counting the number of DAPI stains. The number of cells stained with Caspase-3 was also counted. Percent Caspase-3 staining was found by dividing Caspase-3 staining count by DAPI staining count. A significant number of Caspase-3 was found in 5ng/mL TAT exposure with the addition of EtOH. (N = 4).

2.5.3 Blood-brain Barrier Model

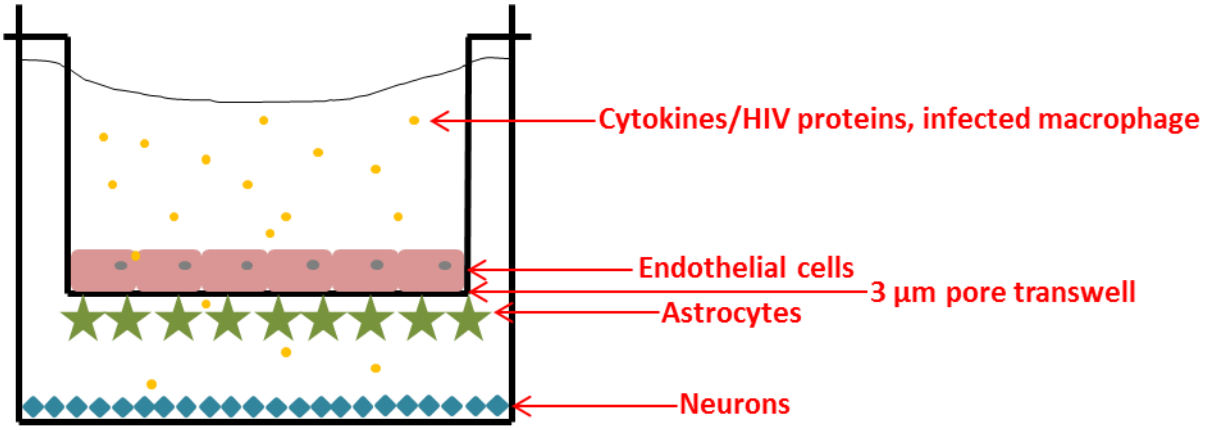

Figure 2.11 In vitro model of the blood-brain barrier. Adapted from Eugenin, E.A. et al. (2003) the model features a tri-culture of endothelial cells, astrocytes, and neurons on a 3µm pore transwell. Astrocytes were cultured on the underside of the transwell while endothelial cells were cultured on the inside bottom. This co-culture was then suspended over cultured neuronal cells. Cytokines/HIV proteins, infected macrophage, or any molecules of interest would be released on the endothelial side of the model, transmigration would then be studied.

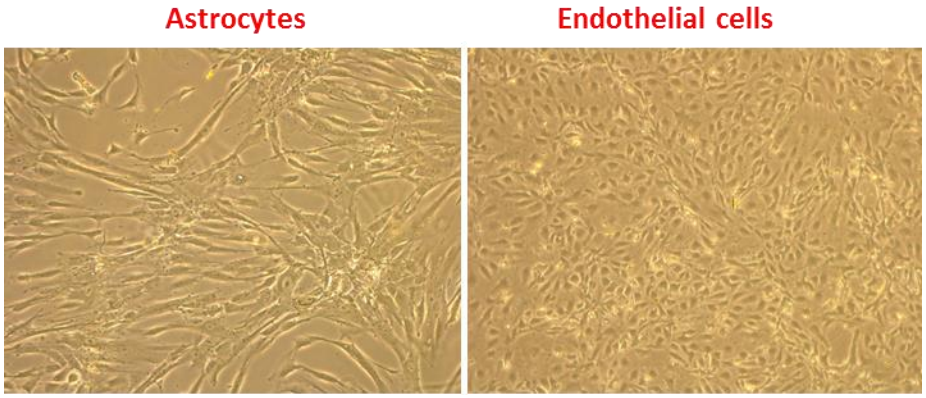

Figure 2.12 Astrocytes and endothelial cells in culture. Phase contrast images on astrocyte and endothelial cultures used for the in vitro blood-brain barrier model.

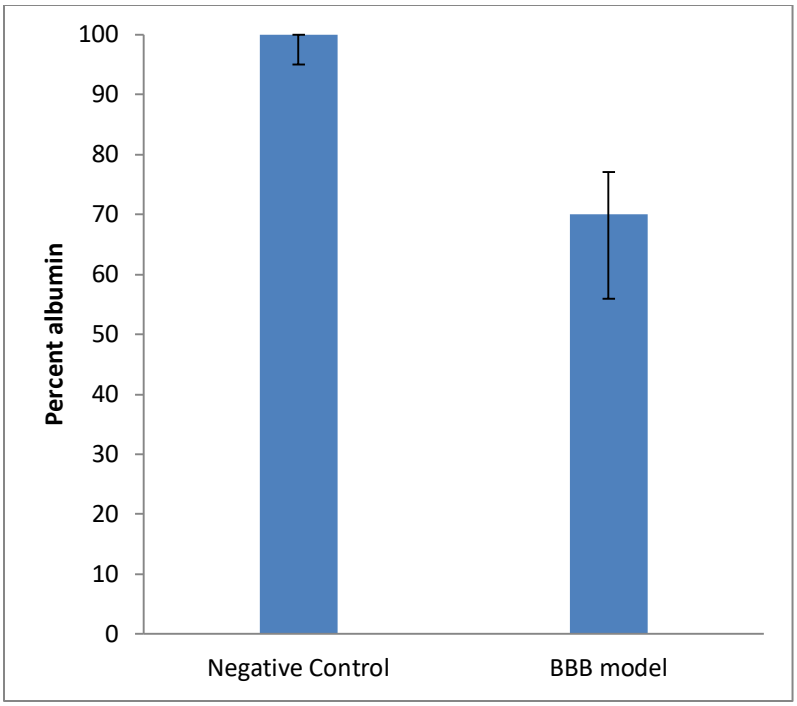

Figure 2.13 Results of permeability assay on the in vitro blood-brain barrier model. 0.5% Evan's blue was conjugated to 0.45% albumin and released on the endothelial side of the model. After 30mins at 37°C the solution on the neuronal side of the model was collected and read with a spectrophotometer. Negative control was a transwell with no cells seeded therefore 100% of the Evan's blue-albumin would pass through the transwell. (N = 4).

2.5.4 Discussion

Complete cell death is certain at TAT concentrations of 5ng/mL and higher however the concentration at which cell death is initiated cannot be definitely ascertained because it is unclear if there was neuronal death at 2ng/mL from the phase contrast and florescent images. MTT and/or MTS assay can be used to find the concentration that initiates death. It is clear that TAT-induced death begins with axon shrinkage and loss of synapse as seen in small neuron aggregates with 5ng/mL TAT exposure. Significant cellular death often means the point at which cells begin dying exponentially; however, significant cellular failure for HIV associated neurological disorders (HAND) might mean the point of

synaptic loss. Overlooking neuronal death instigation and patterns may impede discovery for HAND treatment.

Significant axonal fragmentation was also seen at 5ng/mL. This may mean that TAT-induce axonal death begins with fragmentation; however this was not seen in all axons. Media changes could have washed-away this unseen fragmentation. The neuron cell bodies were more resistant towards TAT toxicity then their axons.

Neuro-spheres are aggregates/clusters of neurons; they can be created in culture with high seeding densities or will occur organically over time. Smaller neuro-spheres, with distinguishable individual neurons, are the result of this organic formation. These neuro-spheres proved to be quite resistant to TAT toxicity because at concentrations greater than 5ng/mL but less than 7ng/mL they showed axon shrinkage, i.e. still projecting neuro-filaments. Both in phase contrast and fluorescent microscopy, these aggregated neurons looked viable because neuro-filament projections were still visible; however this does not affirm that these neurons were actually viable.

Low doses of alcohol have been shown to enhance neurotransmission and thus improve the health of neurons [92]. At 2ng/mL TAT, 2.5mM EtOH exposure improved neuronal culture; this can mean that low doses of alcohol can counter the toxic effects of TAT. However, this protective effect is lost at TAT concentrations that produce complete neuronal death such as 5ng/mL and higher. At such critical concentrations EtOH might exacerbate death or, in the very least, clear away axonal fragmentation and debris. High EtOH dose were not tested because they have been established to have detrimental effects on neurons.

Caspase-3 staining was used to indicate neurons were undergoing apoptosis or programmed cell death. Since little neuronal death was seen with 2ng/mL TAT, it is unsurprising that, upon death, many of these cells would be entering apoptosis. Also, since neurons in their neuro-sphere formation were found to be more resistant to TAT toxicity, it is unsurprising that at the high concentration of 5ng/mL, upon death, neuro-spheres would be entering apoptosis while many surrounding, single cells would not. However the absence of Caspase-3 staining does not signify necrosis, a live/dead assay would have to be performed to establish that unstained dead cells are the result of necrosis or instant death (mitochondrial shut-down).

At 2ng/mL TAT, 2.5mM EtOH had a stimulating effect because neuronal culture looked improved. EtOH's counteractive effects towards TAT toxicity would explain why more Caspase-3 staining of single cell neurons was seen at 5ng/mL with EtOH. However, most neuro-filaments were lost with EtOH than 5ng/mL TAT exposure alone. This means that EtOH concentrations as low as 2.5mM might have a protect effect on the neuronal body while exacerbating TAT toxicity on its extensions This can explain why there are contradictory views in the literature on the correct dose at which alcohol goes from being beneficial to harmful in the brain especially with the presence of toxins.

More sophisticated and quantifiable apoptosis assays, such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), should be performed to confirm neuronal cells are indeed dying from apoptotic signaling cascades even though, exposed to toxins, should in principle undergo cell necrosis. Presence of Caspase-3 staining was dependent on the concentration of TAT exposure, whether neurons were in

single cells or a neuro-sphere formation, the density of the neuro-sphere, and presence of low dose EtOH.

Additionally it would be interesting to find out if and how TAT is cleared away in the brain and if it is cleared away before neurons can experience its toxic effects. Other shedded proteins such as gp120, although only found in picogram concentrations in the serum of HIV infect patients, can also be studied for neurotoxicity. Combination HIV shedded proteins, alcohol, and other cytotoxins can be studied on neuronal death as well.

2.6 Summary

Chapter 2 reviewed exacerbating effects of alcohol on HIV-1 transmission, infection, and the role of metabolic energy imbalance during NeuroAIDS progression in the literature to formulate a possible mechanism for accelerated NeuroAIDS progression. The neurotoxicity of HIV proteins (Tat) in the setting of interactive neuroimmune cell culture (brain endothelial cells, astrocytes, microglia, and neurons) with or without the presence of alcohol was also investigated. These experiments traced the pattern of neuronal death which begins with axon shrinkage and ends with the dislodging of neuron aggregates. Varying effects on TAT neurotoxicity, depending on the TAT concentration used, was observed with the addition of EtOH. Finally exposed cells were stained with Caspase-3 to determine if they were entering apoptosis. Chapter 2 also showed ongoing work on an in vitro blood-brain barrier model, an endothelial cell, astrocyte, and neuronal cell tri-culture on a transwell.

CHAPTER 3

AIM 2: TECHNIQUE FOR RAT EMBRYONIC NEURONAL ISOLATION

3.1 Need for a Superior Isolation Technique

A key material needed for examining TAT neurotoxicity in Chapter 2 was neuronal cells. The most clinically relevant approach to conducting in vitro studies is by using primary cells. Since human fetuses are hard to procure, neuronal cells harvested from rat embryos are the next choice. In terms of sensitivity, there is little difference between human and rat primary neurons. However, when investigating cellular death good quality cells are necessary to prevent false data. It must be clear that the cells have died of TAT exposure and not from poor neuronal isolation; this is especially important when mapping the death cycle. It was found that the rat embryonic neuronal isolation techniques present in the literary did not yield superior neuronal cells. Chapter 3 presents a quick and easy technique that produces consistent, high yield, and very pure neuronal cells.

3.2 Method and Materials

A Sprague Dawley E17 pregnant rat was anesthetized with a combination of ketamine and xylazine according to the recommendations and guidelines of the Institutional Animal Care and Use Committee; after which the rat was pinned down and the abdomen was cut to expose the fetuses. Fetuses were removed one at a time, with the rest remaining in the live mother; the embryo was then extracted from the removed fetus. Holding the embryo in an upright position with the anterior portion of the head faced away, an incision was made long the midline of the skull with a scalpel. Next the skull

was peeled back and the cortices teased out. Successful extraction can be confirmed if the removed tissue has a kidney shape (Figure 3.1). Extracted hemispheres were placed in L-15 media (Thermo Fisher Scientific) on ice, finally meninges were removed. The entire extraction process, from administering anesthesia to removing meninges, was done in less than 10 mins. If many cells were needed, extractions were done by two or more technicians simultaneously to keep total extraction time under 10 mins. One pup with both cortices removed produced almost 2.5 million cells. The whole enzymatic tissue digestion, neuronal isolation techniques, cell count, and cell plating were performed as per our standard laboratory protocols.

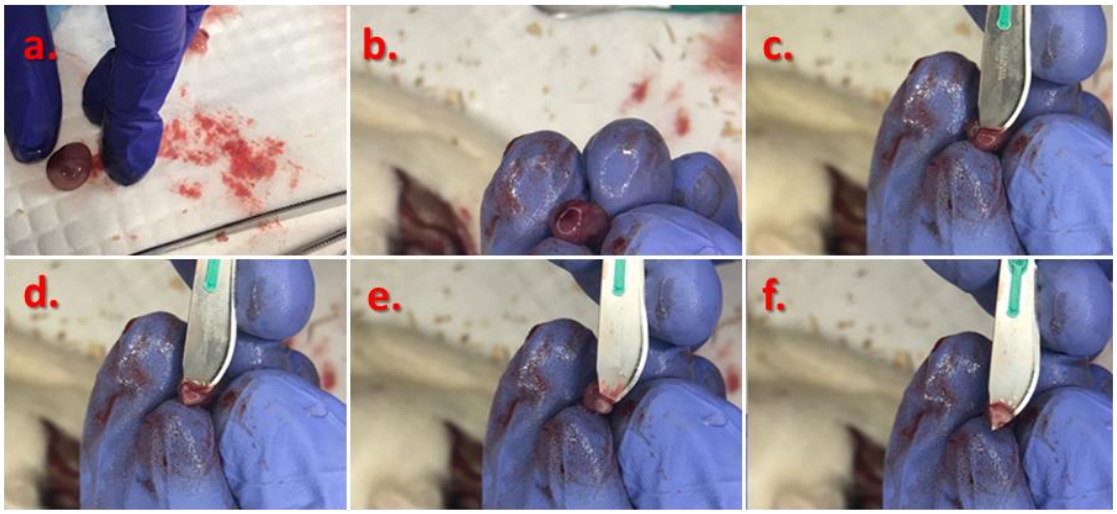

Figure 3.1 Step-by-step procedure of neuronal dissection from freshly extracted fetus. a) Fetus removed from its embryonic sac. b) The embryo held with the brain facing up. The brain of an E17 pup has developed enough while the skull is thin enough so that the hemispheres can be easily distinguished. c) Incision along the midline of the skull being careful not to puncture the brain. d) Peeling back of the skull. e) Gentle back and forth teasing of the cortices from the brain. f) The final kidney shaped tissue extracts.

3.3 Comparison to Established Techniques

Table 3.1 Comparison of techniques found in the literature to the one presented here.

Criteria	Agas	Muramatsu et al. [93]	Pacifici et al. [94]	Xu et al. [95]
Performance	✓	X	X	✓
Yield	✓	✓	✓	✓
Cell Purity	✓	✓	X	X
Cell Sustainability	✓	unknown	X	unknown
Cost Efficient	✓	X	X	X
Time Efficient	✓	X	X	X
Consistency	✓	X	X	✓

Performance was judged by how easy it was to perform the technique. Both the Muramatsu and Pacifici techniques call for extracting the whole brain then require the use of a dissecting microscope (stereomicroscope) to separate the hemispheres, the Agas and Xu techniques do not. Since brain tissue is very gooey, lifting it from the skull and isolating the cortices will prove to be difficult and may even damage the tissue. Also such precise separation and use of a dissection microscope necessitate considerable surgical skill and training; poor isolation decreases the purity of the culture. Finally, a dissection microscope would take away from *Cost Efficiency*. A difficult technique also diminished how consistently it could be performed and the *Consistency* of cell caliber. *Time Efficiency* was judged by how fast the technique could and should be performed. The Muramatsu, Pacifici, and Xu techniques allowed for 2hrs while the Agas technique could be performed within 10mins.

Yield describes both the health of the neurons during culture and number of viable cells obtained from the isolation. Abundant healthy neurons began projecting many strong and long extensions by 5 days in culture (Figure 3.2a and f) while fewer neurons saw weaker and less extensions (Figure 3.2c). *Cell Purity* designates that the cells in

culture are indeed neuronal cells and not any other cell type. In a pure culture, DAPI (blue) staining would overlap with neuronal biomarkers (Figure 3.2b and e); contamination can be inferred with fewer overlaps (Figure 3.2d). Finally, *Cell Sustainability* described neuronal longevity in culture.

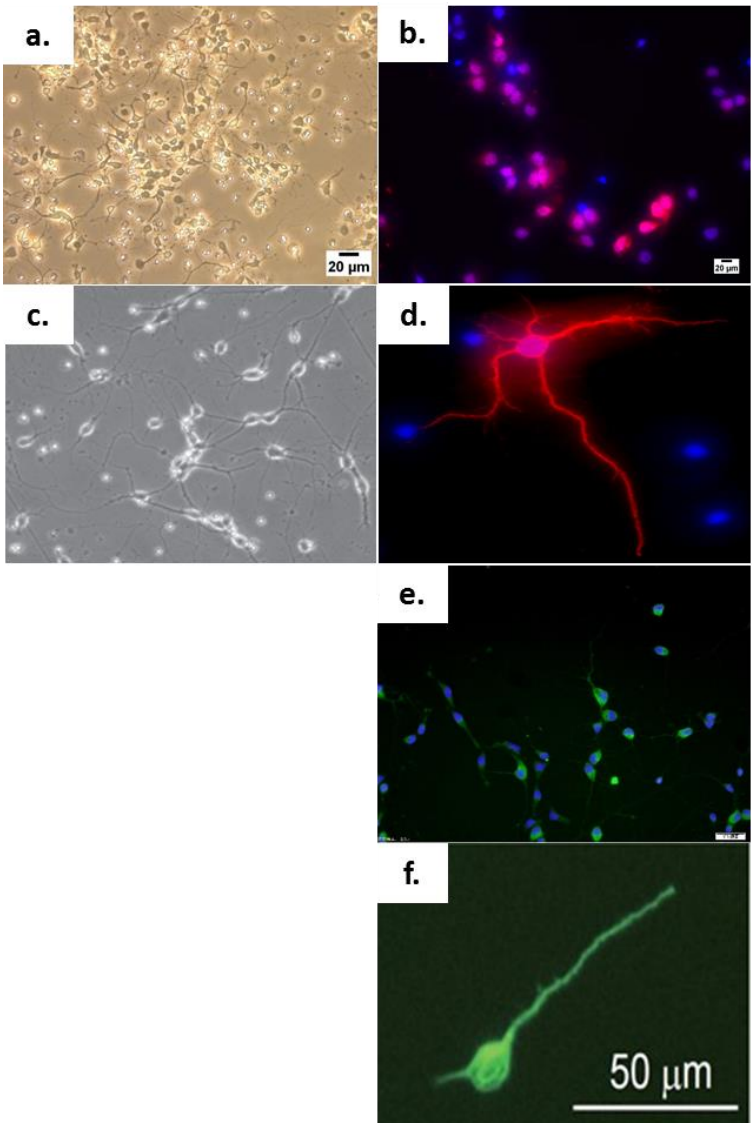

Figure 3.2 Comparison of established techniques to the one present here. Left paneled are phase contrast images and right paneled are fluorescent images (DAPI is stained blue). Not prodivded are phase contrast images for Xu and Muramatsu techniques. a) and b) Agas, Neu-N. c) and d) Pacifici, MAP-2, e) Xu, β-tubulin III+, and f) Muramatsu, β-tubulin III+ immunolabeling.

Sources: [93-95].

3.4 Proof of Concept

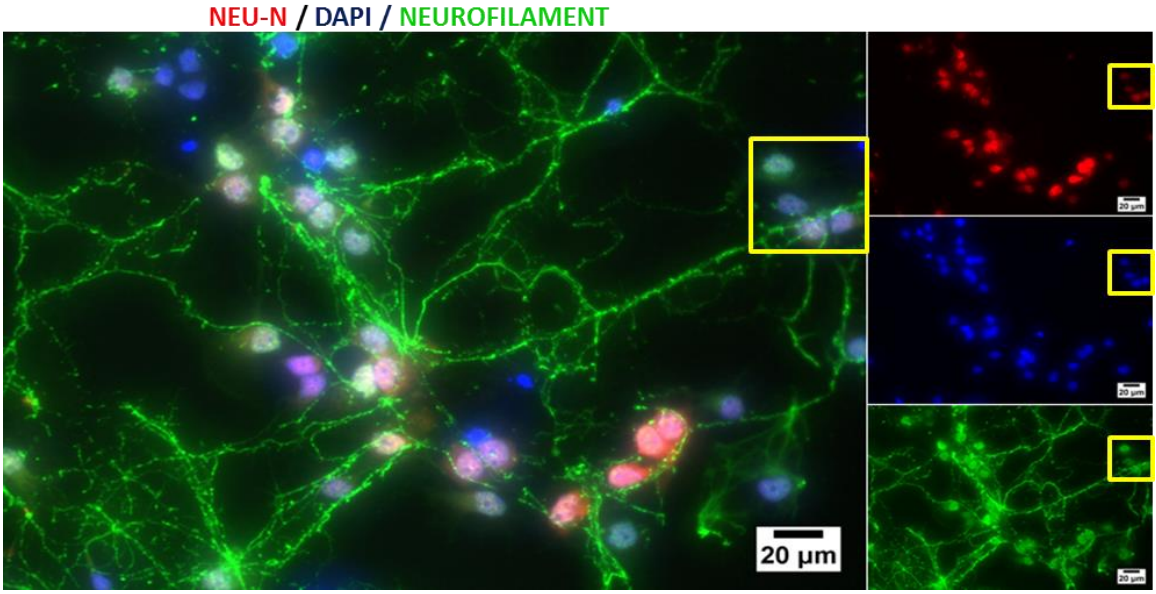

Figure 3.3 Demonstration of culture purity. Cells were cultured for 7 days in cortical media (see Chapter 2.5.1) then fixed with 4% paraformaldehyde and stained. Neuronal biomarkers, Neuro-filament and Neu-N, and DAPI overlap signifies that the cells in culture are indeed neurons and no other cell types.

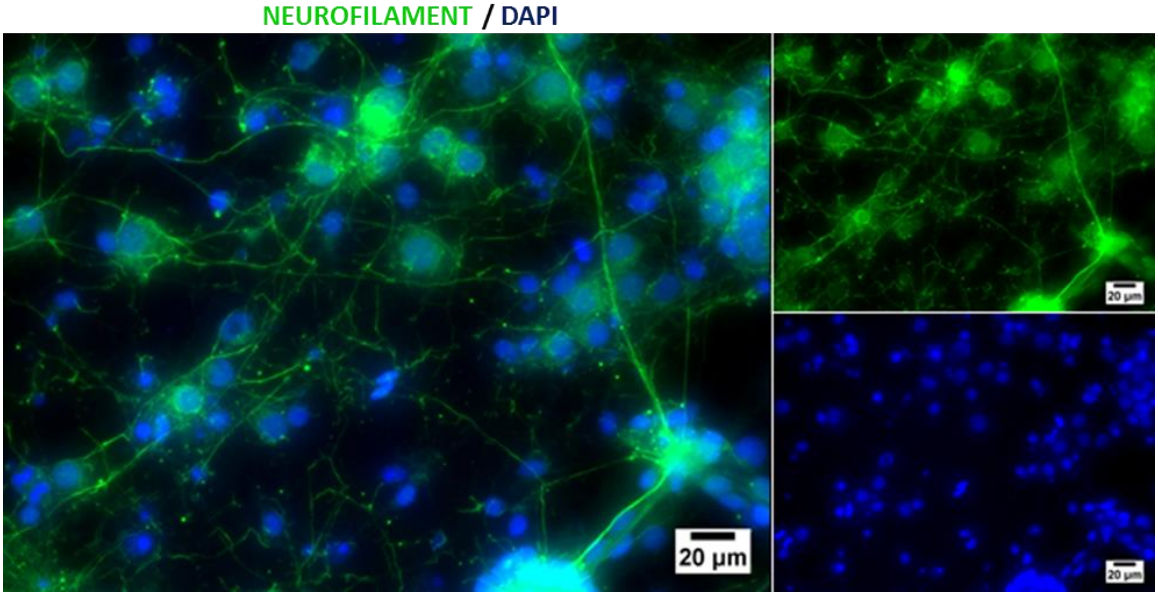

Figure 3.4 Demonstration of cell sustainability. Cells were cultured for 14 days in cortical media (see Chapter 2.5.1) then fixed with 4% paraformaldehyde and stained. There are still some viable cells as revealed by the outwards projections of some single cells and neuro-spheres neuro-filaments towards other cells. Extended projects remain unbroken.

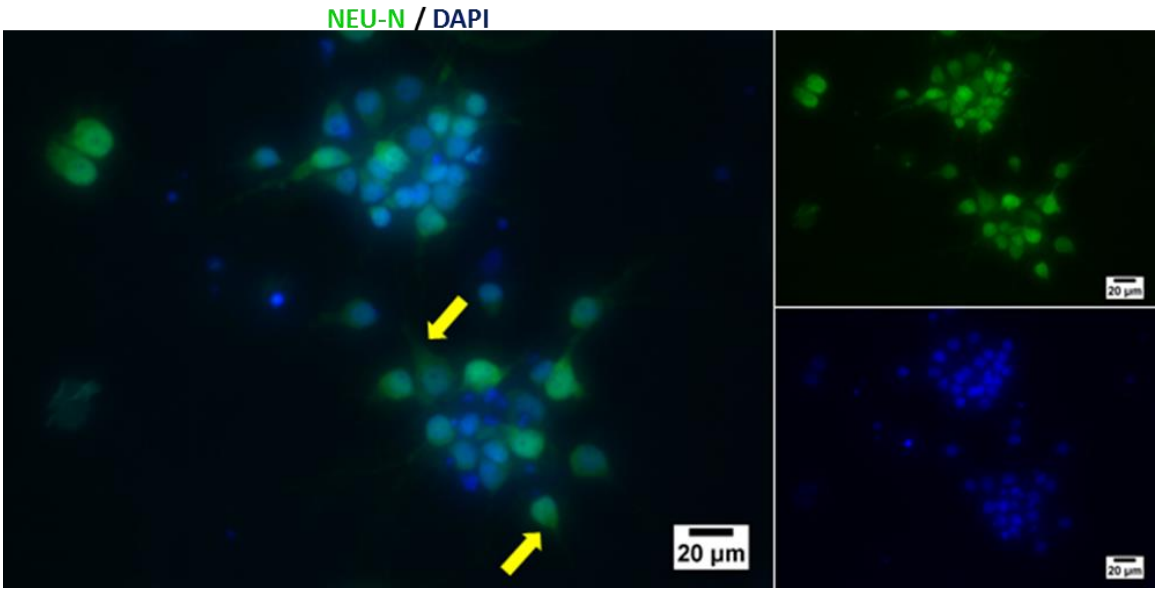

Figure 3.5 Demonstration of cellular health. Cells were cultured for 14 days in cortical media (see Chapter 2.5.1) then fixed with 4% paraformaldehyde and stained. After two weeks culture, these cells are still behaving like neurons because they are coming together into neuro-spheres. Also neuro-sphere formation rather than dissociation can also demonstrate good sustainability.

3.5 Discussion

The cortical isolation technique proved to have a great impact on the yield, purity, and survivability of the final neuronal culture. In keeping the mother alive and removing one fetus at a time for dissection, the remaining fetuses were still linked to the nutrient supply and body warmth of the mother. This created a smaller interruption in the neurons' environment. However, performing the entire dissection in less than 10mins had the greatest positive impact on viability; this was the greatest difference between Muramatsu, Pacifici, and Xu techniques and the technique presented here. The surgeon's rule-of-thumb for time at which the brain experiences fatal damage is 10mins without oxygen [96], therefore the technique was simplified and coordinated so as to perform complete dissection, from anesthetizing the pregnant rat to removing meninges, in less than

10mins. Also, because this technique does not require the use of a surgical microscope, once a feel for this technique is established, it can be applied to and performed on less developed fetuses (younger fetuses would produce healthier neurons). More sophisticated characterizing assays, such as fluorescence-activated cell sorting (FACS) would need to be performed to confirm the superiority of this technique at producing neurons with better yield, purity, and survivability.

3.6 Summary

Chapter 3 presented a quick and easy technique for producing consistent, high yielding, and very pure neuronal cells. This technique proved to be superior to others present in the literature. The advantages of this technique involved anesthetizing the pregnant rat so that remaining fetuses were still linked to the nutrient supply and body warmth of the mother, eliminating the need for a dissecting microscope, and decreasing dissection time from 2hrs to less than 10mins.

CHAPTER 4

CONCLUSIONS

4.1 Overall Summary

This thesis outlined discrepancies in HIV statistics finding highest AIDS-related mortality rates occurring in urban populations where alcohol consumption and heroin use are prevalent. This can further the claim that drugs and/or alcohol cause HIV/AIDS transmission and faster progression. It distinguished NeuroAIDS as a major problem in HIV/AIDS persistence and progression because antiretroviral therapy drugs have difficulty passing the blood-brain barrier and brain-to-blood directing efflux systems purge HIV virions back into circulating blood and re-infect the body. This thesis then postulated that, in a synergistic effect, alcohol and NeuroAIDS can awaken a novel mechanism for accelerated NeuroAIDS progression. The idea behind this mechanism is that as alcohol disrupts energy metabolism, it creates an energy imbalance in the brain in favor of infected cells and, in so doing, progress NeuroAIDS.

One limitation of this thesis was not having access to the live HIV-1 virus; therefore only the neurotoxic effects of shedded HIV-1 proteins were examined. What was originally a study into the exact TAT concentration that initiated neurotoxicity became tracing the pattern of neuronal death which started at axon shrinkage and ended with dislodging neuro-spheres. EtOH was found to have varying effects on TAT neurotoxicity depending upon the TAT concentration used. For 2ng/mL TAT, EtOH had a protective effect while for 5ng/mL and higher TAT, EtOH exacerbated death. However Caspase-3 staining revealed that single cells at 5ng/mL with EtOH were undergoing

apoptosis suggesting that EtOH might have a protect effect on the soma while exacerbating TAT toxicity on its extensions. This relates to the comprehensive hypothesis in the following way: necrosis produces large waste while apoptosis produces fragmented waste; physiologically, microglia use phagocytosis to clear large waste and pinocytosis to clear small waste; if TAT toxicity is causing necrosis, will the route of energy ingestion still be pinocytosis or will it now be phagocytosis? That is, how large of a waste can microglia ingest with its pinocytosis mechanism, and does this amount change with infection? However, this thesis has demonstrated that apoptosis can happen even with TAT exposure; therefore pinocytosis is still a possibility. As an expansion of this project, future work can focus on the effects of 2ng/mL TAT exposure for alterations of axonal diameter thinning and axonal fragmentation. Finally, an efficient neuronal isolation technique was established, which includes keeping the pregnant rat alive under anesthetized condition while removing the pups, and dissecting out cortical brain tissue in less than 10 mins for harvesting viable neurons and better purity yield.

4.2 Future Research

The rationale for future work is based on the literature indication that substance of abuse such as alcohol as well as antiretroviral drugs inhibit the glucose uptake and lipid metabolism in the brain that leads to malnourished hyperglycemia and lipodystrophy in HIV/AIDS patients. Our future research will test the hypothesis that HIV-1 infected microglia hijack high energy utilization during interruption of energy supply by alcohol, promoting malnutrition in non-infected brain cells, thereby accelerate Neuro-AIDS progression in the form of energy wasting.

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