

THE PROTECTIVE ROLE OF ESTROGEN ON HIV-1 MEDIATED
NEUROTOXICITY

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ABSTRACT

Licamied Chalise Macklin: The Protective Role of Estrogen on HIV-1 Mediated Neurotoxicity

(Under the direction of Dr. Jason Paris)

The prevalence of HIV among men have been in a steady inclination over the last several decades, yet the accelerated infection rate among older women is considerably concerning. Not only does the HIV virus affect the immune system but it also affects the central nervous system. One of its viral proteins, Tat, acts as an excitotoxin and disrupts the cell's membrane potential by increasing the concentration of Ca^{2+} inside the cell. This influx of calcium ions leads to apoptosis and secretion of the cell's contents into the surrounding area. Macrophages, along with other innate immune components, gather and induce the release of cytokines or chemokines. However, it has been discovered that estrogens can be used to attenuate these harmful neurotoxic effects. Acknowledging that women over the age of 50 are undergoing menopause, the body's natural levels of estrogen begin to decline. We hypothesized that estradiol (acting at estrogen receptor α or β) would attenuate Tat-mediated formation of reactive oxygen species (ROS), neurotoxicity, and cytokine production in differentiated SH-SY5Y human neuroblastoma cells and primary murine mixed glial cultures. Our findings suggest that any concentration of estradiol (0.01-10 nM) could attenuate Tat-mediated ROS, but cell death involving was not influenced. Estrogen receptor α agonism appeared more effective than β . Tat was also observed to increase cytokine expression. This information may be particularly important for medical treatments concerning aged HIV-infected women. Restoring estrogens post-menopausally may improve the prevalence of HIV-related neurocognitive impairments that occur with age.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
cART	Combined Antiretroviral Therapy
CD4	Cluster of Differentiation 4
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DPN	Diarylpropionitrile
ER-alpha	Estrogen Receptor Alpha
ER-beta	Estrogen Receptor Beta
HIV	Human Immunodeficiency Virus
MCSF	Macrophage Colony-Stimulating Factor
PPT	Propylpyrazoletriol
ROS	Reactive Oxygen Species
Tat	Trans-Activator of Transcription

1. Introduction

a. HIV incidence within the U.S. and worldwide

Human immunodeficiency virus, or HIV, has affected millions of people around the world for decades and continues to affect people today. The beginning of this epidemic in 1981 seemingly only affected the homosexual population, or males who have sex with males (MSM). However, it was clear that the epidemic was spread by 1983 (“A Timeline of HIV and AIDS,” 2018). HIV can be transmitted only through certain bodily fluids such as blood, semen, and anogenital fluids (“HIV Transmission,” 2017). It has been discovered that semen from HIV-infected men can eventually spread the virus through contact via mucosal membranes and barriers during sexual intercourse (Barre-Sinoussi, 2014). In addition, HIV can be transmitted via several routes including the birth canal and contaminated breast milk from the mother (“HIV Transmission,” 2017). This method of transmission has drastically decreased due to an increase in HIV screening of pregnant women, antiretroviral treatment and avoidance of breastfeeding when infected (Brady et al., 2016). Lastly, HIV can also be spread through the sharing of needles and syringes from an infected drug user (“HIV Transmission,” 2017). Like mother-to-child transmission, this route of transmission can also be decreased via the initiation of Syringe Exchange Programs (SEP) such as those that have been successful in major cities like New York City (Amesty et al., 2011). Although all of these routes of transmission have decreased due to recent therapeutic medications and treatments, there is currently no cure available that can resolve the epidemic.

When the epidemic’s first cases were reported in 1981, the Centers for Disease Control (CDC), had identified five relatively healthy, young gay men that had

mysteriously died from a suspected lung infection. Yet, by the end of the year, the total had risen to two hundred and seventy cases and one hundred and twenty-one of them had died. That is equivalent to forty-four percent mortality rate with an unknown etiology. By 1989, the number of cases within the United States had risen to 100,000 (“A Timeline of HIV and AIDS,” 2018). Today, the rate of incidence around the world has slowed but not reversed its relative direction. As reported in the European Union/European Economic Area, the incidence is still between 29,000 and 33,00 new cases every year until 2015 (Pharris et al, 2015). Europe is not the only participant in this phenomenon. East and South Africa host the world’s largest HIV-infected population with a significant difference in prevalence of young women and young men. Specifically, the infection rates in young women are eight times higher than the rate in young men (Harrison et al., 2015). This statistic has remained relatively stable for over a decade (Harrison et al., 2015). A somewhat similar trend has been found within the United States, despite varying opinions. One source written by Lansky et al. claims that new HIV infection rates in the overall women population has decreased by twenty-one percent from 2008 to 2010 (2014). Another source determined different data from around that same time frame. The incidence rate for young men was fifteen times higher than young women during the beginning of the epidemic. However, by 2010, the incidence rate was only three times higher among young men (Breskin et al., 2017). Despite the two slightly opposing ideologies, HIV still wreaks havoc on the central nervous system (CNS) and immune system of both sexes.

b. *HIV effects on the body/progression to AIDS*

The HIV virus can be found within various organs and organ systems throughout the body. This includes the liver, stomach, lungs, and kidneys (Bednar et al., 2015). A prominent site of viral reservoir lies within the central nervous system. As such, this Thesis will focus on the HIV interactions within the central nervous system.

The HIV virus targets the immune system of the individual it affects. Specifically, it predominantly infects monocyte-derived-cells, including macrophages and microglia (the macrophage of the brain) and T-lymphocytes, including CD4⁺ T cells (Nall, 2018). Infection of these cells limits the body's primary adaptive immunological defense mechanism. In the CNS, the virus predominantly activates innate immune responders which produce cytokines (Nasi et al., 2014). Cytokines are signaling proteins that regulate immune responses, increasing or reducing inflammation (Foster, 2001). As a result, massive amounts of cytokines in one area often result in increased inflammation. Inflammation is one of the first signs that the body is attempting to fight off an infection. While this defense mechanism is designed to assist the body, other organ systems begin to degrade under stressors of this magnitude.

The CNS maintains several regulatory mechanisms to prevent invasion given that there is very little adaptive immunity in the brain and spinal cord. Entry to the CNS is regulated by the blood brain barrier (BBB). The BBB provides biologic, physiologic, and immunologic separation between the periphery system and the CNS (Strazza et al., 2011). Due to several different proteins being involved in regulatory processes, the barrier is also considered semi-permeable, meaning only certain chemicals and molecules are able to pass through the barrier. Perturbation of the BBB can promote permeability

(Strazza et al., 2011). Alterations of the surrounding environment can lead to changes in the integrity of the BBB, which ultimately leads to an increase of infected cells migrating across the barrier and into the CNS (McRae, 2016). HIV-infected cells that may gain access to the CNS can produce neuroinflammation, damaging neural cells. It is this effect that is thought to underlie various types of neurocognitive impairments, overall termed “NeuroAIDS.” (Shapshak, 2015).

c. NeuroAIDS Incidence and Current Therapeutic Methods

NeuroAIDS is defined as the result of chronic and persistent HIV infection and inflammation (Shapshak, 2015). This disease includes various degrees of impairments, such as “decreased attention/concentration, psychomotor speed, memory, learning, information processing, and executive function” (Shapshak, 2015). The overall prevalence of this disease has not decreased since the introduction of combined antiretroviral therapy (cART); albeit, cART has reduced the incidence of the most severe form, HIV-associated dementia (Caniglia et al., 2014). cART refers to a therapeutic method of maintaining the HIV infection within the body by introducing various classes of drugs in the body (Boskey, 2018). This method usually includes at least two drug classes in order to properly stabilize the progression of the replicating virus (Boskey, 2018). However, the main issue with this method is the drug’s inability to accumulate within the brain and latent reservoirs, such as macrophages and microglia in the brain. cART is able to infiltrate the BBB due its lipophilicity and by acting as a substrate for several BBB transporters (such as P-glycoproteins). In order for the cART to work optimally and efficiently, the drugs have to be able to enter the BBB and effectively maintain its efficacy within the system and within the reservoirs of latent virus (Zhang et

al. 2015). At present, there is no cART regimen that can eradicate HIV from viral reservoirs within the CNS.

While the HIV infection is one of the major contributors to this disease, there are co-morbid factors that can couple with HIV and further increase the risk of NeuroAIDS. One of the co-morbid factors that will be discussed later is menopause in middle-aged women. To continue with the discussion of NeuroAIDS, it was originally identified with individuals who had progressed to AIDS (Shapshak, 2015). However, within the same year, it was discovered by another research committee that neuroAIDS could also be found within the HIV community (Carvour et al., 2015). In fact, nearly half of HIV-infected individuals who had not progressed to AIDS were diagnosed with neurological complications due to NeuroAIDS (Carvour et al. 2015; Saylor et al., 2016).

d. HIV proteins (Tat) that contribute to NeuroAIDS

One viral protein that also contributes to the progression of NeuroAIDS is an HIV regulatory protein. Known as the trans-activator of transcription, or Tat, it has an important role in the replication cycle of HIV to enhance gene transcription and viral replication (McRae, 2016). In addition to its regulatory function, Tat is soluble, can be secreted from infected cells, and cause several disastrous effects on the CNS. Tat increases in cytokine production and promotes immune cell migration across the BBB (McRae, 2016). Tat also exerts direct neurotoxic effects by acting as an excitatory signal, causing Ca^{2+} channels to open and disrupting cellular ion homeostasis. In disrupted cells, mitochondria work to absorb excess Ca^{2+} which causes the organelle to release reactive oxygen species, which promote cell death (Fitting et al., 2014). Even if the individual begins cART, the issue described earlier arises again, in addition to a new problem.

While on cART, it has been discovered that transcription of viral proteins continues. This results in "cytotoxic stress, inflammatory response and tissue integrity damage, all of which are major contributors to [NeuroAIDS] development and progression" (Bagashev and Bassel, 2013). Thus, production of toxic proteins from persistent reservoirs of HIV within the brain could underlie the cause of neuroAIDS, even in the post-cART era. Additionally, several scientific studies have identified a significant sex difference regarding the overall maintenance and survival rates with this neurodegenerative disease.

e. NeuroAIDS in pre and post-menopausal women

According to previous studies, HIV-infected women perform lower on cognitive tests and survive at lower rates than their male counterparts. One research team made the discovery that women aged 37-38 displayed a lower survival time than men (Carvour et al, 2015). The two groups had several events measured. One of the events measured was the time between diagnoses of HIV and AIDS; women were observed to have less time than men (Carvour et al., 2015). The rate of death was also higher among women compared to men. Lastly, the overall survival time from initial diagnosis to death was lower than men (Carvour et al., 2015). This study provides several examples that there is a factor affecting the survival rates among women.

Another study displayed similar results with different events. Groups of HIV+ and HIV-women were cognitively tested using a memory test. The HIV+ women showcased impairments in verbal learning, verbal delayed recall, figural episodic memory, and working memory (Maki et al., 2009). The average age of these women were slightly higher at 41.95 years old (Maki et al., 2009). This observation of similar results

in slightly older women indicates there is an additional method contributing to the progression and decline of women with NeuroAIDS.

f. Estrogens and their effect on NeuroAIDS

Estrogen is most commonly known as a steroid hormone that is involved in sexual differentiation and development of males and females (Wilson et al., 2006). There are two main types of receptors found throughout the brain and CNS that contribute to memory and other cognitive abilities. ER-alpha, is a receptor that is found throughout the brain, but predominantly in the hypothalamus, the area of the brain that is critical for reproductive signaling. ER-beta expression is distributed throughout the CNS, but predominantly in the hippocampus, a brain region largely associated with emotion and cognition (Wilson et al., 2006). Some evidence suggests that estradiol is neuroprotective. While the exact mechanisms that may underlie its role in neuroprotection and extent to which they interact with sex differences are not fully understood, they are beginning to be examined.

One key difference between HIV-infected men and women is that women have been shown to be more sensitive to the development of neuroAIDS (Maki and Martin-Thormeyer, 2009). One study that was conducted in Europe was indicative that women were twice more likely to develop neuroAIDS than men (Chiesi et al., 1996). Yet, another study conducted in the United States was indicative that there was no sex difference in the development of neuroAIDS (Robertson et al., 2004). Given the variance in these data, further investigation is necessary. Another study by Weber et al. indicated that certain cognitive deficits are believed to be caused by changing levels of estrogen during the menopausal transition (2012). HIV-infected women greater than the age of

forty had a higher incidence of dementia than women who were younger than forty (Wilson et al., 2006). Some have suggested that neurological complications from AIDS is associated with low estrogen levels. Now, it is important to discuss how estrogen interacts with one of the main viral proteins associated with HIV.

g. Estrogen and its interactions with Tat

HIV-1 protein Tat is thought to play an important role in the neuropathology of HIV and hormone levels in women may influence these effects (Adams et al., 2010). Postmenopausal HIV-infected women have a higher risk for experiencing neurocognitive deficits that are associated with the low levels of estrogen (Wallace, 2006). More recent work suggests that the presence of estrogen may slow the progression of HIV and protect against the toxicity of Tat in humans (Wallace, 2006). An experiment performed using neural cells determined that estrogen was able to delay cell death by constricting the apoptotic signal produced by Tat (Adams et al., 2010). The pressing question then arose: which estrogen receptor, if any, was mainly responsible for neuroprotection. Using particular antagonists, it was discovered that ER-beta signals were preferred but ER-alpha signals still contributed to the neuroprotection (Adams et al., 2010).

Yet, another study conducted by Heron et al. determined results that ER-alpha was the primary receptor involved in the neuroprotection against Tat (2009). This research team used different methods, such as using agonists. The team investigated the effects of ER-beta, but the resulting levels were almost negligible compared to the resulting levels of ER-alpha (Heron et al., 2009). As a result, the team decided not to further isolate and investigate the effects of ER-beta. Although these two studies

produced different results, the importance of estrogen remains significant, especially in its effects on the CNS.

h. *Estrogenic Hormone Therapy and its effects on the CNS*

Although estrogen has been considered protective on a concentration dependent interaction, these natural levels deplete on their own after a woman enters menopause. According to a 2016 survey of new HIV diagnoses, seventeen percent were fifty and older and twenty-four percent of that number were women (CDC, 2018). A possible solution to this increasing problem lies in hormone replacement therapy. The procedure involves the woman enhancing her natural levels of estrogen via several routes: pills, skin patches, gels, creams, vaginal creams, vaginal tablets, or vaginal rings (“Hormone Therapy: Is It Right For You?”).

Due to estrogen’s substantial anti-inflammatory and neuroprotective properties, it should be considered as a therapeutic method to counteract inflammation in the brains of HIV-infected menopausal women (Dye et al., 2012). Since there have been various reports and observations of the benefits of estrogen on the CNS, it has led to a suggestion that older women have their estrogen levels maintained throughout and after menopause (Dye et al., 2012). One study demonstrated that a group of older HIV-infected women who had been placed on hormone replacement therapy had a significantly decreased risk of mortality (Dye et al., 2012).

i. *Hypothesis*

To assess the neuroprotective mechanisms of estrogens, we conducted a series of experiments using differentiated human neuroblastoma cells and murine primary mixed glia. We anticipated that exposure to HIV-1 Tat would increase reactive oxygen species

formation and cell death in neurons. In glia, we expected Tat to increase cytokine production. Agonists with selective affinity for ER-alpha (PPT), ER-beta (DPN), or both (estradiol) were expected to attenuate these effects.

2. Materials and Methods

2.1 Description of Cells and Cell Culture

a. SH-SY5Y human neuroblastoma cells

These cells originate from the bone marrow of a four-year old female patient with a metastatic neuroblastoma. The morphology of SH-SY5Y cells are epithelial, however, all of the experiments performed are used with cells that have been differentiated into a neuron-like phenotype. Cells were grown in a 75 cm² cell culture flask in 10 mL of media (89.5% DMEM/F12, 10% fetal bovine serum, 0.5% antibiotics). The flasks were then maintained in an incubator at 37° C with 5% CO₂.

For experimental manipulation, cells were seeded onto 24-or 96-well plates at seeding densities of 50,000 or 5,000 cells per well, respectively. To differentiate SH-SY5Y cells to a neuron-like morphology, retinoic acid (1:500) was supplemented in the media for 5 days. Following this, cells were maintained in serum-free BDNF (1:200) media for additional 3 days per prior protocols (Constantinescu et al.,2007; Encinas et al., 2000). For all experiments, media was fully changed every 48 hours.

b. Primary Mouse Mixed Glia Cells

Primary mixed glia were cultured from 0-1-day old mice (C57BL/6HNSd purchased from Envigo, Indianapolis, IN) as previously described (Paris et al., 2016). Briefly, brains were harvested and minced with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in DMEM and incubated for 30 min (37°C, 5% CO₂). Tissues were triturated and sequentially filtered through 100- μ m and 40- μ m diameter pore cell strainers (Greiner Bio-One, Kremsmünster, Austria). Cells were plated at a density of 50,000 cells/well onto poly-L-lysine-coated 24-well culture plates and maintained for 10-12 days in DMEM

supplemented with (10% fetal bovine serum, 0.5% antibiotics, 0.5% glucose, and 0.13 sodium bicarbonate). Media was fully changed every 48 hours.

Before the cells are collected, multiple 24 well plates were made with a certain chemical mixture called Poly-L-Lysine. The mixture is combined with 50 μ L stock solution (100 mg/mL stored in -80°C freezer) and 10 mL of borate buffer solution. 200 μ L of the mixture was added to each well and left for a 3-hour incubation period. Once that amount of time had passed, the mixture was removed and replaced with distilled H_2O every 15 minutes, for a total of 6 washes. The last wash was removed and then left to air dry in the hood overnight. The mixed glia cells were collected from newborn to 2 days old C57 murine pups and placed into petri dishes. The dishes are then placed into the incubator at 37°C and 5% CO_2 . The cells had their 10% FBM media changed every 48 hours to ensure the optimal growth. For a 200 mL solution, there is 175 mL of DMEM, 3.5 mL of 7.5% Sodium Bicarbonate, 2 mL of 50% glucose, 1 mL of Pen/Strep, and 20 mL of FBS.

c. Process of Treatment

Once the cells in the 24 or 96 well plates were growing at a steady rate, they had to be differentiated into neuronal cells. Several chemicals are added in order to assist this process. First, Retinoic Acid (50 mg retinoic acid, 33.3 mL 95% EtOH) would be added to the flask in a 1:500 dilution with DMEM media. The purpose of this chemical is to begin differentiation of the cells. After approximately 5 days, BDNF (10 μ g BDNF, 1 mL DMEM/F12) was added to the flask to continue the differentiation process. After approximately 3 days, the original cells have completely differentiated into neuronal-shaped cells. This shape consists of a star-shaped cell where the dendrites of the neuron

are connecting and communicating with the other cells around it. This was a clear indication that the cells were ready to be treated with chemicals for an experiment. In addition, the DMEM/F12 media was changed every 48 hours and returned back to the incubator to continue growth.

2.2 Chemicals Involved in Experiments

a. 17- β Estradiol (E2)

Estradiol (Sigma-Aldrich, St. Louis, MO) has been determined through past and current research that estradiol additionally serves as a protective agent in the brain. Estradiol has been proven to be beneficial in memory function. It has equal affinity for ER-alpha (k_i : 0.12 nM) and -beta (k_i : 0.15 nM; Lund et al., 2005). Crystalline estradiol was dissolved in DMSO and diluted to concentration in media.

Propylpyrazoletriol (PPT)

This chemical is an agonist for the estrogen receptor alpha (ER-alpha k_i : 0.5 nM; ER-beta k_i : 700 nM; Lund et al., 2005). Crystalline PPT was dissolved in DMSO and diluted to concentration in media.

b. Diarylpropionitrile (DPN)

DPN is an agonist for the estrogen receptor beta (ER-alpha k_i : 195 nM; ER-beta k_i : 2.5 nM; Lund et al., 2005). Crystalline DPN was dissolved in DMSO and diluted to concentration in media.

c. Transactivator of Transcription (Tat)

The HIV Tat protein exerts indirect neurotoxic effects by promoting neuroinflammation and also produces direct neurotoxic effects via several mechanisms including direct or indirect excitotoxic activation of Ca²⁺ ion channels (NMDA receptors

and voltage-gated L-type Ca^{2+} channels; Eugenin et al., 2007; Haughey et al., 2001; Krogh et al., 2014; Napier et al., 2014), mitochondrial dysfunction which produces reactive oxygen/nitrosative species (Hui et al., 2012; Lecoeur et al., 2012; Malik et al., 2011; Turchan-Cholewo et al., 2006), and leads to subsequent bioenergetic crisis and cell death. Tat (ImmunoDx, Woburn, MA) was diluted to 100 nM in water. The concentration of tat reflects one from a range that elicits functional deficits in glia and neurons similar to those observed in HIV infection (Kruman et al., 1998; Nath et al., 1999).

2.3 Experimental Procedure

a. Reactive Oxygen Species (ROS) Assay

The generation of ROS is an early indicator of mitochondrial stress and cellular dysfunction. Cells were incubated with treatments for 20 hours and then assessed for fluorescence in the presence of 5- (and-6)- chloromethyl-2'7'-dichloro-3',6'-dihydroxyfluorescein diacetate, acetyl ester (CM-H₂DCFDA, Invitrogen, Carlsbad, CA) as previously described (Fitting et al., 2014). Briefly, cells were loaded with 10 μM CM-H₂DCFDA in warm HBSS for 45 min (per manufacturer's protocol), and washed twice. Fluorescence was measured at $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 520 \text{ nm}$ using a Bio Tek (Winooski, VT) Synergy 2 microplate reader. All treatments were performed in duplicate and each plate counted as one observation (the mean of technical replicates per condition). Data are expressed as the percent change from the arbitrary fluorescent units in control wells and represent $n = 4$ independent experiments.

b. Live/Dead Assay

DEAD Red (propidium iodide), a marker of cellular necrosis, and nuclear Hoechst 33342 stain (Invitrogen, Carlsbad, CA) were added to media. Treatments of

Estradiol in 0.01 nM, 0.1 nM, and 1 nM concentrations were applied and plates were imaged 0, 24, and 48 hours later. DEAD Red fluorescence was measured at $\lambda_{ex}= 570$ nm and $\lambda_{em}=602$ nm and Hoechst was measured $\lambda_{ex}=350$ nm and $\lambda_{em}= 461$ nm. Each condition was conducted in duplicate and each plate was counted as one observation (the mean of two technical replicates per condition). Fields were randomly determined and at least 150 cells were counted per field. The proportion of cellular death was determined by the following formula: % dead= (# DEAD Red⁺ cells/ # Hoechst⁺ cells) *100. Data represent n=8-10 independent experiments.

c. Cytokine Array

The purpose of the cytokine protein array was to screen potentially upregulated cytokines and chemokines in order to discover future analytical targets. The array (Protein Profiler Mouse Cytokine Array Kit, #ARY006, R&D Systems, Minneapolis, MN) assessed the protein content of the following analytes. The assay was conducted per manufacturer instructions and blots were read on a LI-COR imager.

3. Results

3.1 ROS Assay

Given that phenol red (a common component included in cell media) contains phytoestrogenic activity, cells were assessed after incubation in phenol red-containing media or phenol-red free media. Altering the growth media from phenol red to phenol-red free had a significant effect on the amount of ROS that was detected [$F(1,108)=43.67$, $p<0.05$] (Fig. 1a and 1b). Cells grown in the phenol red that were treated with additional estrogen agonists displayed less production of ROS than the cells in the phenol-red free media. It appeared that incorporation of estrogen receptor agonists along with the natural estrogen additives of the phenol red media created additional protection against the neurotoxic effects of Tat and significantly decreased cell death. Since the phenol-red free media does not contain any estrogen additives, the minute addition of estrogen receptor agonists was not enough to provide sufficient protection, thus the elevated amount of ROS production and cell death. Although there were no other significant findings, it should be noted that Tat displayed an apparent increase in toxicity.

3.2 Live/Dead Assay

Cells were grown in media with or without estrogenic phenol red. There was a significant interaction [$F(3,66)=3.07$, $p<0.05$] such that Tat significantly increased cell death when grown in phenol red containing media (Fig 2a and b). Cell death was significantly greater in phenol-red free media; as such, no additional death was observed with Tat (Fig. 2c and d). A concentration-response of estradiol was tested to determine if a specific amount provided protection. It appeared the lowest dose of E₂ (0.01 nM)

decreased the Tat toxicity in phenol red media only; however, this did not reach statistical significance ($p=0.14$).

3.3 Cytokine and Chemokine Assay

Cytokines and chemokines are the body's immune hormones. The result of these chemicals secreted by glial cells can cause indirect damage to neurons within the brain. The purpose of testing Tat under these conditions was to determine the indirect neurotoxicity of the viral protein. Application of Tat to primary murine mixed glial cultures significantly increased chemokine (Fig. 3a) and cytokine (fig. 3b) production, albeit these observations were descriptive ($n=2/\text{group}$) and were not statistically-analyzed.

4. Discussion

The initial hypothesis involving the increases of various Tat-induced inflammatory responses and the attenuation of these responses using a concentration dependent interaction of estrogen agonists were partly upheld. The findings from the ROS assay supported that the addition of Tat would cause a direct increase of ROS in SH-SY5Y cells. The findings from the cytokine/chemokine array provide additional information about how Tat indirectly affects and influences the surrounding cells and tissues by stimulating proinflammatory and anti-inflammatory chemicals from glial sources that can damage the neurons. The findings from the live/dead assay demonstrated that Tat can cause direct neurotoxicity on SH-SY5Y cells that estrogenic phenol red was neuroprotective. Albeit, no dose-response protection with estradiol was observed, contrary to expectation.

We anticipated that the addition of Tat to neural cells would increase the production of ROS. Once Tat has become introduced into the cell, it activates excitatory ion channels, such as Ca^{2+} channels, and cause them to open, disrupting the membrane potential (Fitting et al., 2014). Whenever a cell has a dysregulation influx of Ca^{2+} ions, mitochondria attempt to maintain homeostasis, in part by “hiding” the cation charge, storing excess Ca^{2+} within its own membrane. However, as the mitochondria continue to store these cations, ROS is produced as a consequence and mitochondrial membrane potential becomes compromised (Fitting et al., 2014). This event is usually the first to occur following cell death. Extensive ROS production and downstream events eventually lead the cell to undergo apoptosis. These events promote an influx of macrophages to accumulate within the CNS. These macrophages then lead to the stimulation and

secretion of cytokines and chemokines, which were also tested in this experiment. The media of the ROS assay was then manipulated to further contribute to the hypothesis that estradiol would attenuate these effects. By incorporating media that contained additional estrogens, the amount of ROS was significantly decreased compared to the amount of ROS in media without these added estrogens.

While there have already been several studies confirming that estrogen was in fact neuroprotective, there is a paucity of studies identifying the receptor-based mechanism of these actions. One study pursued this question and determined that ER-beta was the primary source of protection (Adams et al., 2010). The method of obtaining these data was contrary to the method used in our experiment in that prior investigations used antagonists whereas the current study uses agonists. It was discovered that ER-beta signals were preferred but ER-alpha signals still contributed to the neuroprotection (Adams et al., 2010). In another study conducted by Bertrand et al., similar results were produced using a chemical called S-Equol to display how estrogen played a role in neuroprotection (2015). By using both enantiomers of the chemical, the team was able to confirm that equol provided neuroprotection in an estrogen-dependent manner and that both acted in an ER-beta-dependent mechanism (Bertrand et al., 2015). According to our results, it appears as if the ER-alpha agonists produced the most neuroprotection against the toxic effects of Tat. Given the research studies mentioned in the introduction that either receptor could be the primary cause, the results of this study coincide with the general concept of estradiol protecting the neurotoxic effects of Tat on neural cells. Additional studies should be performed to further assess the extent of a receptor-dependent mechanism involved in the neuroprotection of microglia cells.

HIV does not infect neurons. Rather, neuronal damage must occur by indirect mechanisms including that caused by toxic viral proteins, such as Tat (King et al., 2006). The secretion of Tat from infected cells into local tissues activates surrounding microglial cells leading to the secretion of defense chemicals: cytokines and chemokines (King et al., 2006). Cytokines and chemokines are a particular type of proteins that are released when the body is under attack from foreign bodies; they can stimulate or inhibit inflammation and contributes to cognitive impairment in HIV-infected individuals (King et al., 2006). Tat induced some anti-inflammatory cytokines, but the majority of the cytokines stimulated were pro-inflammatory. These results directly coincide with the previous studies that state that Tat induces inflammation in the CNS and surrounding tissues. In addition, there was no significant increase among the chemokines with the exception of CCL2 and MCSF. CCL2 is a chemokine that stimulates the immune system to sites of tissue injury or infection (Deshmane et al., 2009). A significant increase of this specific chemokine further supports previous evidence about Tat's neurotoxic effects. Lastly, a significant increase was noted in the chemokine MCSF, which is coincidentally linked to the aforementioned CCL2. This chemokine also stimulates the production of immune-regulated inflammatory proteins (Baran et al. 2007). By implementing the cytokine and chemokine arrays into my experiment, the results can be used to display the indirect, inflammatory effects of Tat on the surrounding tissues and neuronal cells that eventually lead to cognitive deficits.

Although these cognitive impairments may seem inevitable, estrogen can be used to lessen the overall neuronal damage caused by Tat and its toxic effects on the CNS. Through my research, it was determined that ER-alpha was the primary source of

neuroprotection. These findings do not support my original hypothesis; however, they are just as influential towards discovering an alternate method for the HIV community.

Further studies should aim to create a possible treatment method for the HIV-infected community that takes advantage of estrogen's potentially neuroprotective capacity.

The current results produced must be considered with some caveats. Some unforeseen complications were discovered that should be taken into account in future studies. In particular, the live/dead experimental approach should be amended. The protocol used in this experiment was not maintained throughout the duration of the research. A portion of the plates had the fluorescent dye incorporated in the media during all of the time lapses. Once an extremely high death rate was observed, it was discovered that the dye was intended to be added before the 24- and 48-hour time frames. The other portion of the plates were then conducted using the appropriate protocol and recorded that the death rate had been lowered. Future experiments could use the latter protocol, referred to as end-point analysis, when the fluorescent dye is added at the beginning of the 24-hour time lapse. Another option is referred to as time-lapse microscopy, which does not involve fluorescent dyes but analysis of specific areas over a 72-hour period.

Nonetheless, each of the testing methods served an influential purpose: the cytokine/chemokine array uncovered what specific antibodies can be quantitatively tested using ELISA, the Live/Dead assay showed that there should be more increments of estrogen doses across the plate, and the ROS assay showcased that more observations would be more likely to create more viable results.

In conclusion, the findings from these sets of experiments re-solidified essential pieces of information that had been proven through various studies over the years.

Understanding how Tat indirectly, and directly, affects the human body and CNS is mandatory in order to properly create a beneficial method for the HIV community. Through dose-specific incorporations of estrogen, the entire CNS can be partially protected by the detrimental effects of Tat on the nervous tissue in the brain. By continuing research and using updated resources, the next major therapeutic method for the HIV-infected community can be discovered. Not only will this resource be able to help the current group of older women but it will also be able to protect the upcoming generation of women and prolong their lives to its maximum potential.

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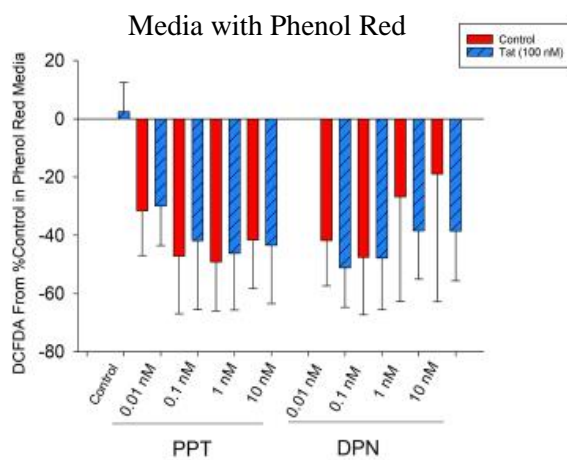


Figure 1a

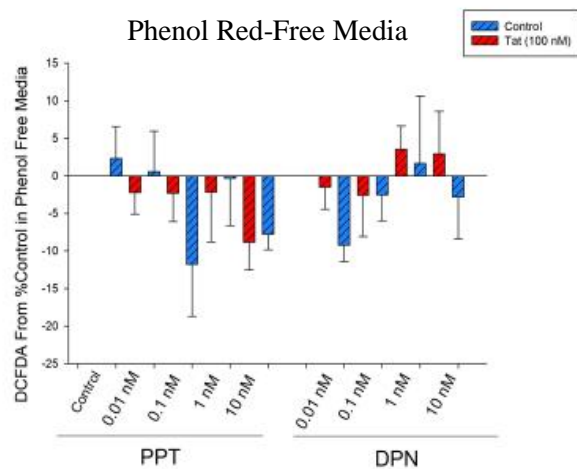


Figure 1b

Figure 1. Differentiated SH-SY5Y cells maintained in media that (a) did or (b) did not contain a phenol-red indicator were exposed to vehicle, Tat (100 nM), and concurrent vehicle, PPT, or DPN (0.01-10 nM). Cells were assessed for the formation of reactive oxygen species using the indicator, DCFDA.

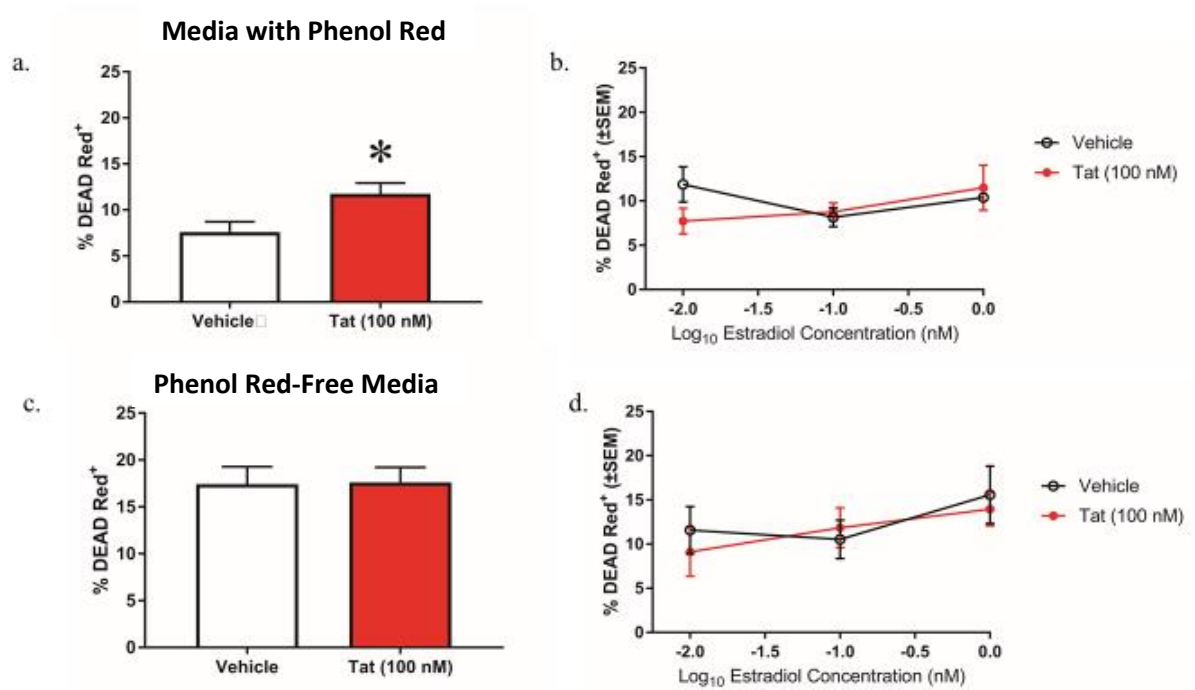


Figure 2. Differentiated SH-SY5Y cells maintained in media that (a,b) did or (c,d) did not contain a phenol-red indicator were exposed to vehicle, Tat (100 nM), and concurrent vehicle or estradiol (0.01, 0.1, or 1.0 nM; presented in Log₁₀ concentration). The proportion of necrotic cells (propidium iodide-positive) was calculated (Hoeschst 33342 used as a nuclear marker) 24 h after treatment. * indicates significant difference from vehicle-treated controls, $p < 0.05$.

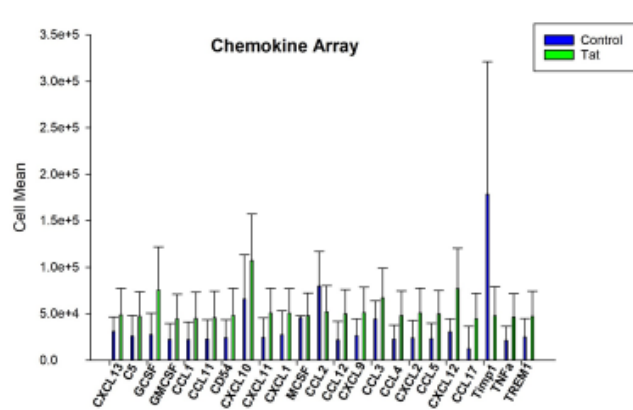


Figure 3a

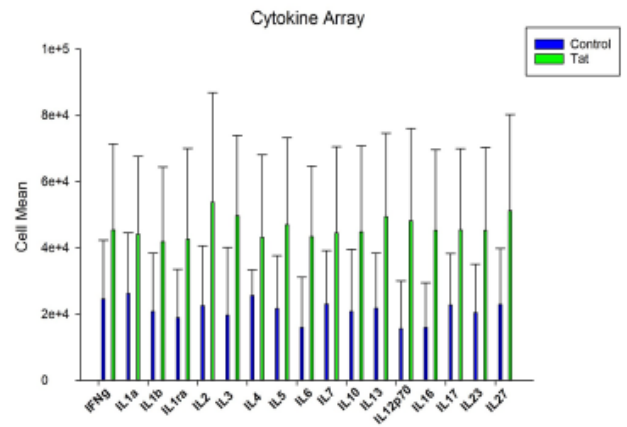


Figure 3b

Figure 3. Primary murine mixed glial cells were exposed to vehicle or Tat (100 nM) and assessed for semi-quantitative (a) chemokine and (a) cytokine content using a Proteome Profiler Mouse Cytokine Array Kit.