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# Endolysosome Involvement In The Protective Effects Of 17σ-Estradiol Against HIV-1 Protein-Induced Neuronal Injury

Nicole Miller

# ENDOLYSOSOME INVOLVEMENT IN THE PROTECTIVE EFFECTS OF 17α-ESTRADIOL AGAINST HIV-1 PROTEIN-INDUCED NEURONAL INJURY

by

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Nicole M. Miller
August, 2021

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## ABSTRACT

Although combined antiretroviral therapy (cART) effectively suppresses HIV-1 infection and increases the lifespan of people living with HIV, up to 50% of HIV-infected individuals are experiencing HIV-associated neurocognitive disorder (HAND), with clinical symptoms ranging from mild cognitive impairment to severe dementia, for which no effective treatment is currently available. Synaptodendritic impairments, such as decreased dendritic spines and synaptic loss, that occur in various brain regions including the prefrontal cortex and hippocampus are key pathological features of HAND that correlates closely with neurocognitive impairments. The development of synaptodendritic impairment in HAND in the post-cART era is complicated and not fully understood; However, chronic presence of viral proteins, such as gp120 and Tat, may disrupt neuronal homeostasis in the CNS could lead to synaptodendritic impairments and functional alterations of neurons.

An important mechanism that is disrupted in HAND is endolysosome function. Endolysosome dysfunction is seen in a variety of neurodegenerative disorders and plays a crucial role in disrupting the homeostasis of the cell. We have shown that gp120 and Tat as well as certain antiretroviral (ART) drugs de-acidify endolysosomes, induce enlargement of endolysosomes, and disrupt the function of endolysosomes. Endolysosomes are important in the maintenance and

neuronal homeostasis of neurons as neurons are long-lived post-mitotic cells that are extremely polarized cells with extensive processes that require constant membrane trafficking to establish and maintain axonal and somatodendritic plasma membrane domains. HIV-1 viral protein induced endolysosome dysfunction could contribute to the development of synaptodendritic impairments in HAND. Thus, preventing endolysosome dysfunction represents a promising therapeutic strategy.

It is well known that estrogen exerts enhancing effects on cognition and synaptic function and 17β-estradiol (17βE2), the primary estrogen in the blood, has been recognized as a potential therapeutic agent against HIV-associated neurocognitive disorders. Previous studies have shown that 17βE2 suppresses HIV-1 replication, protects against HIV-1 protein-induced neuronal cell death, and promotes synapse formation. However, feminizing effects and other side effects such as cancer, thromboembolism, coronary heart disease, and stroke may limit the use of 17βE2 in the larger general population.

Our studies focused on 17α-estradiol (17αE2), a natural non-feminizing estrogen that has neuroprotective effects and is the predominant form of estrogen in the brain. In the prefrontal cortex and the hippocampus, regions that facilitate learning and memory, membrane-bound ER are present and found in dendritic spines while nuclear estrogen receptors (ER) are found to a lesser extent. The presence of extranuclear membrane-bound estrogen receptors in neurons has been implicated in estrogen’s enhancing effect on cognition and synaptic

function. ERα has been detected in hippocampal neurons and located on endolysosomes such that ERα was thought to be degraded. However, as a soluble cytosolic protein, ERα most likely resides on the cytosolic side of the endolysosome membranes rather than in the lumen where it might be degraded. Rather, the endolysosome localization of ERα may alter endolysosome function directly. We have shown that gp120 and Tat induced neurotoxic effects and contributed to the development of HAND, at least in part, by disrupting the function of the endolysosomes. We hypothesize that 17αE2 protects against gp120-and Tat-induced endolysosome dysfunction and synaptodendritic injury via ERα present on endolysosomes.

The present studies were conducted in primary cultured hippocampal neurons and a hippocampal cell line. HIV-1 transgenic (Tg) rats that model HAND were treated with 17αE2. We used a range of different methods to test our hypothesis. Methods used to determine the effect of 17αE2 on HIV-1 protein-induced endolysosome dysfunction and synaptodendritic injury include, ratio-metric endolysosome pH measurement, immunostaining for assessing morphology and function and endolysosomes, live-cell imaging and immunostaining for quantification of changes in dendritic spines, and Golgi-Cox staining for quantification of alteration in dendritic spines in hippocampus of HIV-1 Tg rats. Methods used to assess the involvement of ERα include ERα knockdown with siRNA approach and overexpressing the ERα mutant (C451A) that lacks endolysosome localization.

We found that treating hippocampal neurons with either gp120 or Tat enlarged endolysosomes, elevated endolysosome pH, lowered the percentage of cathepsin D positive endolysosomes, and decreased dendritic spine density. 17αE2 not only prevented gp120-and Tat-induced endolysosome de-acidification and enlargement, but also prevented gp120-and Tat-induced decreases in dendritic spine density. ERα knockdown blocks the protective effects of 17αE2 against gp120-and Tat-induced endolysosome dysfunction and reduction in dendritic spines. Over-expressing the ERα mutant (C451A) that lacks endolysosome localization blocks the protective effects of 17αE2 against gp120-induced endolysosome dysfunction and reduction in dendritic spines. Furthermore, 17αE2 treatment attenuates the development of endolysosome enlargement and reduction in dendritic spines in HIV-1 Tg rats.

Our findings support our hypothesis that 17αE2 protects against gp120-and Tat-induced endolysosome dysfunction and synaptodendritic injury via endolysosome localization of ERα. Our findings suggest that 17αE2 represents a promising therapeutic agent that may slow or prevent the progression of HAND.

# CHAPTER I

# AN INTRODUCTION TO HIV-1 AND ENDOLYSOSOMES

## Overview of HIV

Since the discovery of the human immunodeficiency virus (HIV) in early 1980s, it is estimated that around 26-45.8 million people have died from the virus (UNAIDS, 2021). HIV-1 is a lentivirus that can progress into acquired immunodeficiency syndrome (AIDS), which is life-threatening due to the compromised immune system that develops in individuals infected with HIV (Spudich & Gonzalez-Scarano, 2012). There are two types of HIV, with similarities in their mode of infection, replication as well as the clinical symptoms presented; However, HIV-2 is less transmissible than HIV-1 and less likely to progress into AIDS. HIV-1 is found worldwide, while HIV-2 is located primarily in West Africa (Nyamweya et al., 2013).

HIV infections consists of three stages. The first stage is acute infection, in which the virus is rapidly dividing, and people infected are highly contagious with either flu-like symptoms or no symptoms. A decrease in CD4 cells characterizes this stage, and antibodies are produced. The second stage is called chronic infection; This is also be known as asymptomatic HIV or clinical latency. In this stage,

HIV is reproducing but not as rapidly as stage one. Near the end of this stage, the viral load increases, and the CD4 cell count drops; without combined antiretroviral treatment (cART), people move into stage three, called AIDS. AIDS develops due to the destruction of CD4 T cells, critical immune cells that fight off infection. Once individuals develop AIDS, they are more likely to develop opportunistic infections due to their damaged immune systems and usually have a survival rate of 3 years (“About HIV/AIDS, 2021”).

**HIV-1 Epidemiology**

HIV was the number one cause of death in the 1990s, specifically among the ages of 25-44 in the United States (WebMed, 2020). Currently, HIV-1 infects 38 million people worldwide and, in the U.S., approximately 1.2 million people have HIV (UNAIDS, 2021). In the United States, there were 36,400 new HIV diagnoses during 2018 (HIV.gov, 2021). As of 2018,  770,000 deaths are due to HIV (WHO, 2019), approximately 68% of people with HIV live in sub-Saharan Africa (Avert, 2019). The largest HIV-infected population resides in East and South Africa, with significantly more HIV-infected women than men (Harrison, Colvin, Kuo, Swartz, & Lurie, 2015).  Over the past 15 years, due to cART, the number of deaths worldwide due to HIV/AIDS has decreased by 56% (WHO, 2019).

HIV transmission occurs via body fluids such as blood, semen, rectal fluids, vaginal fluid, breast milk and can be spread through drug use, specifically by sharing needles (Control, 2020). In 1981, the first appearance of HIV was

published by the U.S. Center for Disease Control (CDC). The CDC noted that young homosexual men were infected with opportunistic infections (HIV.gov, 2020). Within eight years, the total number of cases had risen to 100,000 in the United States. The rate of new cases has substantially slowed since the 1980s due to antiretroviral treatment medications and education about HIV.

**HIV-1 Entry to CNS**

HIV infects monocytes, macrophages, microglia, and T-lymphocytes, including CD4+ T cells (Scutari, Alteri, Perno, Svicher, & Aquaro, 2017). HIV adheres to the host cell by binding to different cell attachment factors. Specifically, the HIV envelope glycoprotein, containing gp120 and gp41 subunits, binds to CD4+ T cells, and begins the fusion of the HIV capsid to the membrane. There are two coreceptors, CCR5 and CXCR4, that support HIV-1 fusion (Wilen, Tilton, & Doms, 2012). Once fusion occurs, the HIV capsid can enter the cell, and HIV RNA enters the cell and uses an HIV reverse transcriptase enzyme to convert the RNA into DNA. Once the RNA is converted into DNA, it is incorporated into the host's DNA.

HIV-1 can enter the brain early after infection (Spudich & Gonzalez-Scarano, 2012; Valcour et al., 2012). There are six main cell types in the CNS. Of these, microglia and perivascular macrophages are CD4+, whereas astrocytes, oligodendrocytes, neurons, and brain microvascular endothelial cells do not express CD4. All these cells have chemokine receptors, and perivascular macrophages, microglia, and astrocytes are susceptible to infection from HIV, but

only perivascular macrophages and microglia actively produce the virus (Gonzalez-Scarano & Martin-Garcia, 2005). Neurons are affected by HIV but not by the virus itself, as there is no evidence that HIV-1 infects neurons. However, macrophages and microglia in the brain are infected by HIV-1 and release neurotoxins (Adle-Biassette et al., 1995). These cells produce and release cytokines and chemokines, which can affect neurons and astrocytes.

The blood-brain barrier (BBB) is selectively permeable and composed of brain microvascular endothelial cells connected by tight junctions. The Trojan horse hypothesis is that HIV enters the BBB through immune cells such as T cells and monocytes (R. Peluso, Haase, Stowring, Edwards, & Ventura, 1985; Saili et al., 2017). A schematic of how the HIV virus can enter the CNS through the BBB is illustrated (Figure 1).

**CNS as HIV Reservoir**

HIV is systemic as it impacts the entire body and the central nervous system (CNS). HIV can be found throughout the body, this includes the stomach, lungs, kidneys, and liver (Bednar et al., 2015) There is evidence to suggest that the brain is a viral reservoir for the HIV (Gray et al., 2014; Marban et al., 2016) and the ability to eradicate the virus from the brain has become a new research strategy. HIV infects macrophages and microglia, and they actively produce virions and viral proteins (Crowe, Zhu, & Muller, 2003). A CNS HIV reservoir may be linked to astrocytes, which harbor HIV. Astrocytes make up over 60% of all cells within the brain. Then, even a small percentage of these cells

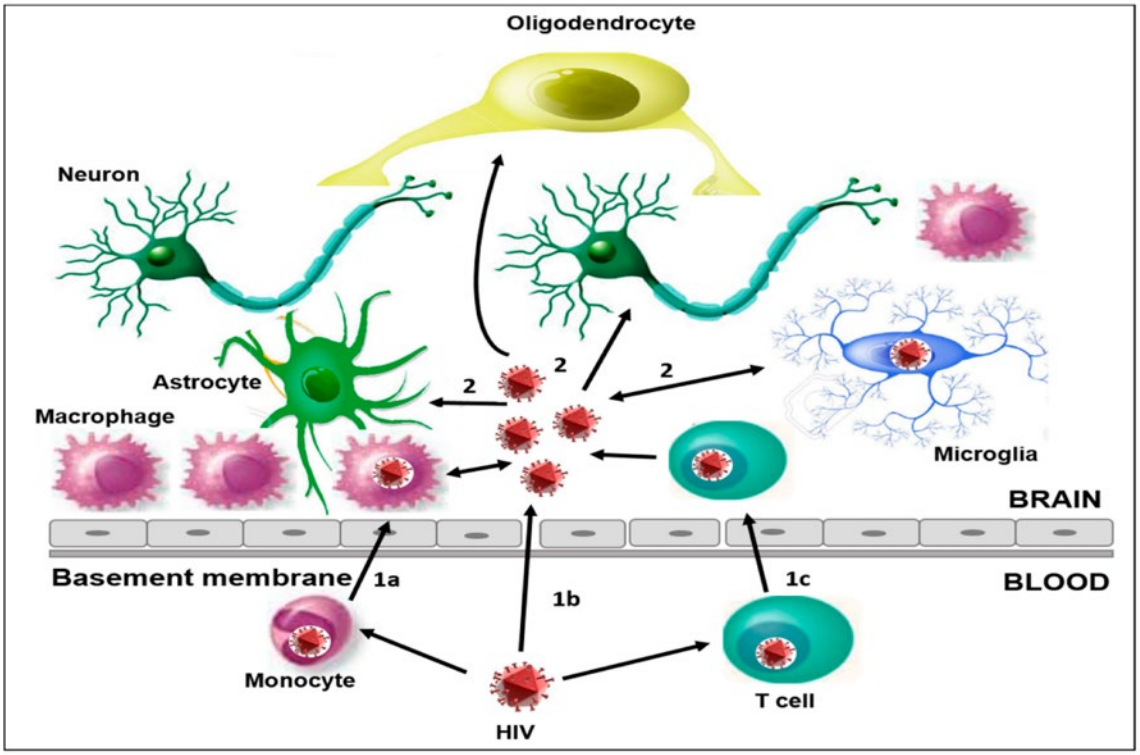


Figure 1. HIV-1 infection in the CNS.
HIV-1 infects CNS during the first week of systemic infection. HIV-1 could cross the BBB and enter CNS via infected monocytes, infected T cells, and the virus itself. In CNS, HIV-1 virus and released viral proteins as well as inflammatory responses affect the cells of the CNS that include microglia, neurons, oligodendrocytes, and astrocytes (Scutari, R., Alteri, C., Perno, C. F., Svicher, V., & Aquaro, S. (2017). The Role of HIV Infection in Neurologic Injury. Brain sciences, 7(4), 38. https://doi.org/10.3390/brainsci7040038).

containing HIV could serve as a reservoir for HIV. A study found that astrocytes with HIV replicate the virus in vivo in mice with transplanted HIV infected astrocytes, and from this, the virus can move from the brain to the peripheral tissue. The researchers also found that HIV-infected individuals, while taking cART, show integrated HIV gag DNA in 0.4-5.2% of astrocytes and HIV mRNA in 2-7% of astrocytes (Lutgen et al., 2020). Other evidence suggests that astrocytes serve as a reservoir as a study found the virus in 19% of astrocytes in the autopsy brain tissue of HIV+ individuals (Churchill et al., 2006).

**Antiretroviral Therapy and Challenges**

In 1983, the HIV virus was discovered as the cause of acquired immune deficiency syndrome (AIDS). The first HIV drug used was called Zidovudine, known as azidothymidine (AZT) or Retrovir. This drug was available in 1987, five years after the first known cases but was originally discovered in 1964. However, AZT had toxic side effects that affected the liver and blood counts. AZT is classified as an NRTI. Saquinavir was released in 1995 and was the first protease inhibitor. The next antiretroviral was NNRTI, called nevirapine. These drugs used together were called combination antiretroviral therapy (cART) and were established as the new standard for HIV patients in 1996 (WebMed, 2020). Instead of living only about three years once AIDS developed, people with HIV have lived longer with treatment. A study looked at the difference between monotherapy and cART and found that at age 20, with HIV, a person’s life

expectancy increased from 11.8 years to 54.9 years with cART (Gueler et al., 2017).

The development of combined antiretroviral therapy (cART) has significantly decreased the progression to AIDS ever since the treatment became readily available in 1996. Due to cART, there was a 50% decrease in the death rate due to AIDS, a reduction in the opportunistic infections occurring, and about a 50% decrease in the HIV acquired dementia (HAD), the most severe form of HIV-1 induced-cognitive impairment (Maschke et al., 2000). The cART regimens consist of using a combination of drugs with different mechanisms of action. The regimen requires either a protease inhibitor (PI) and two nucleoside reverse transcriptase inhibitors (NRTIs) or two NRTIs and one non-nucleoside reverse transcriptase inhibitor (NNRTIs) (Quashie, Mesplede, & Wainberg, 2013). Each of these antiretrovirals has a different mechanism of action. NRTIs inhibit reverse transcription by leading to chain termination, while NNRTIs prevent reverse transcription by binding to the enzyme directly and thereby prevent the enzyme from functioning. PIs bind to the active site of the protease and inhibit the enzyme from cleaving Gag and Gag-Pol polyprotein precursor that produce mature active proteins. Other drugs such as integrase inhibitors prevent integrase from inserting the HIV DNA into the human DNA. Another of theses antiretroviral is called an entry inhibitor (Enfuvirtide) which prevents the fusion of the viral envelope with the cell membrane (Pau & George, 2014).

Although the immune system will typically clear pathogens; cells can harbor HIV, and the immune system cannot target them. A new strategy to treat HIV infection in these latent cells is known as "Shock and Kill," that essentially reactivates the virus by inducing transcription. Then, the immune system can eliminate those cells and antiretrovirals can be used to prevent replication (Y. Kim, Anderson, & Lewin, 2018). There was no significant change in HIV positive individuals using the strategy when compared to HIV positive individuals on ARTs (Fidler et al., 2020). Another strategy is called "Lock & Block," which attempts to inhibit the expression of viral RNA, either by preventing the latent cells from activating or editing the genome to stop the reinfection (Darcis, Van Driessche, & Van Lint, 2017; Kwarteng, Ahuno, & Kwakye-Nuako, 2017).

**HIV-associated Complications**

HIV-associated complications consist of myelopathy, a spinal cord dysfunction resulting in impaired gait and weakness in the lower extremities, myopathy, and overall progression of weakness, inflammatory demyelinating polyradiculoneuropathy, headache disorders, CNS lesions, and opportunistic infections. HIV-infected individuals may experience cardiomyopathy, endocarditis, myocarditis, pericarditis, HIV-associated pulmonary arterial hypertension, pneumonia, HIV-associated neurocognitive disorders (HAND), and tuberculosis. Due to cART and the immunosuppression of HIV, HIV infected individuals can also develop infectious colitis, esophagitis, and gastroenteritis. Individuals can develop acute kidney injury, which is linked to antiretrovirals.

They may develop metabolic and endocrine disorders. HIV infected individuals may develop alopecia, infectious dermatoses, along with noninfectious dermatoses. Individuals infected with HIV may also develop myalgia/myopathy, rhabdomyolysis, and asymptomatic creatine kinase elevation, linked to the virus itself but is also associated with antiretroviral medications (Chu, Pollock, & Selwyn, 2017).

## HAND

HAND refers to different subsets of neurocognitive impairment due to HIV infection. This includes asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HAD. HAND is diagnosed by using functional status assessments and neuropsychological testing (Antinori et al., 2007). In the cART era, severe cases of HAND are low, but MND and ANI cases remain common (Heaton et al., 2011). A meta-analysis study found that HAND had a prevalence of 39.7-42.6% in cART era . The prevalence of ANI was 20.3–26.8%, MND was 10.6-16.3%, and HAD was 3.5-6.8%. The study found that HAND was found in individuals with a low CD4 nadir. Most cases of HAND were found in sub-Saharan Africa, roughly 72% of the cases of HAND (Y. Wang et al., 2020).

About 50% of HIV-infected individuals have HAND. HIV infected individuals that develop neurocognitive impairment have a statistically significant higher risk of dying than those that were considered of normal cognition (R. J. Ellis et al., 1997). Individuals with ANI are more likely to develop symptomatic HAND

compared with HIV infected individuals that were neurocognitively normal (Grant et al., 2014).

Before cART, HAND presented similar to Parkinson's disease, such as slowed movements, bradykinesia, gait imbalance, and loss of coordination. The more common clinical symptoms due to HAND, after cART was implemented, are memory and learning impairment, such as attention, impulse control, and memory retrieval similar to AD (Heaton et al., 2011). HIV encephalitis in autopsies has decreased from 54% to 15% due to the implementation of cART (Vago et al., 2002). Neuronal loss and encephalitis were typical for neurological dysfunction before the cART era, but this has been less common since cART (Gelman, 2015).

There are multiple risk factors for HAND that increase the likelihood of developing this disorder. Cardiovascular troubles, tobacco use, and hyperlipidemia were associated with decreased cognitive performance (Fabbiani et al., 2013). Older age was shown in the Hawaii Ageing Cohort and South African cohorts to be associated with a risk of developing HAND compared to younger HIV-infected individuals (Fazeli et al., 2014; Joska et al., 2012; Joska et al., 2011; Valcour et al., 2004). Other risk factors include substance and alcohol abuse, viral co-infections, vitamin deficiencies, traumatic brain injury, sleep apnea, insomnia, diabetes, hypertension, obesity, hepatitis C infection, and psychiatric illnesses (Saylor et al., 2016).

## Pathogenesis of HAND

HIV can enter through the BBB itself, or infected lymphocytes or monocytes can enter once inside HIV infects microglia, macrophages, and astrocytes. HIV does not directly infect neurons, however, the chronic presence of neuroinflammation and HIV-1 proteins damage neurons. This can lead to clinical symptoms such as, forgetfulness, mood disorders behavioral changes, confusion, and headaches (NINDS, 2019).

The underlying pathogenesis of HAND is still being investigated, but CNS inflammation seems to be relevant. Inflammation in the brain continues even when cART, which suppresses the virus, is introduced (Nguyen, Farrell, & Gunthel, 2010; M. J. Peluso et al., 2013). The virus can stimulate innate immune cells in the CNS, producing and releasing cytokines (Nasi, Pinti, Mussini, & Cossarizza, 2014). Usually, the release of cytokines is a defense mechanism. However, in HIV-1 infection, a substantial release of cytokines, which helps produce chronic inflammation, seems to play an essential role contributing to the neurotoxicity of the CNS.

Another factor in the pathogenesis of HAND is antiretrovirals; while keeping the virus under control, these drugs may be neurotoxic. The neurotoxicity of 15 antiretrovirals was investigated, and abacavir, efavirenz, etravirine, nevirapine, and atazanavir were the most neurotoxic in terms of neuronal loss and damage (Robertson, Liner, & Meeker, 2012). Efavirenz (EFV) and metabolites were found to damage neurons; specifically, 8-OH-EFV evoked

calcium influx and led to dendritic spine damage similar to the concentration found in the cerebrospinal fluid (CSF) of HIV infected individual's neurons (Tovar-y-Romo et al., 2012). Long-term usage of EFV was associated with worse neurocognitive effects (Ma et al., 2016).

Eradicating the CNS reservoir is a therapeutic strategy for HAND and using antiretrovirals that penetrate the CNS has been investigated as a potential treatment. Dolutegravir is a high CNS penetrating antiretroviral and patients taking Dolutegravir had a reduced CSF viral load (Letendre et al., 2014). However, a randomized study was completed using CNS targeted or non-CNS target antiretrovirals. The study found no neurocognitive difference for the CNS-targeted antiretrovirals compared to the non-CNS antiretrovirals (R. J. Ellis et al., 2014).

HIV-1 viral proteins are also a factor in the pathogenesis of HAND. HIV-1 Tat is a transcription factor known to enhance HIV-1 replication, to disrupt the integrity of the BBB, and is present in the CSF of HIV-infected individuals on cART that have no detectable HIV viral load in the CSF or the blood (Banks, Robinson, & Nath, 2005; A. T. Das, Harwig, & Berkhout, 2011; T. P. Johnson et al., 2013). HIV-1 gp120 assists with the entry of the virus into the cell and can release calcium leading to neuronal damage (P. W. Halcrow et al., 2021; Yoon et al., 2010; Zhang, Green, & Thayer, 2019).

## Synaptodendritic Damage in HAND/AIDS

AIDS patients, some of whom experienced dementia, display characteristics of the pathology of HAND consistent with ventricular dilation and sulcal widening. AIDs patients also experience lesions in the brain, myelin pallor, microglial nodules with multinucleated giant cells (Everall, Luthert, & Lantos, 1993; Navia, Cho, Petito, & Price, 1986; Navia, Jordan, & Price, 1986; Wallace, 2006) along with neuronal loss in the pyramidal neurons in the hippocampus and non-pyramidal cells of the basal ganglia (Fox, Alford, Achim, Mallory, & Masliah, 1997). The viral load is greater in these areas and correlates to the pathological changes; however, this still needs to be elucidated (Wilson, Dimayuga, et al., 2006). A 40% loss of dendrites and a 40-60% decrease in spine density in the frontal cortex were observed using Golgi-cox analysis in postmortem HIV-positive patients with encephalitis (Masliah et al., 1997; Wilson, Dimayuga, et al., 2006). Spine density and neuronal number were dramatically decreased in AIDS patients. Furthermore, a correlation between poor cognitive performance and reduced dendritic spines was observed (Everall et al., 1999).

In postmortem prefrontal cortexes of individuals with HAND and HIV-negative individuals, neurons were beaded, and the presynaptic marker synaptophysin was decreased in the HAND patients. Golgi-Cox staining was also completed and found that the dendritic spines were substantially diminished (R. Ellis, Langford, & Masliah, 2007). In our research, we have found that there is altered

neurite morphology in HAND individuals compared to HIV-positive individuals that were cognitively normal (Figure 2).

As stated above, multiple risk factors play into the pathology changes in the brain of HAND individuals; however, HIV-1 viral proteins may contribute to the underlying pathogenesis of HAND. HIV-1 infected SK-N-MC cells had significant spine density and dendritic morphology changes. These changes resulted in a significant decrease in number of spines, spine density, total dendrite area, spine length, dendrite diameter, and the spine area (Atluri, Kanthikeel, Reddy, Yndart, & Nair, 2013). The changes in dendrites and dendritic spines could be a potential factor in the increased cases of ANI and MND.

## Synaptodendritic Changes in Neurodegenerative Diseases

Loss or changes in the morphology of these spines have been described in individuals with neurodegenerative disorders. In Alzheimer's patients, evidence showed significant loss of neurons in the septohippocampal pathway, along with substantial decreases in the spine density (Gertz, Cervos-Navarro, & Ewald, 1987). A study looked at patients with Down syndrome compared to neurologically normal individuals and found a significant decrease in dendritic length, a decrease in spine density, and a decrease in the complexity of dendritic branching (Takashima, Ieshima, Nakamura, & Becker, 1989). Dendritic length has been used to determine the neurotoxicity and dendrite injury (Liu et al., 2018).

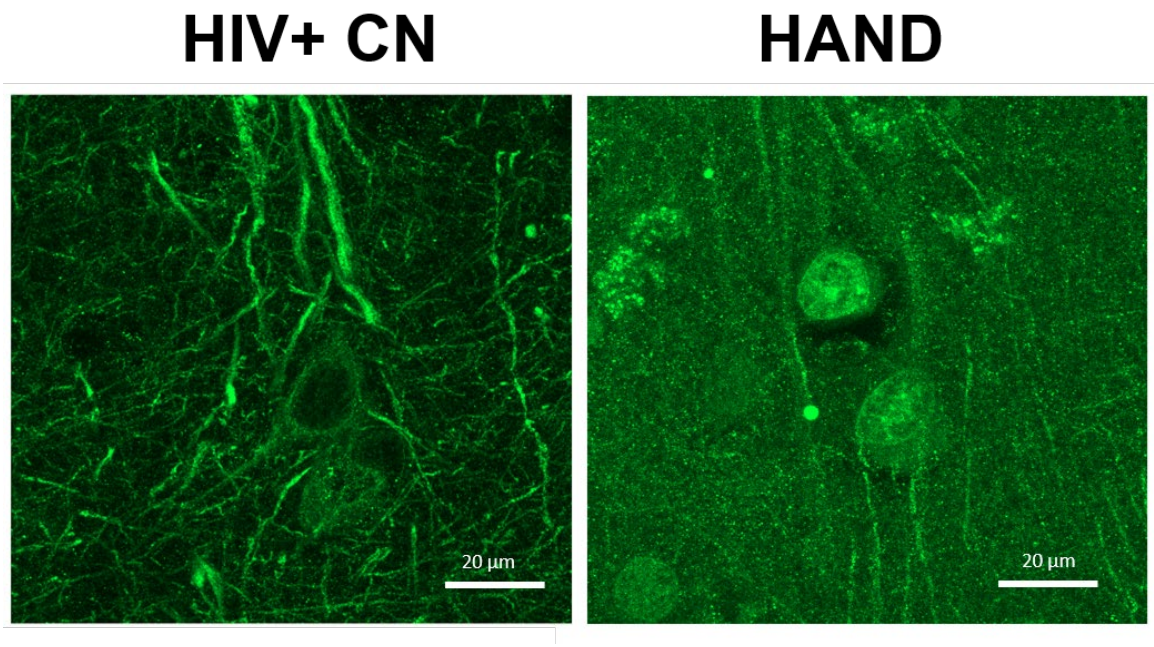


Figure 2. Altered neurite morphology in HAND.
Neuronal dendrites were stained with MAP2 antibody (green) in post-mortem prefrontal cerebral cortex from HIV-1 positive cognitively normal individuals (HIV+CN) or HIV-associated neurocognitive disorders (HAND). Neurites were less complex and MAP2 signal was diminished in HAND compared to HIV-CN.

Postmortem brain samples of AD patients showed a significant decrease in dendritic spines in the cortex and hippocampus compared to controls (DeKosky & Scheff, 1990; Knobloch & Mansuy, 2008; Walsh & Selkoe, 2004a, 2004b). Another study found compared to age-matched controls; AD patients had 45% dendritic spine loss in both the hippocampus and neocortex (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). In Parkinson's disease, there is a significant decrease in dendritic spine loss and dendritic length (Chidambaram et al., 2019; Villalba & Smith, 2013). Individuals with Intellectual disabilities show altered morphology and markedly decreased dendritic spine number (Fiala, Spacek, & Harris, 2002; Torres, Garcia, Tang, & Busciglio, 2018). In Down syndrome patients, the Golgi-Cox staining method showed a significant decrease in the dendritic spine number in the CA1 and CA2-3 segments of the hippocampus (Ferrer & Gullotta, 1990). These patients also show an altered morphology with a significantly higher number of thin spines (Torres et al., 2018). In the prefrontal cortex of schizophrenic subjects, there was a decreased dendritic spine density on these pyramidal neurons (Glantz & Lewis, 2000).

**Synaptic Plasticity/Dendritic Spines**

For the brain to be active, neural circuit activity is essential (Srivastava, Woolfrey, & Penzes, 2013). Synaptic plasticity are the changes that occur over time that can strengthen or weaken the synapse activity, which can alter due to multiple components (Wei et al., 2010). Structural dendritic spines are associated with cognitive function, and that long-term potentiation will increase spine-volume

growth and receptor trafficking, while long-term depression is associated with the opposite (Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010).

Most of the excitatory neurons contain dendritic spines in the mammalian brain. Dendritic spine density in the adult hippocampus of the CA1 is around 2-4 spines per μm of the dendrite (Chidambaram et al., 2019; Harris, Jensen, & Tsao, 1992). According to Zuo (et al., 2005), spine turnover decreases with aging, and the highest level of spine density occurs during adolescents (Zuo, Lin, Chang, & Gan, 2005; Zuo, Yang, Kwon, & Gan, 2005). Changes in dendritic spines and synapses occur during aging, and these changes in dendritic spine pathologies are unique depending on the region. During aging, cerebellar Purkinje cells experience a decrease in dendritic spine density, while in the amygdala, there was an increase in dendritic length; however, spine density was not significantly impacted. (Rogers, Zornetzer, Bloom, & Mervis, 1984). An age-related increase in dendrite length occurred in the rat amygdala, and they had unchanged spine densities (Marcuzzo, Dall'oglio, Ribeiro, Achaval, & Rasia-Filho, 2007). Associated with normal aging, in the prefrontal cortex of rhesus monkeys, there was a decrease in total dendritic length, spine density, number of branches, and dendritic branch order (Cupp & Uemura, 1980).

Ramón Y Cajal discovered neuronal dendritic spines in 1888. Dendritic spines are tiny protrusions along neuronal dendrites, commonly located in excitatory synapses and undergo remodeling, an activity-dependent process. Dendritic spines contain excitatory synapses that are associated with essential processes

such as memory and learning. Dendritic spines contain a head and a neck, of which spines are classified by the size of the head and the narrow neck. The number of dendritic spines and the shape and size vary depending on the type of spine and the developmental stage. In a mature brain, dendritic spines are less than 3 μm in length with a bulbous head anywhere from 0.5–1.5 μm in diameter, attached to the neck, which is less than 0.5 μm in diameter (Chidambaram et al., 2019). There are four types of dendritic spines which are classified by their morphology. The first and most stable spines are called mushroom spines with a large head and narrow neck, followed by stubby spines. The least stable spines are spines called long thin spines with a smaller head (Chidambaram et al., 2019). However, there are some variations in the literature about the classification of spines. Classifications of dendritic protrusions are mushroom, stubby, thin, and filopodia-like spines (Peters & Kaiserman-Abramof, 1970). According to a study published in 2017, 25% of dendritic spines are mushrooms, while greater than 65% are thin shaped in the adult cortex and hippocampus. Mushroom spines have a larger synapse per surface area, while thin spines have a smaller postsynaptic density (PSD) but greater potential to strengthen. Therefore, this has led to the hypothesis that thin spines are considered learning spines while mushrooms are memory spines (Berry & Nedivi, 2017).

Filopodia-like structures, immature spines, and mature spines are different in dynamics, lifetime, prevalence, synaptic function, and PSD-95. Filopodia-like structures are highly dynamic, they have a lifetime of minutes to hours, and in the mature brain, they are 2-3% of dendritic spines (Berry & Nedivi, 2017). Filopodia

also have little synaptic function and lack PSD-95. (Chidambaram et al., 2019). Filopodia can measure anywhere from 3-40 μm in length, and these are transient dendritic spines that may turn into mushroom or thin dendritic spines. However, not all Filopodia spines transform; only 0.2% mature (Majewska, Newton, & Sur, 2006). Immature spines are dynamic, they have a lifetime of about two days, and about 20% of spines are immature. Immature spines have a low AMPA to NMDA ratio and no PSD-95. Mature spines have an average lifespan of over a year, and about 70-80 % of spines are present as mature spines. Mature spines have a high AMPA to NMDA ratio, and they contain PSD-95. Dendritic spines fluctuate their morphology depending on the synaptic strength (Hotulainen & Hoogenraad, 2010).

Dendritic spines contain the cytoskeleton (actin), cytoskeleton proteins, small GTPase, postsynaptic density (PSD) region, micro-RNA, transcriptions factors, and cell membrane receptors which include metabotropic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and N-methyl-D-aspartate (NMDA) receptors (Chidambaram et al., 2019). Alterations in the morphology of spines are due to actin polymerization. The PSD region is an electron-dense structure where synaptic contacts are made. Modulation of actin filaments is the major contributor to new dendritic spines or changes in dendritic spines during LTP. Actin-binding proteins and signaling pathways can control actin polymerization (Chidambaram et al., 2019).

Another major contributor to the maintenance and modulation of dendritic spines are endolysosomes (endosomes and lysosomes) as these vesicles transport proteins and degrade, recycle cargo of the dendritic spines (Marisa S. Goo et al., 2017; Nikoletopoulou & Tavernarakis, 2018; Padamsey et al., 2017; Yap, Digilio, McMahon, Garcia, & Winckler, 2018). Once endolysosomes fuse with the plasma membrane, they release cathepsin B, increasing the matrix metalloproteinase 9 (MMP-9), which is involved in the synaptic plasticity and extracellular matrix (Padamsey et al., 2017). Endolysosomes are especially important for neurons, because neurons are long-lived post-mitotic cells that require active endolysosomes to maintain neuronal homeostasis, and because neurons are extraordinarily polarized cells with extensive processes that require constant vesicular membrane trafficking to establish and maintain axonal and somatodendritic plasma membrane domains. Thus, more research needs to be completed to determine the extent to which endolysosomes contribute to the synaptic plasticity of dendritic spines.

## Endolysosome and Lysosomes (Endolysosomes)

### A Brief History of Endolysosomes

Lysosomes were discovered in 1955 by Christian de Duve (de Duve, 2005), and in 1985, endosomes and endocytosis were discovered (Motulsky, 1986). Lysosomes are the primary digestive vesicles of the cells, but they also contribute to different vital functions in the cell. Lysosomes are essential for the day-to-day operations of the cell, such as plasma membrane repair, cell death,

cell signaling, tissue and bone remodeling, cholesterol homeostasis, pathogen defense, and the degradation and recycling of intra- and extracellular material (Zerial & McBride, 2001). Lysosomes receive their material through autophagy, phagocytosis, or endocytosis. They are heterogeneous vesicles that alter their distribution patterns and their positioning within the cell by responses to changes in pH and insults (Pu, Guardia, Keren-Kaplan, & Bonifacino, 2016; Wartosch, Bright, & Luzio, 2015). The lysosome system contains multivesicular bodies, autophagolysosomes, autophagosomes, and endosomes (Huotari & Helenius, 2011).

**Structure and Functions of Endolysosomes**

The processing of the endocytic pathway starts with the early endosome, which is an organelle that is mildly acidic and receives material by endocytosis (Appelqvist, Waster, Kagedal, & Ollinger, 2013). The endocytic pathway starts with the cargo (plasma membrane elements and extracellular components) being endocytosed and trafficked into early endosomes. Early endosomes then sort the cargo for recycling or degradation. Cargo destined for lysosomal degradation stays in the early endosomal vacuole, while the cargo that is going to be recycled goes to the tubular sorting endosome. Rab5 is located at the cytoplasmic surface of the plasma membrane and on early endosomes, and Rab7 is localized to late endosomes. Rab5 and Rab7 are exchanged once the early endosome matures into the late endosome; as early endosomes become late endosomes, their size, morphology, and pH change. Existing lysosomes can fuse with late endosomes

where the cargo can be degraded. This fusion produces endolysosomes, and then they can be converted back into lysosomes (Huotari & Helenius, 2011). Autophagy transports intracellular cargo to the lysosome for degradation (Settembre, Fraldi, Medina, & Ballabio, 2013). Figure 3 illustrates how the endolysosomal pathway process endocytosed cargo in early endosomes, then how late endosomes become lysosomes. This figure also illustrates the autophagy process and the fusion of the lysosomes and amphisomes, resulting in autolysosomes (Hansen & Johansen, 2011).

Lysosomes are heterogeneous in size (range 0.5 to 1.5 nm), are single membrane acidic organelles whose intraluminal pH values range from 4.5 to 6.0. Lysosomes contain over 60 acidic hydrolase enzymes (Xu & Ren, 2015). The membrane ion channels and the vacuolar-ATPases regulate the pH of lysosomes for the hydrolase enzymes to be in the optimal environment to catalyze the degradation of cargo (Colacurcio & Nixon, 2016; Huotari & Helenius, 2011; Mindell, 2012; Steinberg et al., 2010).

There are two classes of proteins in the lysosome necessary for its function: acid hydrolases and integral lysosomal membrane proteins (LMPs). Fifty acid hydrolases have specific targets that they degrade. LMPs are located in the lysosomal membrane and have various functions, including acidification of the lysosome, membrane fusion, importing protein from the cytosol, and transport of

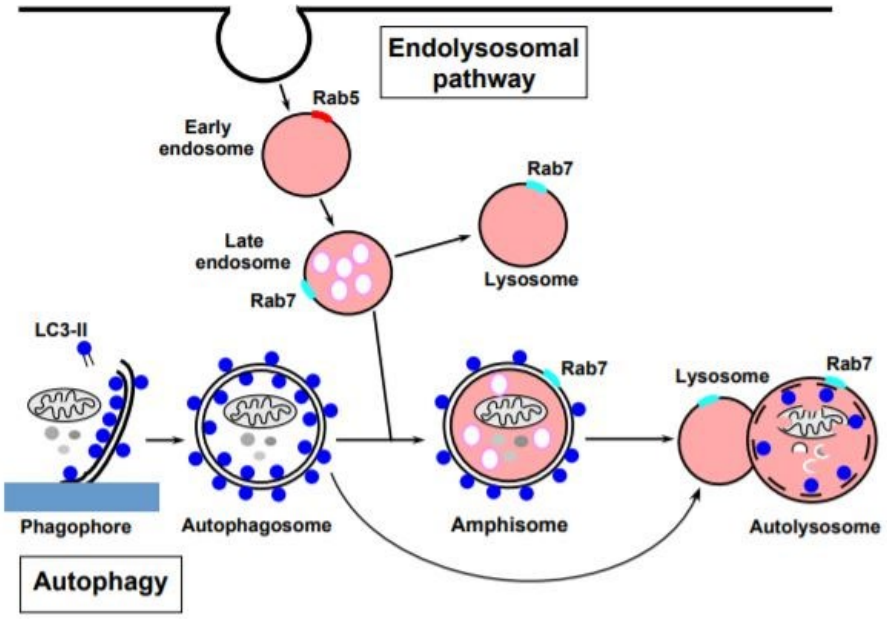


Figure 3. Endolysosomal Pathway.
The diagram depicts the endolysosome system that include endosomes, lysosomes and autophagosomes. Endoysosomes are important for sorting and trafficking of internalized (endocytosis) or transported (autophagy) macromolecules and/or membranes to their intended destinations for proper processing and function (Hansen, T.E., Johansen, T. Following autophagy step by step. BMC Biol 9, 39 (2011). https://doi.org/10.1186/1741-7007-9-39)).

degradation cargo to the cytoplasm. LAMP1, LAMP2, lysosome integral membrane protein 2, are some of the most abundant LMPs (Zerial & McBride, 2001).

Autophagy is essential for the clearance and degradation of cytoplasmic material. Autophagosomes arise when double-membrane vesicles surround intracellular cargo. Autophagosomes can fuse with lysosomes forming autolysosomes which are essential for clearing damaged proteins and organelles (Galluzzi et al., 2017; Ravikumar et al., 2010; Yim & Mizushima, 2020).

The endocytic pathway is important in essential cellular processes, including intracellular transport, regulation of cell surface receptor signaling, and immune response (Lakpa, Khan, Afghah, Chen, & Geiger, 2021). When the endocytic trafficking is disrupted, this can affect the homeostasis of the cell, which can lead to detrimental changes in the cell's physiology. This is very important in neurons as they are post-mitotic cells and need active endolysosomes to maintain their environment (Kuijpers, Azarnia Tehran, Haucke, & Soykan, 2020).

**Endolysosome Dysfunction**

Endolysosome de-acidification is an increase in the endolysosome pH that causes a variety of changes in the cell, such as the positioning of endolysosomes. De-acidified endolysosomes are positioned near the membrane of cells, while acidic endolysosomes are found near the nucleus (D. E. Johnson, Ostrowski, Jaumouille, & Grinstein, 2016; Pu et al., 2016). One change that

occurs due to the de-acidification is an increase of the size of the endolysosome and results in an accumulation of undigested intracellular cargo (Myers, Prendergast, Holman, Kuntz, & LaRusso, 1991; Ohkuma & Poole, 1981; Yoshimori, Yamamoto, Moriyama, Futai, & Tashiro, 1991). Endolysosome de-acidification can lead to autophagosomes and lysosomes not fusing, and which may result in cell death (Doherty & Baehrecke, 2018; Mauthe et al., 2018).

Endolysosomes contain releasable stores of biological essential divalent cations. These cations are calcium, iron, copper, and zinc (Lakpa et al., 2021). An efflux of calcium and iron occurs when endolysosomes are de-acidified, which can contribute to the impairment of endolysosomes (K. A. Christensen, Myers, & Swanson, 2002; Hui et al., 2015; Kiselyov et al., 2011; Kurz, Eaton, & Brunk, 2011; Lee et al., 2015; Xu & Ren, 2015). Endolysosome dysfunction has been observed in multiple neurodegenerative diseases and seems to be a common factor.

**Endolysosome Dysfunction in Neurodegenerative Diseases**

In lysosome storage disorders, there are mutations in the lysosome hydrolases (Platt, d'Azzo, Davidson, Neufeld, & Tifft, 2018; Sun, 2018) and impaired autophagosome-lysosome (Tanaka et al., 2000). In AD, lysosome dysfunction has been observed, including impaired autophagy, altered expression of cathepsin D, and increased lysosomal pH (Bi et al., 2000; Cataldo et al., 1995; Wolfe et al., 2013). In HIV infected brains and HIV gp120 in human neuronal cells observed that Aβ peptides were found in lysosomes of neurons. Transient

receptor potential mucolipin 1 (TRPML1) agonist resulted in calcium efflux, lysosomal acidification and cleared out the Aβ from lysosomes (Bae et al., 2014).

In PD, Lewy bodies are one of the hallmarks. A major genetic mutation in GBA1 (encoding for lysosomal hydrolase glucocerebrosidase) is linked to Lewy bodies (Wallings, Humble, Ward, & Wade-Martins, 2019). These Lewy bodies are made up of alpha-synuclein, which can be internalized into endolysosomes and leads to changes in the lysosome structure and function. For example, studies found that when lysosome morphology changes, there were decreases in cathepsin D activity, impaired fusion with autophagosomes, and increased pH (Hoffmann et al., 2019; Winslow et al., 2010). Huntington's disease (HD) is an autosomal-dominant disease that is caused by HTT gene mutation. This leads to impaired autophagy and alters cathepsin activity. (del Toro et al., 2009; Ratovitski, Chighladze, Waldron, Hirschhorn, & Ross, 2011; Ravikumar et al., 2010; Rubinsztein, 2006; Trajkovic, Jeong, & Krainc, 2017; Wallings et al., 2019).

**Endolysosome Dysfunction in HAND**

Endolysosome dysfunction has been observed by the expansion of lysosomes in white matter in HIV-infected individuals (Gelman et al., 2005). Macrophages release more cathepsin B, which leads to neuronal apoptosis when infected with HIV (Cantres-Rosario et al., 2015; Rodriguez-Franco et al., 2012; Zenon et al., 2015; Zenon, Segarra, Gonzalez, & Melendez, 2014). In patients with HAD, brain tissue and plasma showed increased cathepsin B. When infected with HIV, phagosome-lysosome fusion is impaired (Moorjani, Craddock, Morrison, &

Steigbigel, 1996). HIV-1 infection positions the lysosomes towards the cell's periphery, which suggests that the lysosomes are de-acidified (Cinti et al., 2017; D. E. Johnson et al., 2016). Consistent with morphological changes of lysosomes in people with AIDS (Gelman et al., 2005), we have shown that endolysosomes were enlarged in HAND individuals compared to HIV-positive individuals who were cognitively normal (Figure 4).

HIV-1 viral proteins have been implicated in the impairment of endolysosomes. One study found that HIV-1 Vpr protein led to a decrease in lysosomal acidification, accumulation of a-synuclein, indicating impaired lysosomal clearance (Santerre et al., 2021). Both HIV-1 gp120 and Tat can be endocytosed (Vendeville et al., 2004; Wenzel et al., 2017). HIV-1 gp120 increased the pH of endolysosomes, increased lysosomal exocytosis, and positioned the lysosomes toward the cell's periphery (Datta, Miller, Afghah, Geiger, & Chen, 2019; P. W. Halcrow et al., 2021). HIV-1 viral protein Tat increased the size and pH of endolysosomes, increased membrane permeability of endolysosomes, and inhibited autophagy (Hui, Chen, Haughey, & Geiger, 2012). Therefore, the endolysosome system, which is responsible for intracellular and extracellular material clearance and cell homeostasis, is impaired in individuals with HIV and HAND.

Another factor that could lead to endolysosome dysfunction is antiretroviral drugs. Our lab looked at a series of antiretrovirals and the effect that they have

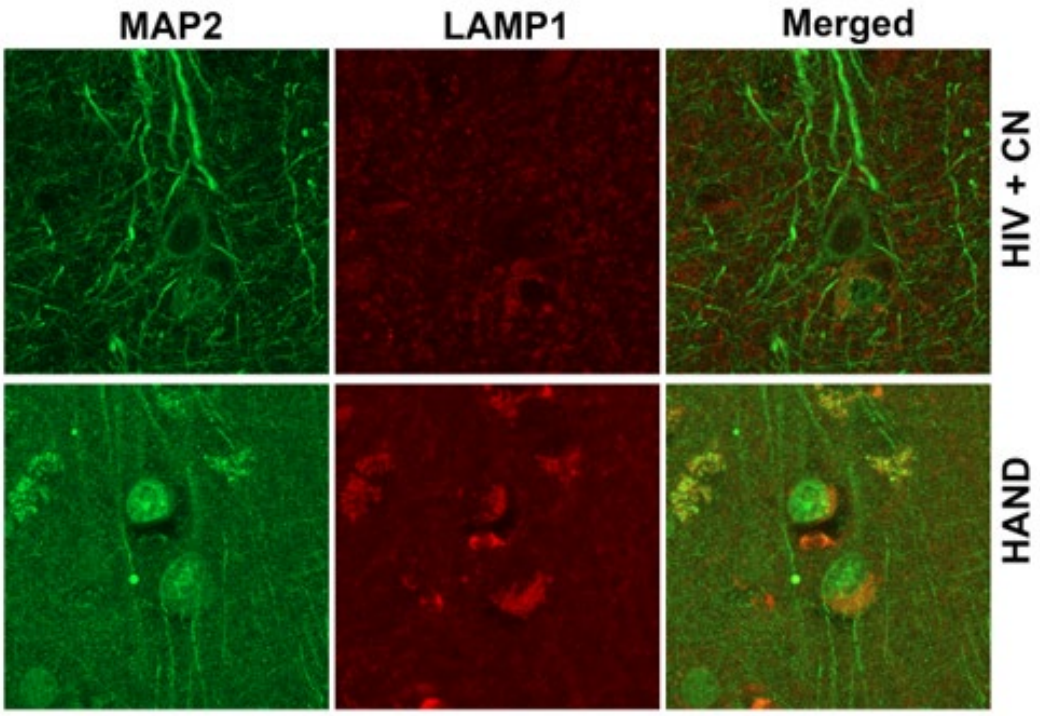


Figure 4. Endolysosome enlargement in HAND.
Lysosomes were stained with LAMP1 (red) in post-mortem prefrontal cerebral cortex from HIV-1 positive individuals with cognitive normal (HIV-CN) or HIV-associated neurocognitive disorders (HAND). Neuronal dendrites were stained with MAP2 antibody (green). LAMP1 positive signals (red) were dramatically increased in HAND compared to HIV-CN.

on the endolysosome system. Antiretrovirals are either weak acids or weak bases, and weak bases were shown to de-acidify endolysosomes, increase endolysosome size, and increase Aβ protein within endolysosomes. Antiretrovirals that are weak acids acidified endolysosomes and resulted in a decrease of Aβ protein of endolysosomes. Such endolysosome dysfunction induced by various HIV-1 viral related factors could contribute to the development of synaptodendritic impairments in HAND. Conversely, enhancing endolysosome function represents a promising therapeutic strategy. Our lab had shown that the endolysosomes can be acidified by activating TRPML1 or BK channels (Khan et al., 2019). Acidifying endolysosomes using TRPML1 agonist led to enhanced Tat degradation (Khan et al., 2019), and attenuated antiretroviral-induced Aβ production (Hui et al., 2021).

As mentioned earlier, HIV-1 gp120 and Tat has been shown to de-acidify endolysosomes (P. W. Halcrow et al., 2021; Hui et al., 2012). HIV-1 Tat and gp120 also lead to decreased dendritic spine density (Fitting et al., 2013; Iskander, Walsh, & Hammond, 2004; Liu et al., 2000). However, the causal relationship between HIV-1 gp120 and Tat induced endolysosome dysfunction and decreased dendritic spine density has not been studied, which is the main focus of my dissertation. In my dissertation, I have determined the effects of HIV-1 gp120 and Tat on endolysosome structure and function and dendritic spine density. I have determined effects of 17α-estradiol on endolysosome structure and function as well as dendritic spines and explored the involvement of endolysosome-localized estrogen receptors. To demonstrate a causal

relationship, I determined whether acidifying endolysosomes with 17α-estradiol prevented HIV-1 gp120 and Tat induced dendritic spine density. Furthermore, I determined the effects of 17α-estradiol on the size of endolysosomes and dendritic spines in HIV-1 Tg rats that model closely HAND.

# CHAPTER II

# HIV-1 VIRAL PROTEINS TAT AND GP120 INDUCE ENDOLYSOMAL DYSFUNCTION AND SYNAPTODENDRITIC IMPAIRMENT IN CELL CULTURES

## Introduction: HIV-1 Viral Proteins

HIV-1 viral proteins Tat and gp120 induce neurotoxic and synaptodendritic damage, which is associated with and may contribute, at least in part, to the development of synaptodendritic impairment in HAND (Bertrand, Mactutus, Aksenova, Espensen-Sturges, & Booze, 2014; Festa, Gutoskey, Graziano, Waterhouse, & Meucci, 2015; Fitting et al., 2013; Hargus & Thayer, 2013; Nath & Steiner, 2014; Speidell et al., 2019). Tat and gp120 contribute to neuronal injury through both direct and indirect mechanisms. Both HIV-1 viral proteins directly induce neuronal damage, in part, from internalization via endocytosis (Bachis, Aden, Nosheny, Andrews, & Mocchetti, 2006; Berth, Caicedo, Sarma, Morfini, & Brady, 2015; Wenzel et al., 2017).

### HIV-1 Tat

HIV-1 Tat is a transcription factor and an RNA binding protein that binds to transactivator response element (TAR) located on the HIV-1

RNA molecule (Bagashev & Sawaya, 2013) and is known to enhance HIV-1 replication (A. T. Das et al., 2011). Two-thirds of Tat can be actively secreted from HIV-1 infected cells (Agostini et al., 2017; H. C. Chang, Samaniego, Nair, Buonaguro, & Ensoli, 1997; Ensoli, Barillari, Salahuddin, Gallo, & Wong-Staal, 1990; Rayne et al., 2010). Current ART does not block the secretion of Tat in HIV-1 infected individuals (T. P. Johnson et al., 2013; Mediouni et al., 2012). Tat is present in the CSF from HIV-infected individuals on cART with no detectable HIV viral load in the blood or the CSF (Tat levels anywhere from 200 pg/ml to 6.5 ng/ml), indicating Tat is still produced even when replication of the HIV virus is well controlled (T. P. Johnson et al., 2013).

Tat has been shown to inhibit proteasomal activity, alter the cytoskeleton, lead to cell death, and alter neuronal receptors (Ajasin & Eugenin, 2020; Apcher et al., 2003). In addition, Tat can disrupt the endothelial tight junctions of the BBB, resulting in a loss of integrity, leaving the brain open to immune cells leading to potential neuroinflammation (Banks et al., 2005). In Tat transgenic mice, Tat has been shown to damage the cerebellum and cortex, leading to neuronal apoptosis and increase the number of immune cells entering the brain (B. O. Kim et al., 2003). Tat treatment increases gliosis and apoptosis, neuronal death, calcium dysregulation, and the levels of TNF-α and NF-*κ*B, and production of nitric oxide (M. Y. Aksenov et al., 2001; Michael Y. Aksenov et al., 2003; Bansal et al., 2000; Haughey, Holden, Nath, & Geiger, 1999; Hu, 2016; New, Maggirwar, Epstein, Dewhurst, & Gelbard, 1998; Polazzi, Levi, & Minghetti, 1999).

HIV Tat protein is released into extracellular space from cells actively producing the virus. Secreted Tat can bind to chemokine receptors, heparan sulfate proteoglycans, lipoprotein receptor-related-protein-1 and integrins, allowing Tat to be endocytosed (Ajasin & Eugenin, 2020; Liu et al., 2000; Tyagi, Rusnati, Presta, & Giacca, 2001). Tat can affect uninfected cells and can be endocytosed by macrophages, cardiomyocytes, microglia, astrocytes, neurons, $CD4^{+}$T lymphocytes, and monocytes (Ajasin & Eugenin, 2020). Tat can be endocytosed via the clathrin-mediated endocytosis pathway and the caveolar pathway (Fittipaldi et al., 2003; Richard et al., 2005; Vendeville et al., 2004). Once endocytosed, Tat enters endolysosomes (Vendeville et al., 2004), from where Tat has to escape to the cytosol and enter nucleus for viral transcription (Khan et al., 2020). Once endocytosed, Tat has been found to escape the endolysosome system by altering calcium homeostasis, using the two-pore channels on the endolysosome to release calcium and Tat (Khan et al., 2020).

Tat is detected in neurons in HIV-1 infected individuals (J. R. Chang et al., 2011). The direct neuronal damaging effect of Tat depends, in part, on its internalization into endolysosomes (Chen, Hui, Geiger, Haughey, & Geiger, 2013; Debaisieux, Rayne, Yezid, & Beaumelle, 2012; Frankel & Pabo, 1988; Gaskill, Miller, Gamble-George, Yano, & Khoshbouei, 2017; Hui et al., 2012; Liu et al., 2000; Mann & Frankel, 1991; Tyagi et al., 2001). Our lab has published findings showing that Tat induces endolysosome deacidification, endolysosome enlargement, and endolysosome and autophagy dysfunction in neurons (Chen et al., 2013; Hui et al., 2012).

**HIV gp120**

HIV-1 gp120 is an envelope protein located on the outer part of the virus and plays an essential role in infection as it assists the entry of the virus into the cell (Yoon et al., 2010). HIV-1 gp120 can be shed from infected cells (Moore, McKeating, Weiss, & Sattentau, 1990; Schneider, Kaaden, Copeland, Oroszlan, & Hunsmann, 1986). Despite cART, antibodies against gp120 are still present in the CSF (Burbelo et al., 2018; Trujillo et al., 1996). HIV-1 gp120 has consistently been shown to contribute to neuronal injury through both direct and indirect mechanisms (Bezzi et al., 2001; Jana & Pahan, 2004; Kraft-Terry, Buch, Fox, & Gendelman, 2009).

HIV-1 gp120 can be endocytosed. Endocytosis of gp120 is a vital process for gp120 neurotoxic effects, as researchers blocked the endocytosis of gp120 and found that this prevented gp120-induced damage. However, when gp120 was delivered through nanoparticles, the neurotoxic effect was present and not due to chemokine receptor; therefore, preventing endocytosis of gp120 may be a therapeutic target (Wenzel et al., 2017). HIV-1 gp120 can release calcium leading to neuronal damage by impairing axonal transport (Dreyer, Kaiser, Offermann, & Lipton, 1990; Mocchetti, Bachis, & Avdoshina, 2012; Zhang et al., 2019). Our lab has published findings showing that HIV-1 gp120 induces endolysosome dysfunction; de-acidifying endolysosomes (Bae et al., 2014), disrupting iron homeostasis in the endolysosomes (P. W. Halcrow et al., 2021),

and leads to the lysosomes moving toward plasma membranes and induces lysosomal exocytosis in Schwann cells (Datta et al., 2019).

The neuronal damaging effects of Tat and gp120 are, in part, due to the internalization of these proteins in the endolysosome system, and both Tat and gp120 induce endolysosome dysfunction. Endolysosomes are essential for maintaining dendritic spines as they contribute to the transport and degradation of proteins spines (Marisa S. Goo et al., 2017; Nikoletopoulou & Tavernarakis, 2018; Padamsey et al., 2017; Yap et al., 2018). Therefore, endolysosome dysfunction resulting from HIV-1 gp120 and Tat may lead to synaptodendritic impairment. Here, I determined the effects of HIV-1 gp120 and Tat on endolysosome function and dendritic spines in primary cultured neurons.

## Methods

### Cell Culture

Mouse embryonic hippocampal E-18 cells CLU199 (Cellutions Biosystems, Cedarlane, Ontario, Canada) were grown and maintained in 1x DMEM. The DMEM was supplemented with 10% fetal bovine serum (FBS), 25 mM glucose and 1% penicillin/streptomycin and maintained at 37°C with 5% $CO_2$ following manufacturer's instructions. Only cells from passages 3-7 were used in the experiments.

## Cultured Primary Rat Hippocampal Neurons

Primary hippocampal neurons were cultured from embryonic day 18 rats. Pregnant Sprague Dawley rats were sacrificed $CO_2$ followed by decapitation. The fetuses were removed, decapitated, and the meninges were removed from the brain. The hippocampal area was located and dissected from the brain, and trypsin was used for enzymatic digestion. Neurons were plated on 35-$mm^2$ culture dishes coated with poly-D-lysine. Neurobasal media with 1% L-glutamine, 2% $B_{27}$ supplement, and 1% penicillin/streptomycin were added to grow the neurons. Neuronal media was changed every 4-5 days and the cells were maintained at 5% $CO_2$ and 37°C for 10-21 days depending on the experiment.

## Cultured Primary Mouse Hippocampal Neurons

Primary mouse embryonic hippocampal neurons (C57EHP, Brainbits LLC, Springfield, IL) were obtained from E18 mouse cortex. The manufacturer's instructions were followed for the primary neuron culture protocols. The papain was prepared, and tissue was transferred to digest in the papain (2mg/ml) for 10 minutes. The tissue was then centrifuged at 200g reconstituted for 1 minute and plated at 100,000 cells per well on either 35 $mm^2$ ploy-D-lysine coated glass bottom dish (P35GC-0-10-C, MatTek Life Sciences, Ashland, MA), 12 mm poly-D-lysine coverslips (GG-12-PDL, Neuvitro Corporation, Vancouver, WA) or in 24 well plates. NbActiv1 media (Brainbits) was used for both plating and maintenance. Neuronal media was changed every 4-5 days and were maintained at 5% $CO_2$ and 37°C for 10-21 days depending on the experiment.

**Measurement of Endolysosome pH with LysoSensor**

Endolysosome pH was measured using a ratio-metric lysosome pH indicator dye LysoSensor (Yellow/Blue DND-160 from Invitrogen). LysoSensor is a dual excitation dye that enters acidic organelles. Neurons were pretreated with 2 μM LysoSensor and incubated for 5 minutes at 37°C. The LysoSensor is excited at 340 nm and 380 nm and the light is emitted at 520 nm. The excitation was measured by a filter-based imaging system (Zeiss) for 20 msec every 30 seconds. The ratio of light 340/380 nm excitation and the 520 nm emission was converted to pH by the calibration curve. This curve was established using 10 μM of the $H^+/Na^+$ ionophore monensin and 20 μM of the $H^+/K^+$ ionophore nigericin dissolved in 20 mM 2-(N-morpholino) ethane sulfonic acid (MES), 110 mM KCl, and 20 mM NaCl. The pH was adjusted between 3.0 and 7.0 with HCl/NaOH. At least 5 cells were imaged per condition, per replicate.

**Measurement of Endolysosome pH with Dual-Dextran Labeling**

Endolysosome pH of CLU199 cells were measured using a combination of dextran labelling as described (Nash el al., 2019). CLU199 cells were plated on poly-D-lysine 35 $mm^2$ glass bottom dishes and then 10 μg/mL each of pH sensitive pHrodo Green Dextran (P35368, Thermo Fisher) and pH insensitive Dextran, Texas Red (D1863, Thermo Fisher) were added. After 24 hours, the cells were washed twice with PBS, and transferred into Hibernate E Low Fluorescence Medium (HELF, Brainbits). HIV-1 gp120 (0.5 nM), HIV-1 heat inactivated gp120, or 17αE2 (10 nM) was added to the cells. The pH sensitive-

pHrodo Green Dextran emits at 533 nm and the pH-insensitive Dextran, Texas Red emits at 615 nm. The ratio 533/615 was converted to pH by using a intracellular calibration pH kit (P35379, Thermo Fisher) with the addition of 10 μM nigericin and 20 μM monensin in Hibernate E Flow Fluorescence Media adjusted to different pH with HCl or NaOH. For Rab7 positive vesicle pH, the CLU199 cells were loaded with pH-sensitive and pH-insensitive dextran for 6 hours and then placed for 2 hours in fresh media before being imaged. For the pH measurements, 5 fields were located under 40X on a Zeiss LSM800 confocal microscope. At least 30 cells were imaged per condition, per replicate.

**Immunofluorescence Staining in Fixed Cells**

Neurons (div 14-21) were treated, rinsed with warm PBS and fixed with 4% PFA for 20 minutes at room temperature and then washed in PBS three times. Neurons were permeabilized with 0.05 % Triton X-100 for 10 min, incubated in blocking buffer 3% BSA (Sigma) and 1% goat serum (Thermo Fisher) in PBS for 1 hour. Primary antibodies were incubated overnight at 4°C followed by secondary antibodies incubating for 1-2 hours at 4°C. The cells were mounted on microscope slides (Fisher Scientific) with ProLong Gold Antifade (P36930, Thermo Fisher) or DAPI (Vectorsheild). Controls included staining neurons with primary antibodies without fluorescence-conjugated secondary antibodies (background controls). Staining neurons with only secondary antibodies allow for the elimination of auto-fluorescence in the different channels and control bleed-through between channels. The following primary antibodies were used in

immunofluorescence staining; LAMP1 (D2D11) (1:500, 9091S, Cell Signaling), LAMP1 (1:200, ab21428, Abcam), Rab7 (1:500, ab137029, Abcam), MAP2 (1:500, ab32454, Abcam), MAP2 (1:500, ab92434, Abcam). Alexa Fluor 594 goat anti-rabbit, 488 goat anti-rabbit, 594 goat anti-mouse, 488 goat anti-mouse secondary antibodies were purchased from Thermo Fisher. All secondary antibodies were used at 1:250 dilutions. To visualize dendritic spines, neurons were stained for actin by incubating cells with (Alexa Fluor 594 Phalloidin) for 1 hour at room temperature after blocking buffer and visualized with the Zeiss LSM 800 confocal microscope. Dendritic morphology and spines were analyzed from either the same or different set of images from at least 10 neurons per condition, per replicate.

**Live Imaging of Dendritic Spines**

BacMam GFP Transduction Control (B10383, Thermo Fisher) was added to mouse primary hippocampal neurons (div 12-18) for 48 hours. Neurons had media removed and warm (37°C) Hibernate E Low Fluorescence Medium (HELF, Brainbits) was added before being placed on the Zeiss LSM800 Confocal microscope. Following treatment, cells were imaged using z-stack intervals of 0.5 μm using the 63X objective. The dendritic spines were identified with GFP and analyzed with Imaris 9.5 at 0 min (t-0) and 10 min (t-10) using the filaments module with IMARIS. The spines were plotted as a percentage of spines lost/gained. Spine classification criteria was used per published studies. This classification was kept the same for Golgi-Cox-stained brain tissue and

fluorescently stained neurons. Recombinant HIV-1 IIIB gp120 protein (Baculovirus expression) was obtained from ImmunoDx (1001, ImmunoDx LLC, Woburn, MA). Recombinant Tat HIV-1 IIIB (E. coli) was obtained from ImmunoDx (1002, ImmunoDx, Woburn, MA). Gp120 and Tat were heat-inactivated by heating at 95°C for 15 min. At least 5 dendrite segments were imaged, per condition, per replicate.

**Live-Imaging of Endolysosome Morphology**

Primary hippocampal neurons were stained with LysoTracker Red DND-99 (50 nM, Invitrogen) for 30 mins at 37°C to visualize endolysosomes. Live cell images were taken using a Zeiss LSM 800 confocal microscope, and Z-stacks were taken at 0.5 μm intervals of five random fields. At least 15 cells were imaged per condition, per replicate.

**Active Cathepsin D Staining**

Active Cathepsin D in both CLU99 cells and primary neurons were identified using BODIPY-FL Pepstatin A (P12271, Thermo Fisher). BODIPY-FL was added at 1 μM for 30 min, along with LysoTracker Red DND-99 at 10 nM at 37°C. The plates were washed twice with PBS and then transferred into Hibernate E Low Fluorescence (HELF) media for imaging. A total of 15-20 cells per treatment group were imaged using the Zeiss LSM 800 confocal microscope. Z-stacks were taken at 0.5 μm. LysoTracker Red and cathepsin D positive endolysosomes were reconstructed as spots using the spot module in Imaris 9.6 (Oxford

Instruments, Bitplane, Zurich, Switzerland). Object based colocalization was determined using a distance of 0.2 μm. At least 20 cells were imaged per condition, per replicate.

### Statistical Analyses

All data was expressed as means and SEM. Statistical analyses was performed using GraphPad Prism 9.0 software (GraphPad Software, Inc.). Statistical significance was calculated by either Student's *t*-test between two groups, one-way analysis of variance or two-way analysis of variance between groups among multiple groups with Tukey *post-hoc* tests. A p value $< 0.05$ was considered to be significant.

## Results

### HIV-1 gp120 and Tat Induce Endolysosome Dysfunction

Both HIV-1 gp120 and Tat can be endocytosed (Fittipaldi et al., 2003; Richard et al., 2005; Vendeville et al., 2004; Wenzel et al., 2017). Our lab has shown that both HIV-1 gp120 and Tat can induce de-acidification of endolysosomes in various types of cells (P. W. Halcrow et al., 2021; Hui et al., 2012). Therefore, we decided to confirm and extend our findings that gp120 and Tat affect endolysosome structure and function in neurons.

By measuring endolysosome pH with a combination of pH-sensitive (pH rodo) and pH-insensitive (Texas Red) dextran, we were able to demonstrate the effect

gp120 has on primary hippocampal neurons (div 9-12). HIV-1 gp120 (0.5 nM) was added to cells and endolysosome pH was determined for 10 min. HIV-1 gp120 was shown to de-acidify endolysosomes (Figure 5A). As a further measure of function, we determined effects of gp120 on lysosome hydrolase activity. We determined the number of active endolysosomes by calculating the percentage of cathepsin D (CatD) positive endolysosomes compared to total endolysosomes, which were stained with LysoTracker Red. HIV-1 gp120 significantly decreased the percentage of CatD positive endolysosomes (Figure 5B).

By measuring endolysosome pH using a ratio-metric method with LysoSensor DND-160, we determined the effect of HIV-1 Tat on endolysosome pH in primary hippocampal neurons (DIV 9-12). Tat (100 nM) was added to cells and endolysosome pH was determined for 15 min. Tat was shown to de-acidify endolysosomes (Figure 6A). As an additional measure of function, we determined the impact of Tat on lysosome hydrolase activity. Tat significantly decreased the percentage of CatD positive endolysosomes (Figure 6B). Our findings suggest that both gp120 and Tat induced endolysosome dysfunction in primary cultured neurons.

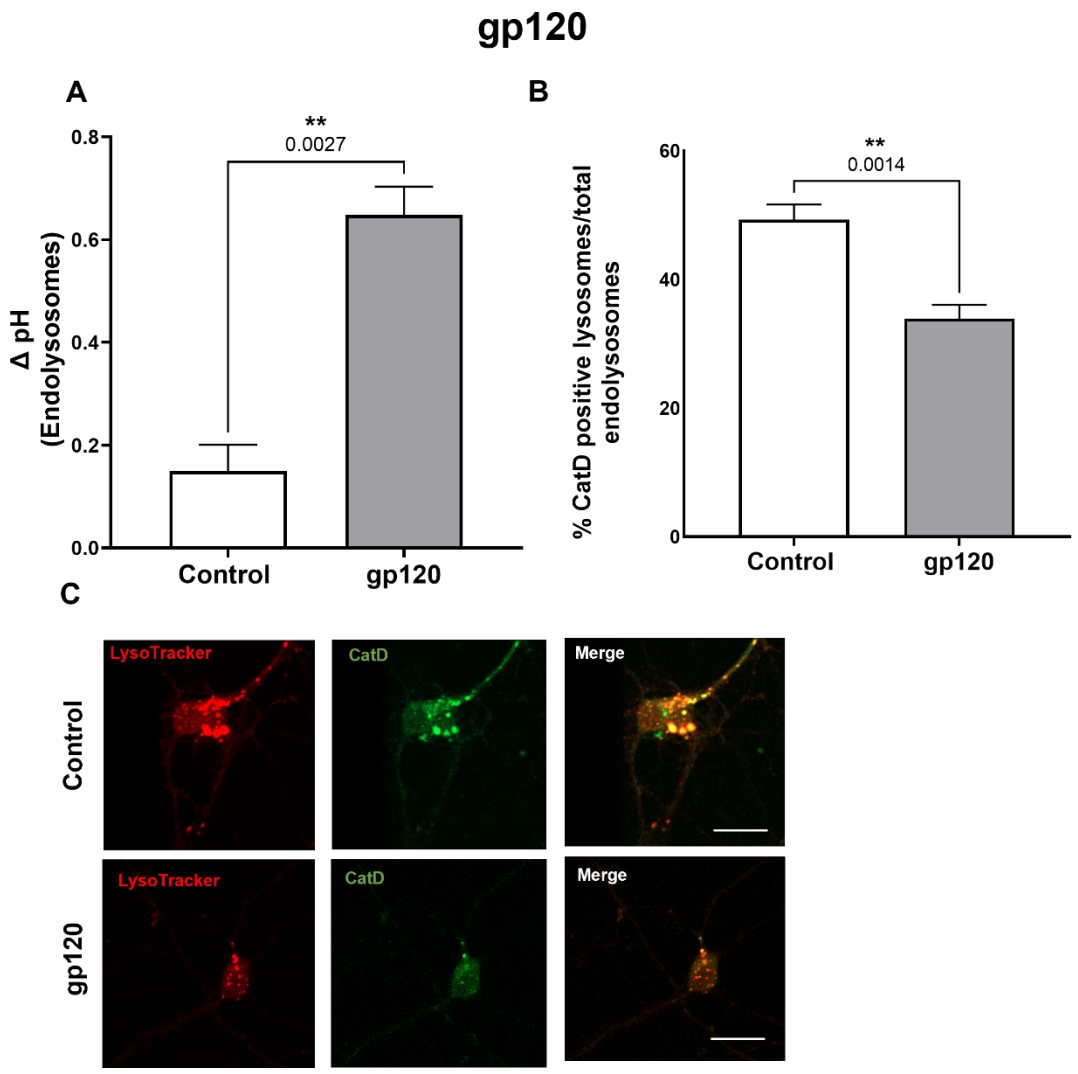


Figure 5. Endolysosome dysfunction induced by HIV-1 gp120.
**(A)** Endolysosomal pH was measured in primary hippocampal neurons (DIV9-12) using a ratio-metric methods with a combination of pH sensitive (pH rodo) and pH insensitive (Texas Red) dextran. HIV-1 gp120 (0.5 nM) de-acidified endolysosomes. Data were presented as mean ± SEM (n=3 replicates, **p<0.01, Student's t-test, two-tailed). **(B)** Bar graphs show changes in the percentage of active cathepsin D (CatD) positive endolysosomes to total endolysosomes. HIV-1 gp120 decreased the percent of CatD in endolysosomes. Data were presented as mean ± SEM (n=3 replicates, ***p<0.001, Student's t-test, two-tailed). **(C)** Representative confocal images of DIV9 hippocampal neurons show endolysosomes identified with LysoTracker Red (red) and active endolysosomes identified with active CatD (green). Active CatD was measured using BODIPY FL-Pepstatin A in hippocampal neurons for live imaging.

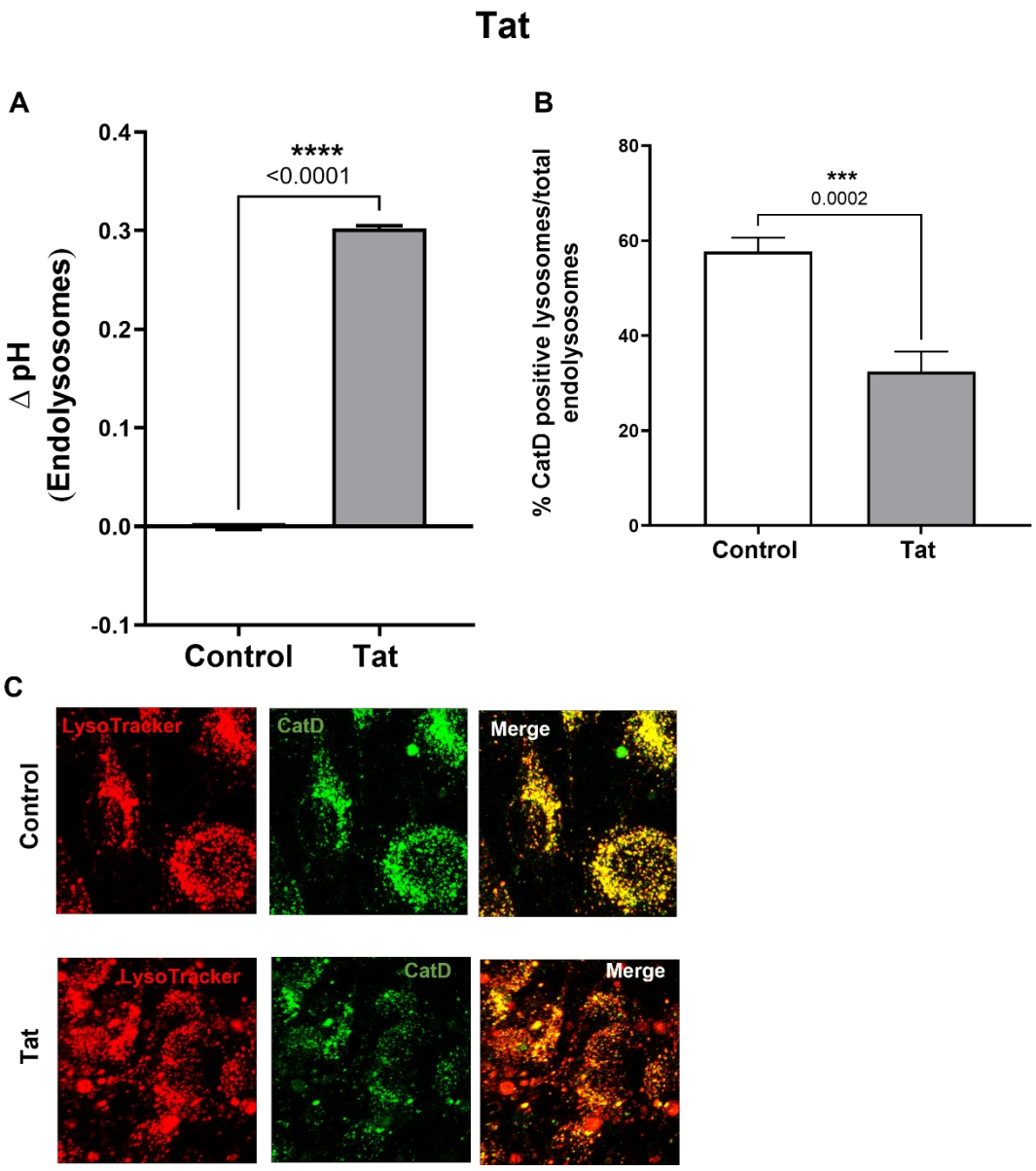


Figure 6. Endolysosome dysfunction induced by HIV-1 Tat.
**(A)** Endolysosomal pH was measured in CLU199 cells using a ratio-metric method with LysoSensor DND-160. Tat (100 nM) de-acidified endolysosomes. Data were presented as mean ± SEM (n=3 replicates, ****p<0.0001, Student's t-test, two-tailed. **(B)** Bar graphs show changes in the percentage of active cathepsin D (CatD) positive endolysosomes to total endolysosomes. Tat decreased the percent of CatD in endolysosomes. Data were presented as mean ± SEM (n= 2 replicates, ***p<0.001, Student's t-test, two-tailed).
**(C)** Representative confocal images of DIV9 hippocampal neurons show endolysosomes identified with LysoTracker Red (red) and active endolysosomes identified with active CatD (green). Active CatD was measured using BODIPY FL-Pepstatin A in hippocampal neurons for live imaging.

### HIV-1 gp120 and Tat Induce Endolysosome Enlargement

Endolysosome size has been shown to be increased in individuals with AIDS (Gelman et al., 2005) and in primary neurons treated with Tat (Hui et al., 2012). Due to the change in endolysosome pH and CatD percentage, we wanted to determine if the morphology of endolysosomes is being compromised due to gp120 and Tat treatment. LysoTracker Red was used to stain acidic vesicles such as endosomes and lysosomes, and the endolysosomal area was analyzed. HIV-1 gp120 (0.5 nM for 48 hr) significantly increased the size of endolysosomes (Figure 7B). This experiment was repeated with HIV-1 viral Tat protein. Tat (100 nM for 48 hr) significantly increased the size of endolysosomes (Figure 8B). These findings indicate that the both endolysosome function and endolysosome structure are altered with HIV-1 viral proteins, gp120 and Tat. Endolysosome enlargement may be due to the impaired breakdown of intra- and extracellular material (Zerial & McBride, 2001).

### HIV-1 gp120 and Tat Reduces Total Dendritic Spine Density

Endolysosome dysfunction resulting from HIV-1 gp120 and Tat may be more damaging to neurons than any other cells in the brain, because neurons are long-lived post mitotic cells and they are polarized cells with highly extensive processes. Endolysosomes are required to maintain the neuronal processes and are important for trafficking to repair plasma membranes. When lysosomes are inhibited, it changes their movement, and a decrease in the number of dendritic spines occurs (M. S. Goo et al., 2017). Therefore, it is probable that

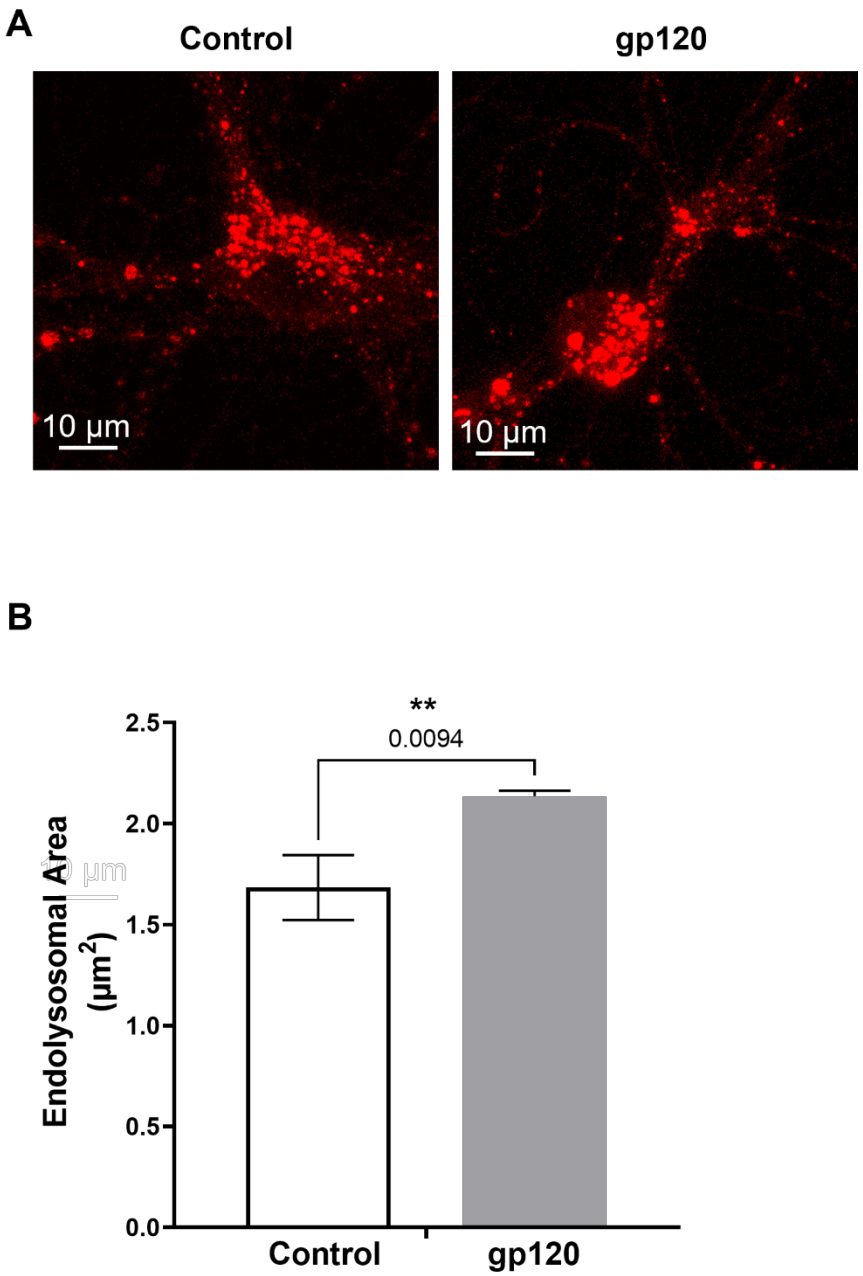


Figure 7. HIV-1 gp120 induced enlargement of endolysosomes. **(A)** Representative confocal images of rat hippocampal neurons (DIV14-19) show distribution and morphology of endolysosomes as identified with LysoTracker Red DND-99 (red) following treatment with HIV-1 gp120 for 48 hours. **(B)** HIV-1 gp120 increased the size of endolysosomes per neuron. Data were presented as mean ± SEM (n=2 replicates, **p<0.01, Student's t-test, two-tailed).

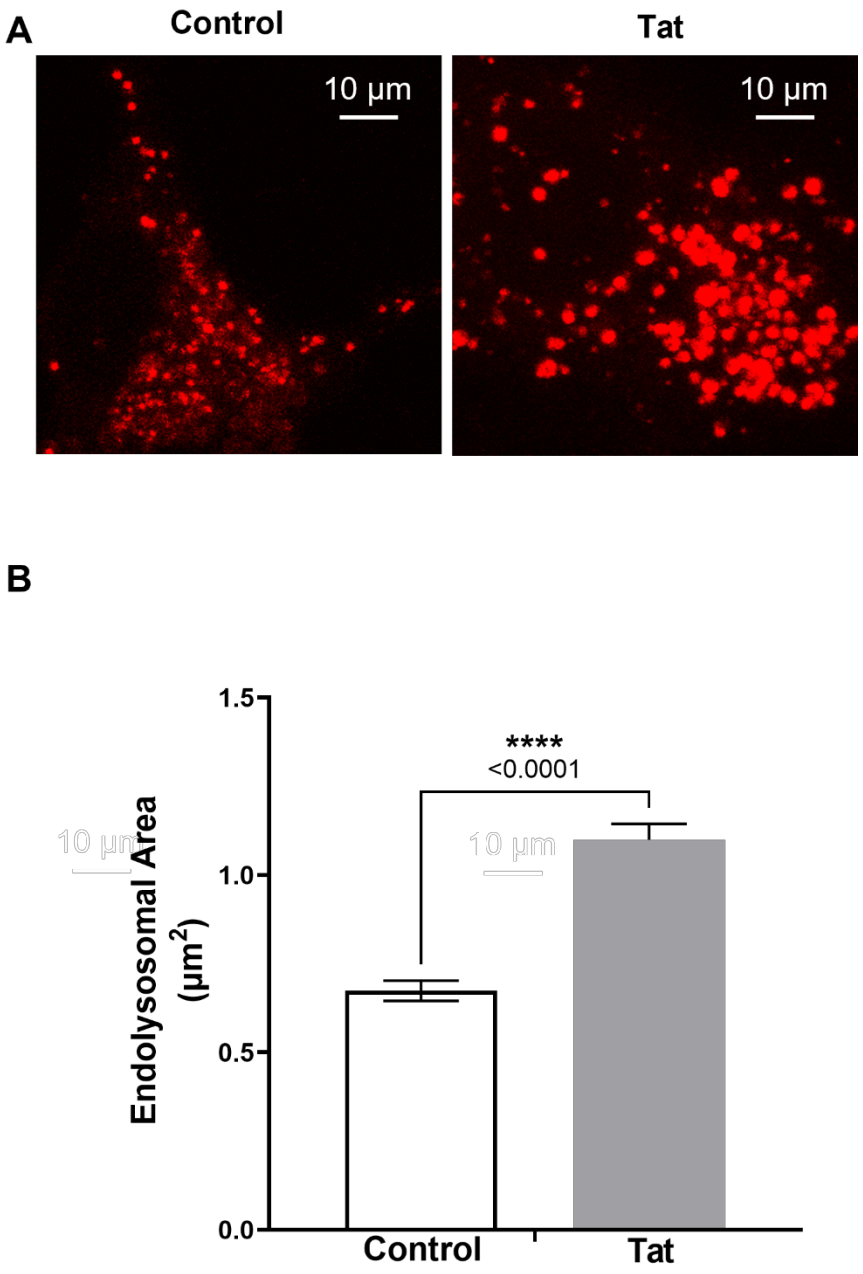


Figure 8. HIV-1 Tat induced enlargement of endolysosomes.
**(A)** Representative confocal images of primary rat hippocampal neurons (DIV14-19) show distribution and morphology of endolysosomes identified with LysoTracker Red (red) following treatment with HIV-1 Tat (100 nM) for 48 hours. **(B)** HIV-1 Tat increased the sizes of endolysosomes. Data were presented as mean ± SEM (n=2 replicates, ****p<0.0001, Student's t-test, two-tailed).

endolysosome dysfunction, stemming from the endocytosis and internalization of gp120 and Tat, can lead to changes in the morphology of dendritic spines.

Primary hippocampal neurons (DIV 15) were stained with MAP2 to determine dendritic length, and actin was stained with phalloidin to determine spine density. Neurons were treated with different concentrations of gp120 (0.2 nM, 0.5 nM, and 1.0 nM) for 48 hours. Gp120 significantly decreased the dendritic length at a concentration of 1 nM (Figure 9B). The spine density significantly decreased at 0.5 nM gp120 when compared to control (Figure 9B).

Similarly, we determined effects of Tat on dendritic length and dendritic spine density. The dendrite length was measured following incubation with 10 nM, 50 nM, and 100 nM of Tat. There was no significant change to dendrite length, although lengths decreased slightly as the concentration increased (Figure 10B). The density of dendritic spines was also measured using increasing concentrations of Tat. At 100 nM of Tat, there was a significant decrease in dendritic spine density (Figure 10C).

**HIV-1 gp120 and Tat Alter Subtypes of Dendritic Spines**

While dendritic spine density can be decreased, we also know that different spine morphology is associated with neurodegenerative decreases, intellectual

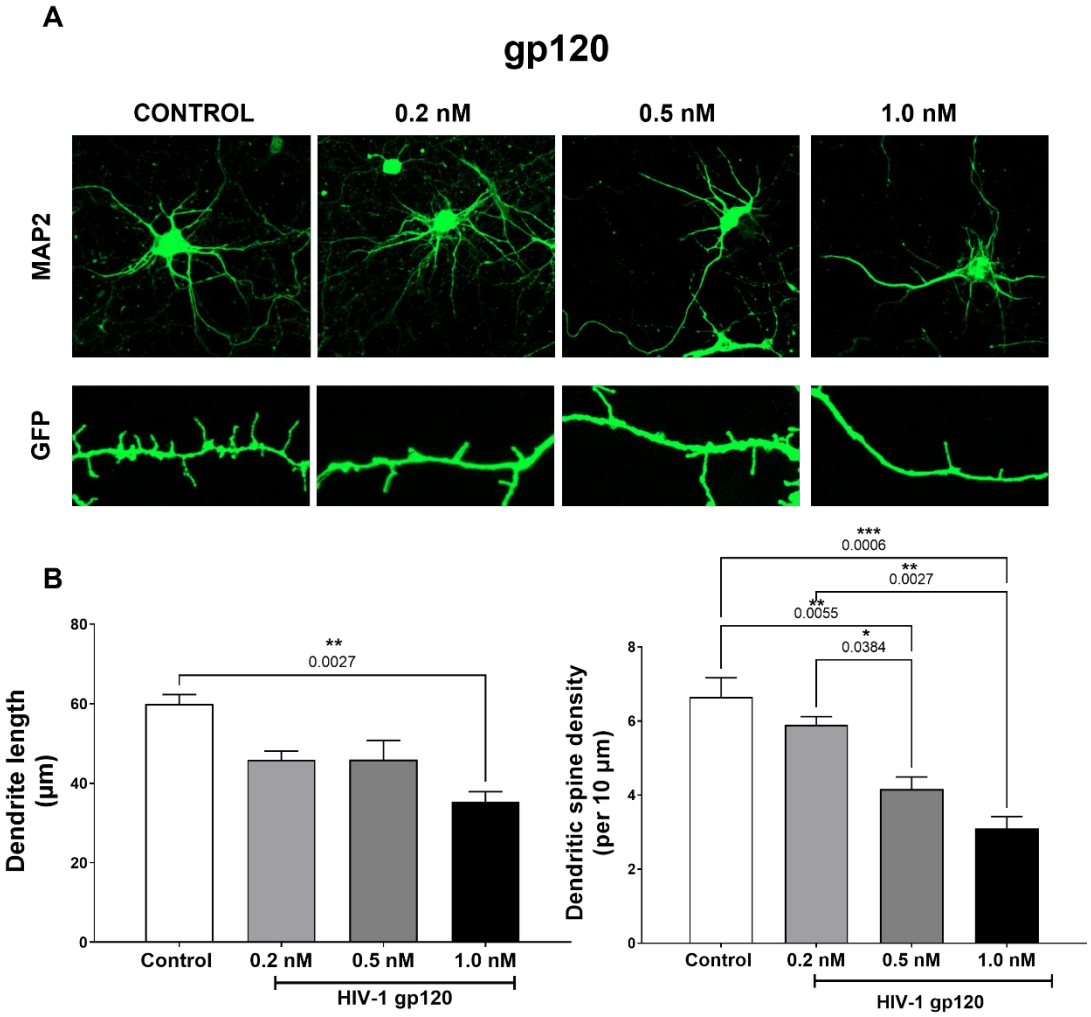


Figure 9. HIV-1 gp120 decreased dendritic length and reduces dendritic spine density.
**(A)** Representative confocal images of mouse primary hippocampal neurons (DIV 15) following treatment with HIV-1 gp120 (0.2-1 nM) for 48 hrs. Dendrites were identified with MAP2 (green) staining and dendritic spines were identified in neurons transduced with EGFP.
**(B)** HIV-1 gp120 significantly decreased dendritic length and reducesd dendritic spine density in a concentration-dependent manner. Data were presented as mean ± SEM (n=3 replicates, *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's post-hoc test).

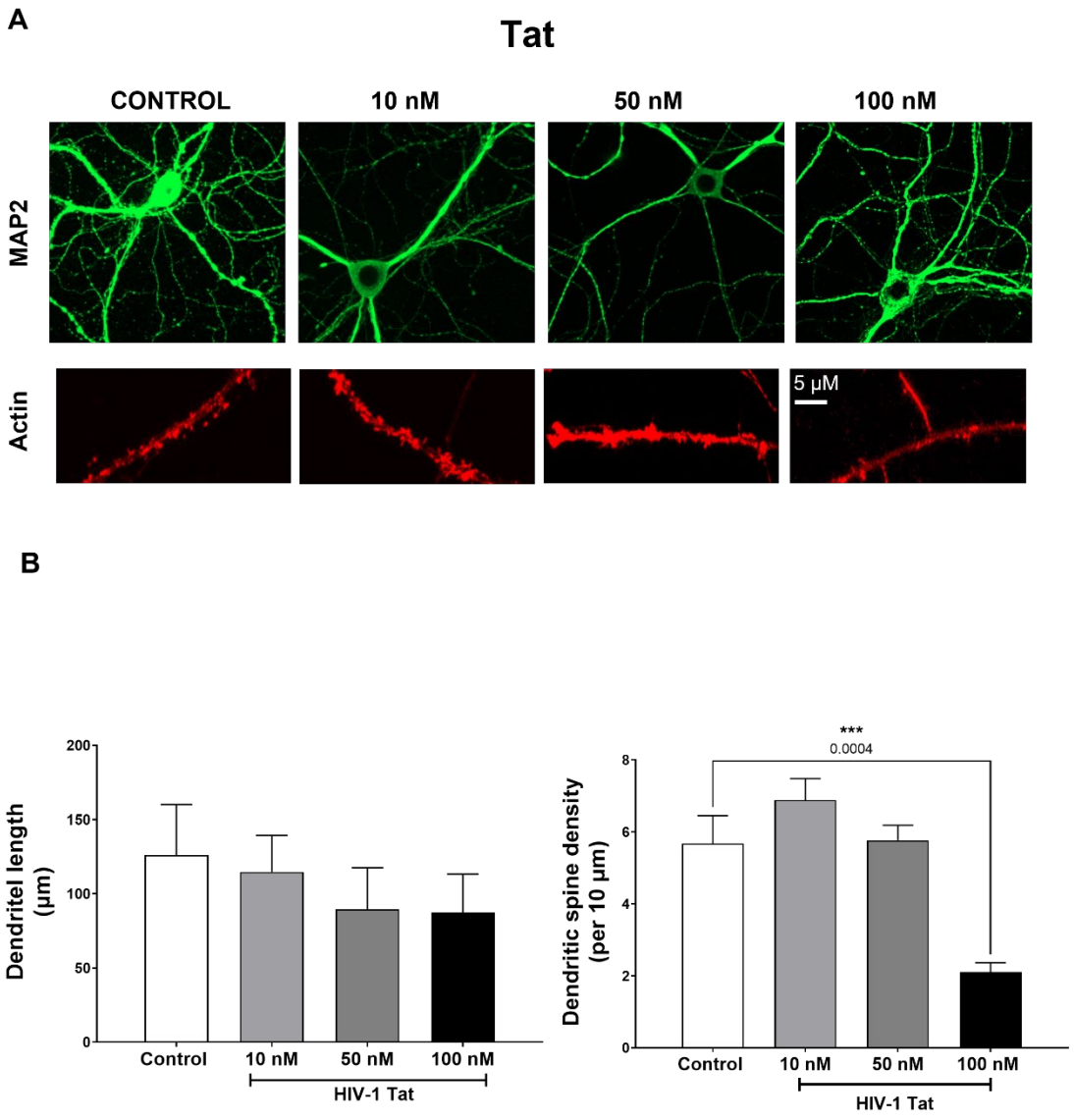


Figure 10. HIV-1 Tat decreased dendritic spine density.
**(A)** Representative confocal images of mouse primary hippocampal neurons (DIV 15) following treatment with HIV-1 Tat (10-100 nM) for 48 hrs. Dendrites were identified with MAP2 (green) staining and dendritic spines were identified in neurons stained for actin with phallodin. **(B)** HIV-1 Tat did not decrease dendritic length. Tat significantly reduced dendritic spine density in a concentration-dependent manner (n= 2 replicates, ***p<0.001, one-way ANOVA with Tukey's post-hoc test).

impairment, and psychiatric disorders (Boros, Greathouse, Gearing, & Herskowitz, 2019; R. Ellis et al., 2007; Forrest, Parnell, & Penzes, 2018; Kaufmann & Moser, 2000; Torres et al., 2018). Spines are classified according to their size and morphology, immature spines are long and have less surface area at the head compared to mature spines. Immature spines are classified as long thin spines, while mature spines are classified as stubby and mushroom spines (Figure 11A). Golgi-Cox staining is a well-known method for staining neurons and observing dendritic spines for quantification (Figure 11B). In addition, dendritic spines are very dynamic. Thus, we determined the effects of gp120 and Tat on the turnover of dendritic spines.

Primary hippocampal neurons (DIV 14) were transduced with EGFP, and live images were taken of the same dendrite at 0 mins and then again after 10 mins of treatment. Dendritic spine turnover was calculated as a percentage of net gain/loss in different subtypes of spines over the 10 min treatment window. HIV-1 gp120 (0.5 nM) treatment led to a significant decrease in both stubby and mushroom spines but led to an increase in long thin spines (Figure 12B). This experiment was repeated with HIV-1 Tat (100 nM). Tat led to a significant decrease in stubby, mushroom, and long/thin spines (Figure 13B).

## DISCUSSION

We observed a direct association between endolysosome dysfunction and altered dendritic spines induced by gp120 or Tat treatment. Our findings

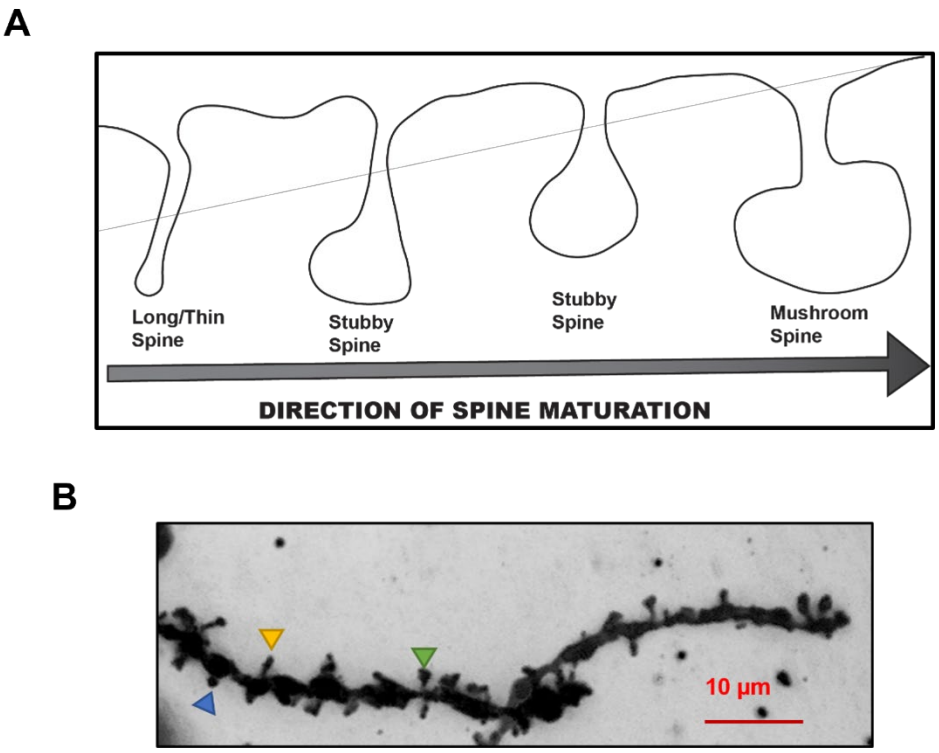


Figure 11. Dendritic spine classification.
**(A)** Different types of dendritic spines found on neurons were portrayed above. Immature spines followed by mature spines are as follows; long thin spines (yellow), stubby spines (blue), and mushroom spines (green). **(B)** Golgi-Cox-stained neurons show dendritic spines. Arrows were labeled with different colors to represent the spine subtype.

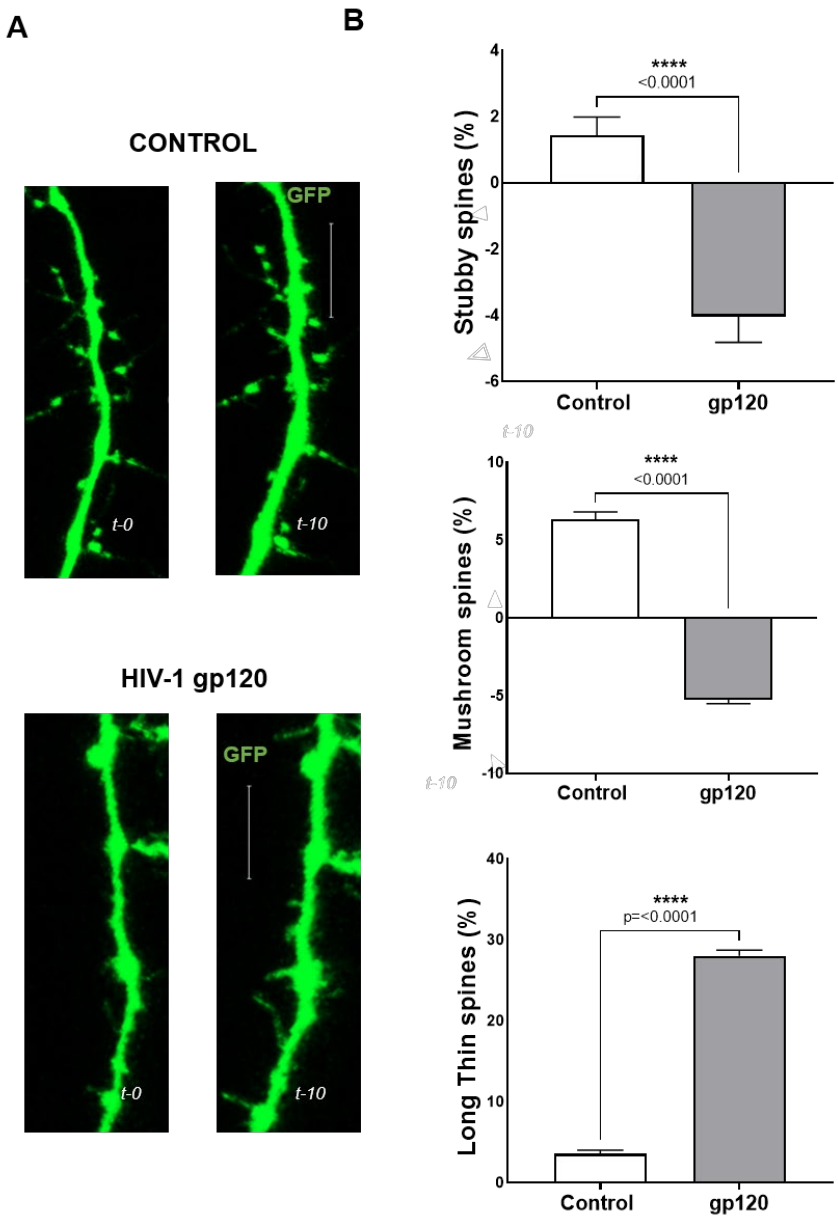


Figure 12. HIV-1 gp120 induced dendritic impairments.
**(A)** Representative confocal images show the rapid change in the dendritic spine morphology of mouse hippocampal neurons (DIV14) transduced with EGFP on the same dendrite imaged at 0 and 10 min with different treatments. Scale bar- 5 µm. **(B)** Bar graphs show changes in dendritic spine turnover over 10 min with different treatments. Positive values indicate spine formation, while negative values indicate spine elimination between 0- and 10-min. HIV-1 gp120 (0.5 nM) treatment led to a significant reduction in stubby and mushroom spines. HIV-1 gp120 (0.5 nM) treatment led to a significant increase in long/thin spines. Data were presented as mean ± SEM (n=3 replicates, ****p<0.0001, Student's t-test, two-tailed).

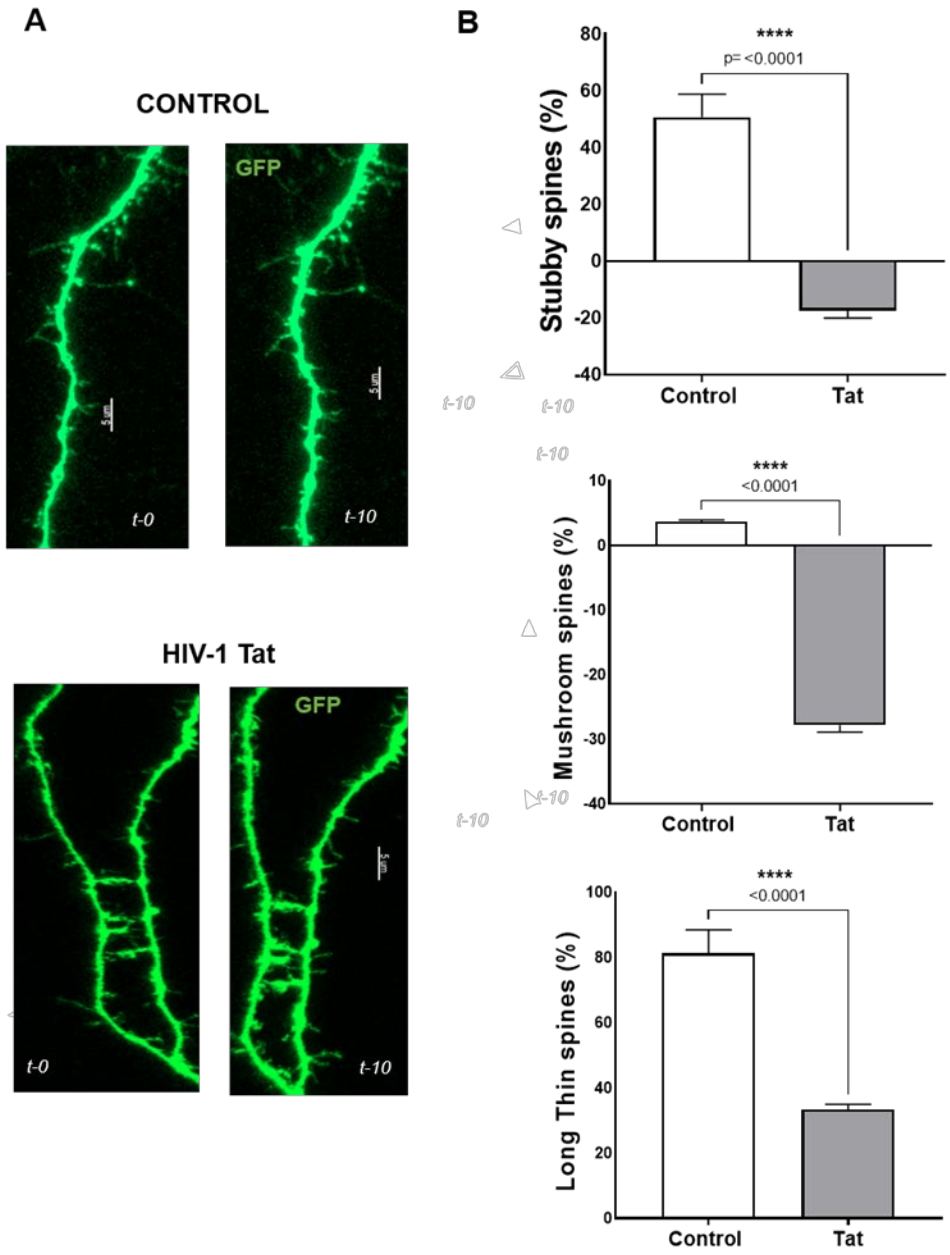


Figure 13. HIV-1 Tat induced dendritic impairments.
**(A)** Representative confocal images show the rapid change in the spine morphology of mouse hippocampal neurons (DIV14) transduced with EGFP on the same dendrite imaged at 0 and 10 min with different treatments. Scale bar- 5 μm. **(B)** Bar graphs show changes in dendritic spine turnover over 10 min with different treatments. Positive values indicate spine formation, while negative values indicate spine elimination between 0- and 10-min. HIV-1 Tat (100 nM) treatment led to a significant reduction in stubby spines and mushroom spines, as well as long/thin spines. Data were presented as mean ± SEM (n=2 replicates, ****p<0.001, Student's t-test, two-tailed).

indicate that endolysosome dysfunction, which occurs early in the direct neuronal injury process induced by gp120 and Tat, could lead to altered dendritic spines. HIV-1 gp120 and Tat are both endocytosed by neurons (Fittipaldi et al., 2003; Richard et al., 2005; Vendeville et al., 2004; Wenzel et al., 2017). Thus, internalized gp120 or Tat could directly disrupt the structure and function of the endolysosomes. Both HIV-1 gp120 and Tat led to an increased endolysosome pH (de-acidification). Endolysosomes need to be at an acidic pH to have fully functional acidic hydrolases. Endolysosomes that are de-acidified also present with a decrease in lysosomal enzyme activity; as shown in this study, CatD was observed to decrease both HIV-1 gp120 and Tat treatment. Depending upon the pH of the endolysosomes, cathepsin activities are altered (Claus et al., 1998). Therefore, the decrease in CatD positive endolysosomes induced by gp120 and Tat follows de-acidification of the pH.

De-acidification results in an increase of the size of the endolysosome followed by an accumulation of undigested intracellular cargo (Myers et al., 1991; Ohkuma & Poole, 1981; Yoshimori et al., 1991). Endolysosome de-acidification can lead to impairment in fusion of autophagosomes with lysosomes, which may result in cell death (Doherty & Baehrecke, 2018; Mauthe et al., 2018). Human brains infected with HIV-1 have observed an increase in the size of lysosomes in neurons (Gelman et al., 2005). Consistent with these findings, treatment with gp120 and Tat led to robust endolysosome enlargement. Such gp120- and Tat-induced morphological changes of endolysosomes could be resulting from accumulating cargo (internalized or autophagy), because increased

endolysosome pH and decreased cathepsin D activity could lead to impaired degradation of cargos.

Lysosomes are found along dendrites and in dendritic spines, as they are essential in regulating synaptic plasticity through the dendritic spines (Cheng et al., 2018; Marisa S. Goo et al., 2017; Padamsey et al., 2017; Yap et al., 2018). When lysosomes are inhibited, their mobility is altered, and the density of dendritic spines are decreased (M. S. Goo et al., 2017). We found that HIV-1 viral proteins, gp120 and Tat, induce endolysosome dysfunction and alter dendritic spines. The total spine density is decreased significantly by either gp120 or Tat treatment, whereas only gp120 treatment led to a significant decrease in dendritic length. Tat has been shown to reduce the length of dendrites in cultured neurons (Liu et al., 2018). Our study did not show a significant decrease in dendritic length, and this could be due to the different concentrations of Tat. Liu et al, used Tat (1-72 aa) from DG Peptides Co., Ltd (China) at a concentration of 2 μmol/L while our Tat was from ImmunoDx (1002-F) and used at 0.1 μmol/L (100 nM).

In HIV infected neuroblastoma cells, dendritic spine morphology was altered; spine volume was decreased (Atluri et al., 2013). In AD there is increased spine length, increased filopodia density, and reduced spine head diameter (Boros et al., 2019); similar morphology changes are found in neurodegenerative, psychiatric, and intellectual disorders. The morphologic changes in the spine types are associated with cognitive decline and memory impairment. Both gp120

and Tat resulted in a decrease of stubby and mushroom spines, which are considered mature spines. HIV-1 gp120 increased the percentage of long/thin spines, while Tat decreased the percentage of long thin spines, which are considered immature spines. Tat and gp120 produce different effects in the percentage of immature spines. Although it still needs to be elucidated, one potential difference is that internalized Tat can escape endolysosomes and enter cytosol, whereas gp120 has not been shown to escape from the endolysosome.

These findings suggest that both HIV-1 proteins gp120 and Tat induce endolysosome dysfunction and altered dendritic spines. Because internalization of gp120 and Tat occurs early in the direct neuronal damaging effects of these viral proteins, the observed association between endolysosome dysfunction and altered dendritic spines indicate endolysosome dysfunction, at least in part, leads to alterations in dendritic spines. Although the causal relation remains to be determined, targeting the endolysosome system and preventing endolysosome dysfunction may avoid the development of synaptodendritic impairment as occur in HAND.

# CHAPTER III

# 17α-ESTRADIOL TREATMENT PROTECTS AGAINST TAT AND gp120 INDUCED ENDOLYSOMAL DYSFUNCTION AND SYNAPTODENDRITIC IMPAIRMENT IN CELL CULTURE

## Introduction

### Overview of Estrogen

In women, ovaries and developing follicles are the primary producers of estrogen, but several extragonadal tissues produce estrogen. These tissues include the brain, osteoblasts, vascular endothelium, aortic smooth muscle cells, breast, and chondrocytes. In premenopausal women, sources of 17βE2 are ovaries and the adrenal cortex. In postmenopausal women, the primary sources of 17βE2 synthesis are the liver and adrenal glands (Simpson, 2003). The plasma level of 17βE2 in premenopausal women ranges from 0.11-2.20 nM. In postmenopausal women, sources of 17βE2 are produced by extragonadal tissue. The plasma level of 17βE2 in postmenopausal women is around 0.04 nM. In men, sources of 17βE2 synthesis are extragonadal tissue and the testes. The plasma level of 17βE2 in men is around 0.10 nM (Vrtacnik, Ostanek, Mencej-Bedrac, & Marc, 2014). Males possess a higher estrogen level in blood when compared to postmenopausal women (Janicki & Schupf, 2010). Currently, the location and tissue content of 17αE2 in humans are not well documented;

however, endogenous 17αE2 has been detected in the urine and serum of humans (Santos, de Fatima, Frank, Carneiro, & Clegg, 2017)

Estrogens are present in both men and women in significant amounts; however, estrogens are higher in women after menarche and drop significantly during menopause. The primary significance estrogen has in males is the maturation of sperm (Schulster, Bernie, & Ramasamy, 2016). In women, estrogen helps develop the female secondary characteristics such as breasts, regulation of the menstrual cycle, and the endometrium. In general, estrogens reduce bone resorption, which increases bone formation. Estrogens increase platelet adhesiveness, HDL, triglycerides and decrease LDL levels (Ruggiero & Likis, 2002).

**Biochemistry of Estrogen**

There are three major estrogens, estrone, estradiol, and estriol. Estradiol is the most common estrogen in nonpregnant women, with estrone next most common. Estrone is produced in women during menopause but is a weak estrogen. Estriol is the weakest estrogen and is increased during pregnancy (Sison, 2020).

Estrogens are C18 steroid hormones and contain a phenolic structure. Conversion of estradiol to estrone or estrone to estradiol is catalyzed by different 17β-hydroxysteroid-dehydrogenases (HSD17B) (Kuhl, 2005). The synthesis of estrogen starts with cholesterol and is converted into estradiol via multiple steps. (Olivo-Marston et al., 2010).

Physiological estradiol levels in humans range from 1-20,000 pg/ml. Serum estradiol levels ranged from 20-300 pg/ml in adolescent girls, women during their menstrual cycle ranged from 30-800 pg/ml, and in pregnancy the levels were around 20,000 pg/ml. Postmenopausal women had serum estradiol levels of less than 20 pg/ml, and men had less than 40 pg/ml. Postmenopausal women on estrogen therapy had serum levels that ranged from 20-100 pg/ml (Stanczyk, Archer, & Bhavnani, 2013; Stanczyk & Clarke, 2014).

Estradiol is a naturally occurring estrogen, an agonist of estrogen receptors. Estradiol can be administered in various ways; oral, sublingual, transdermal (patch or gel), intranasal, vaginal, buccal, intramuscular, and subcutaneous injections. The bioavailability of oral estradiol is 5% (Kuhl, 2005). Most of the estrogens circulating are bound to serum proteins. 37% of estradiol is bound to sex hormone-binding globulin (SHBG) , 61% is bound to albumin, and 2% is free. The half-life of estradiol is about 20–30 min. Due to the metabolism in the gut and liver occurring at a high rate, there is a high estrone/estradiol ratio (oral estradiol is 5:1). The levels of estradiol, after oral administration, are elevated for 12 hours and then slowly decrease (Hall & McDonnell, 1999).

Intranasal administration of estradiol resulted in a rapid increase of serum estradiol that contained 250 $\mu$g estradiol in 70 $\mu$l. This has been used for hormone replacement therapy (HRT) and leads to high estradiol levels in the serum for 10-30 mins and then decreases rapidly (Kuhl, 2005). Intranasal application of 200-400 $\mu$g estradiol had similar clinical effects in reducing

menopausal symptoms similar to oral administration of 1-2 mg oral estradiol valerate (Lopes, Rozenberg, Graaf, Fernandez-Villoria, & Marianowski, 2001; Studd et al., 1999). Intranasal administration is a direct pathway to the cerebrospinal fluid (CSF) from the nasal cavity (Kuhl, 2005). Studies have shown increased levels of drug concentrations in the CSF with intranasal administration versus intravenous administration. As the lipophilicity of the drugs increased, the greater the concentration of the drug was present in the CSF (Sakane et al., 1991; Wattanakumtornkul, Pinto, & Williams, 2003). Intranasal estradiol administration avoids the metabolism of the liver (Studd et al., 1999).

**Estradiol**

17αE2 is an enantiomer of 17βE2, neurons synthesize both in the rodent brain, but 17αE2 was shown to be the dominant form. 17αE2 has a 40 times lower affinity for estrogen receptors than 17βE2 but has still shown to be neuroprotective (Kaur, Bansal, & Chopra, 2015). 17αE2 was found to alleviate age-related metabolic dysfunction and inflammation without causing feminization or cardiac dysfunction in male mice (M. B. Stout et al., 2017). Endogenous levels of 17αE2 and 17βE2 were identified in the brains of adult mice. Significant endogenous levels of 17αE2 were found in brain regions compared to low serum levels, suggesting that 17αE2 is synthesized locally in the brain (C. D. Toran-Allerand, A. A. Tinnikov, R. J. Singh, & I. S. Nethrapalli, 2005) (Figure 14). 17αE2 levels were also elevated in animals that had been gonadectomized, adrenalectomized, and ovariectomized, while 17βE2 was not detected. In the

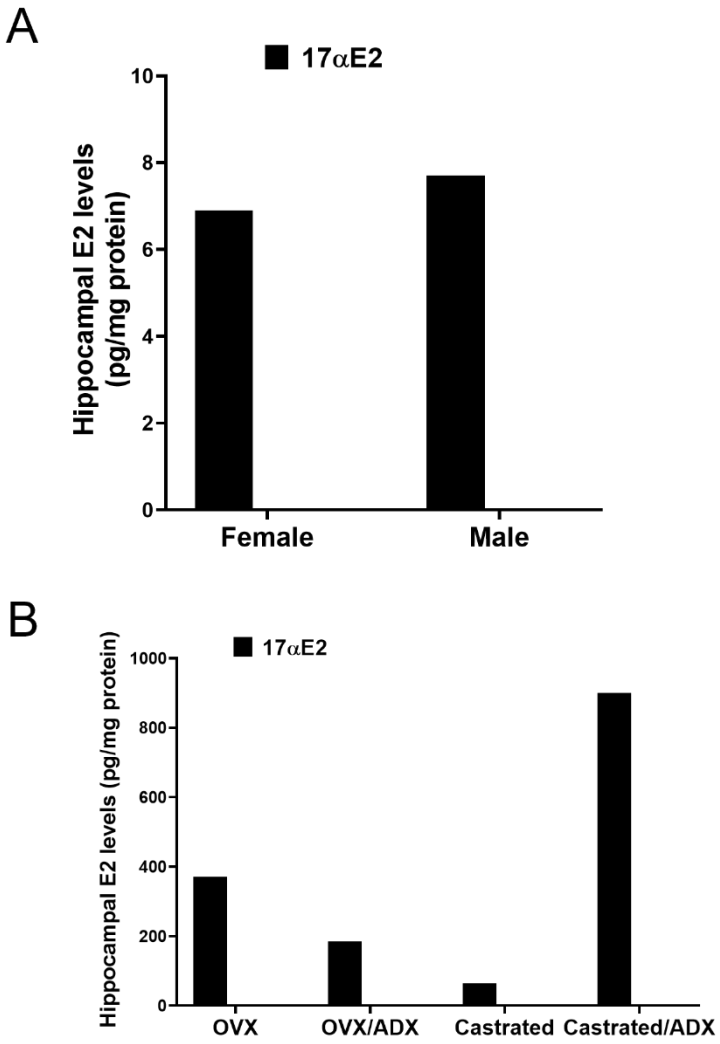


Figure 14. 17αE2 is the main estradiol present in brain of adult mice HIV-1 Tat induces dendritic impairments.
**(A)** 17αE2 was detected in the hippocampus of female and male mice, whereas 17βE2 was not detected (Data extracted from Toran-Allerand,C. Endocrinology. (2005). **(B)** 17αE2 is produced in the brain as evidenced by findings that 17αE2 was detected in the hippocampus of ovariectomized (OVX) female mice, ovariectomized and adrenalectomized (ADX) female mice, castrated male mice, and castrated and adrenalectomized male mice.
(C. Dominique Toran-Allerand, 17α-Estradiol: A Brain-Active Estrogen?, Endocrinology 2005, 146, 9, 3843-3850, by permission of Oxford University Press)

hippocampus of females in adult mice, the 17αE2 was 0.9 pg/mg, 17βE2 was not detected, while males had a 7.7 pg/mg level and 17βE2 was not detected (C. D. Toran-Allerand et al., 2005). This evidence suggests that estrogen is made in the brain de novo through cholesterol and specifically that 17αE2 is synthesized locally in the brain. 17αE2 and 17βE2 act on estrogen receptors to induce their neuroprotective effects.

**Estrogen Receptors**

Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are part of the nuclear receptor family. These receptors act as transcription factors by either homo- or heterodimers. Estrogens are steroid compounds; therefore, they diffuse across the cell membrane, and then they can bind to the estrogen receptors in the nucleus. This is called the genomic pathway that regulates the expression of different genes by estrogen that binds to the receptor and translocates to the nucleus, thereby activating the transcription of multiple target genes (Bjornstrom & Sjoberg, 2005).

The non-genomic pathway of activation estrogen receptors results in intracellular signaling that produces rapid effects. There are estrogen receptors located on extranuclear membranes and responsible for cytosolic signaling. When estrogen binds to estrogen receptors, this can result in activation of the phosphoinositol 3-kinase signaling pathway, causing the release of intracellular calcium, cyclic adenosine monophosphate production, activation of adenylate cyclase activity,

and activation of membrane tyrosine kinase receptors (Bjornstrom & Sjoberg, 2005).

ERβ is found in the prostate, colon, testes, vascular endothelium, bladder, lungs, ovaries, skeleton, salivary glands, and brain. ERα is located in the brain, breasts, uterus, liver, epididymis, white adipose tissue, kidneys, prostate, ovaries, testes, and the skeleton. GPER1 (GPR30) is distributed in the the central and peripheral nervous system, mammary glands, uterus, spermatogonial cells, ovaries, testes, kidneys, gastrointestinal system, pancreas, liver, pituitary and adrenal glands, bone tissue, immune cells, and cardiovascular system (Vrtacnik et al., 2014). ERα and ERβ are both estrogen receptors that are expressed in the brain, but ERα was located in multiple regions of the brain (including the prefrontal cortex and hippocampus), and ERβ is found in the cerebellum and the hippocampus (Warfvinge et al., 2020). ERα was expressed in the human hippocampus in neurons and astrocytes (Heron, Turchan-Cholewo, Bruce-Keller, & Wilson, 2009).

Estrogen receptors have been found to localize on different organelle membranes. ERβ has been found to colocalize with the mitochondria in primary rat neurons, murine hippocampal lines, and primary cardiomyocytes (Yang et al., 2004). GPR30 is an intracellular transmembrane estrogen receptor that is located in endoplasmic reticulum and on plasma membranes (Gaudet, Cheng, Christensen, & Filardo, 2015; Revankar, Cimino, Sklar, Arterburn, & Prossnitz, 2005). ERα has been located on plasma membranes and endolysosomes (Levin,

2009; Totta, Pesiri, Marino, & Acconcia, 2014). Endolysosome localization of ERα has been implicated in its degradation (Sampayo et al., 2018). However, as a soluble cytosolic protein (Schlegel, Wang, Katzenellenbogen, Pestell, & Lisanti, 1999), ERα most likely resides on endolysosome membranes facing the cytosol rather than facing the lumen. Thus, endolysosome localized ERα may directly affect endolysosome function.

**Neuroprotective Effects of 17αE2**

Although not as extensively studied as that of 17βE2, 17αE2 has been shown to exert neuroprotective effects. Synaptic density in the hippocampus is at its peak when estrogen levels are high and when estrogen is low following ovariectomy, the hippocampus neurons experience a decrease in synaptic spine density. This can be repaired by estrogen replacement (Gould, Woolley, Frankfurt, & McEwen, 1990; MacLusky & Naftolin, 1981; Woolley & McEwen, 1992). A study using 11-month-old Alzheimer's mice (amyloid-β protein precursor (AβPPswe) and presenilin 1 (PS1ΔE9)) showed a loss of neurons in the CA1 hippocampal region due to amyloid-β aggregation. Female mice were ovariectomized, treated for 60 days with 17αE2. These female mice were given 1.5 mg of 17α-estradiol or placebo. The treated females showed that 17αE2 reduced the loss of neurons in CA1 (Manaye et al., 2011). In a study using SK-N-SH cells, human neuroblastoma cell line, cells were treated with 17βE2 (0.2 or 2 nM) or with 17αE2 (0.2 or 2 nM). Cultures were starved of serum; however, both 17αE2 and 17βE2 protected the cells from cell death (Green, Bishop, & Simpkins, 1997).

## Estrogen as a HAND Therapy

Although not without controversy in epidemiological studies, sex differences have been documented in the development of HIV-associated neurocognitive impairment (Carvour et al., 2015; Rubin et al., 2019; Sundermann et al., 2018). Hormonal changes throughout a woman's life could impact their prognosis of developing HAND (Quinn & Overbaugh, 2005). Cognitive deficits in women could be attributed to changing estrogen levels in both HAND as well as during menopause (Weber, Mapstone, Staskiewicz, & Maki, 2012). Hippocampal memory is associated with estrogen levels and the sharp increase in neurocognitive impairments in HIV-positive post-menopausal women may be explained by the declining neuroprotection offered by estrogen (Maki et al., 2021).

Experimental studies have shown that estrogen is protective against HIV-1 infection and HIV-associated neuropathology. 17βE2 is the primary estrogen in the blood and suppresses the replication of HIV-1 (Szotek, Narasipura, & Al-Harthi, 2013). 17βE2 is neuroprotective and has been shown in many studies to protect against effects of Tat and gp120, such preventing the release of pro-inflammatory mediators, oxidative stress, cell death, and preventing the loss of dopamine transporter function (Wilson, Dimayuga, et al., 2006). 17βE2 reduced the neuronal apoptosis in the rat neocortex and hippocampal neurons caused by gp120 (Corasaniti et al., 2005). Therefore, estrogen replacement therapy (ERT) could be a possible solution in preventing cognitive defects as estrogen appears

to have neuroprotective and inflammatory properties (Vegeto et al., 2003; Zemlyak, Brooke, & Sapolsky, 2005). However, 17βE2 has feminizing effects and other side effects such as cancer, thromboembolism, coronary heart disease, and stroke (Bassuk & Manson, 2016; Cushman et al., 2018; D'Alonzo, Bounous, Villa, & Biglia, 2019). Therefore, this makes 17βE2 a poor candidate for HAND. The isomer of 17βE2, 17αE2, is less potent but still produces the same desired effects as 17β-E2. 17αE2 has neuroprotective effects by protecting against neuronal loss and behavioral defects (McClean & Nunez, 2008). 17βE2 and 17αE2 were shown to protect at the same level against gp120 neuronal toxicity (Howard, Brooke, & Sapolsky, 2001). Thus, 17αE2 is a promising alternative to 17βE2 as it is a natural non-feminizing estrogen (Kaur et al., 2015).

## Hypothesis

We know that HIV-1 viral proteins Tat and gp120 are endocytosed into the endolysosome system (Ajasin & Eugenin, 2020; Fittipaldi et al., 2003; Liu et al., 2000; Richard et al., 2005; Tyagi et al., 2001; Vendeville et al., 2004; Wenzel et al., 2017). Tat and gp120 have been previously shown to affect endolysosomes (P. W. Halcrow et al., 2021; Hui et al., 2012). Tat and gp120 has been shown to decrease spine density (Festa et al., 2015; Fitting et al., 2013; Speidell et al., 2019). In the previous chapter, we have shown that endolysosome dysfunction resulting from HIV-1 Tat and gp120 plays a role in HIV-1 Tat- and gp120-induced reduction in dendritic spines. Thus, preventing endolysosome dysfunction may prevent HIV-1 Tat- and gp120-induced reduction in dendritic spines. Because

ERα is localized on endolysosomes, 17αE2 might interact with endolysosome localized ERα and affect endolysosome function and exert neuroprotective effects.

The overall hypothesis is that 17αE2 exerts enhancing effects on endolysosomes and dendritic spines and protects against HIV-1 Tat- and gp120-induced endolysosome dysfunction, reduction in the number of dendritic spines, altered morphology of dendritic spines, and that such protective effects of 17αE2 are mediated via endolysosome localized ERα (Figure 15). In these experiments I determined the effects of 17αE2 on endolysosomes and dendritic spines as well as HIV-1 Tat- and gp120-induced endolysosome dysfunction and dendritic spine impairment.

## Methods

### Cell Culture

Mouse embryonic hippocampal E-18 cell line CLU199 (Cellutions Biosystems, Cedarlane, Ontario, Canada) were grown and maintained in 1x DMEM. Described in detail in Chapter II.

### Cultured Primary Rat Hippocampal Neurons

Primary mouse hippocampal neurons were cultured from embryonic day 18 rats. Described in detail in Chapter II.

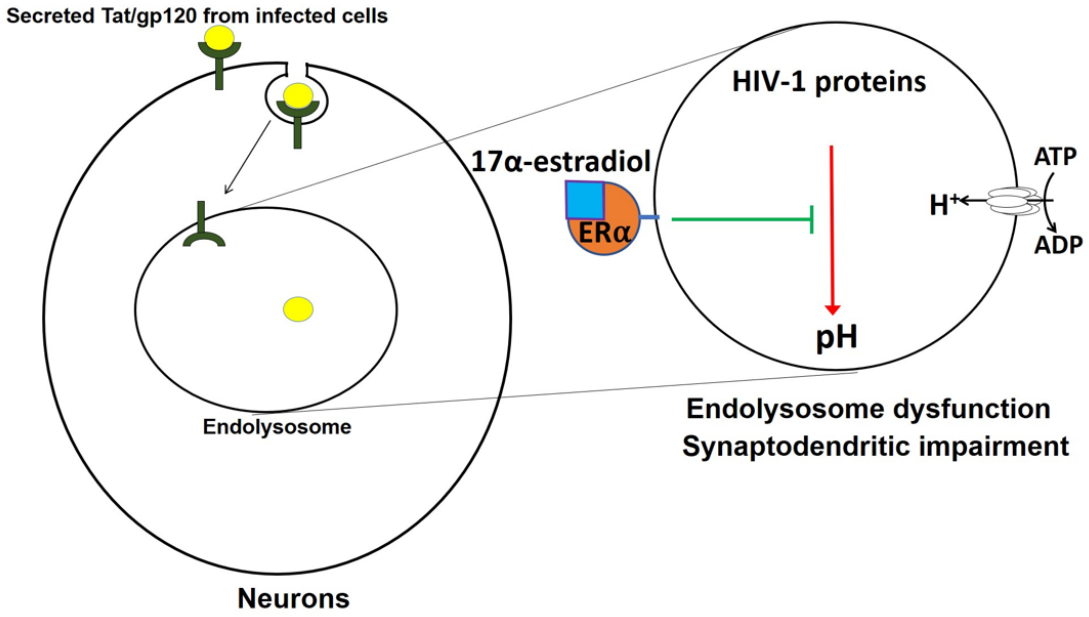


Figure 15. Proposed mechanism whereby ERα is necessary for the protective effects of 17αE2.
HIV viral proteins (gp120 and Tat) are internalized via receptor mediated endocystosis. Internalization of these proteins leads to a deacidification of the endolysosome pH, disrupting the endolysosome funtion. Endolysosome dysfunction could lead to synaptodendritic imapirement. These effects could be prevented by 17αE2 via ERα activity localized on endolysosomes.

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### Cultured Primary Mouse Hippocampal Neurons

Primary mouse embryonic hippocampal neurons (C57EHP, Brainbits LLC, Springfield, IL) were obtained from E18 mouse cortex. Described in detail in Chapter II.

### Measurement of Endolysosome pH with LysoSensor

Endolysosome pH was measured using a ratio-metric lysosome pH indicator dye called LysoSensor (Yellow/Blue DND-160 from Invitrogen). Described in detail in Chapter II.

### Measurement of Endolysosome pH with Dual-Dextran Labeling

The pH of CLU199 cells were measured using a combination of dextran labelling as described (Nash el al., 2019). The treatments of HIV-1 gp120 (0.5 nM), HIV-1 Tat (100 nM), HIV-1 heat-inactivated gp120, HIV-1 heat-inactivated Tat or 17$\alpha$E2 (10 nM) were added. Described in detail in Chapter II.

### Immunofluorescence Staining in Fixed Cells

The following primary antibodies were used in immunofluorescence staining; ER$\alpha$ (1:50, sc-8002 Santa Cruz), LAMP1 (D2D11) (1:500, 9091S, Cell Signaling), LAMP1 (1:200, ab21428, Abcam), Rab7 (1:500, ab137029, Abcam), MAP2 (1:500, ab32454, Abcam), MAP2 (1:500 ab92434, Abcam). Alexa Fluor 594 goat anti-rabbit, 488 goat anti-rabbit, 594 goat anti-mouse, 488 goat anti-

mouse secondary antibodies were purchased from Thermo Fisher. All secondary antibodies were used at 1:250 dilutions. Described in detail in Chapter II.

**Live Imaging of Dendritic Spines**

BacMam GFP Transduction Control (B10383, Thermo Fisher) was added to mouse primary hippocampal neurons (div 12-18) for 48 hours. Recombinant HIV-1 IIIB gp120 protein (Baculovirus expression) was obtained from ImmunoDx (1001, ImmunoDx LLC, Woburn, MA). Recombinant Tat HIV-1 IIIB (E. coli) was obtained from ImmunoDx (1002, ImmunoDx, Woburn, MA). The gp120 and Tat was heat-inactivated by heating at 95°C for 15 min. Treatments used included; vehicle-PBS, control-heat inactivated HIV-1 gp120, control-heat inactivated HIV-1 Tat, HIV-1 gp120 (0.5 nM), HIV-1 Tat (100 nM), and 17$\alpha$E2 (10 nM). Described in detail in Chapter II.

**Live-Imaging of Endolysosome Morphology**

Primary hippocampal neurons were stained with LysoTracker Red DND-99 (50 nM, Invitrogen) for 30 mins at 37°C to visualize endolysosomes. Live cell images were taken using a Zeiss LSM 800 confocal microscope, and Z-stacks were taken at 0.5 $\mu$m of five random fields.

**Active Cathepsin Staining**

Active Cathepsin D in both CLU99 cells and primary neurons were identified using BODIPY-FL Pepstatin A (P12271, Thermo Fisher). BODIPY-FL was added

at 1 μM for 30 min, along with LysoTracker Red DND-99 at 10 nM at 37°C. Described in detail in Chapter II.

**Nuclear-Activation of ERα**

TransAM ER assay (Active Motif, Carlsbad, CA) was used to determine the nuclear activation of ERα follow manufacturer's instructions. CLU199 cells (3-5 million) were treated for 30 mins, washed with cold PBS twice, transferred in HB buffer and incubated for 20 mins. 0.5% NP-40 (Sigma) was added to the HB buffer and the cells were vortexed and centrifuged for 10 min at 300 g. This allowed for the separation of the cytoplasmic (supernatant) and nuclear (pellet) fractions. The pellet was dissolved in 50 μL lysis buffer and centrifuged for 15 minutes at 14,000 g to obtain the nuclear extract. Precision Red Advanced Protein Assay (ADV02, Cytoskeleton Inc., Denver, CO) was used to estimate the protein. The TransAM-ER ELISA was measured using the Spectra Max Plate Reader (Molecular Devices, CA, USA) measuring the absorbance at 450 nm. The experiment was conducted from three wells per condition, per replicate.

**Statistical Analyses**

All data was expressed as means and SEM. Statistical analyses was performed using GraphPad Prism 9.0 software (GraphPad Software, Inc.). Statistical significance was calculated by either Student's *t*-test between two groups, one-way analysis of variance or two-way analysis of variance between groups among

multiple groups with Tukey *post-hoc* tests. A p value < 0.05 was considered to be significant.

## Results

### ERα is Present on Endolysosomes in Neuronal Cells

ERα is expressed in human hippocampus on neurons (Heron et al., 2009) and on endolysosomes (Levin, 2009; Totta et al., 2014). Using double staining methods, we determined the endolysosome localization of ERα. ERα was co-immunostained with either Rab7 (marker for late endosomes) or LAMP1 (marker for lysosomes) antibodies using CLU199 cells. ERα and Rab7 colocalized at a higher percent than ERα and LAMP1 (Figure 16A). ERα was co-immunostained with Rab7 in mouse hippocampal neurons (DIV 9). We observed the co-localization of Rab7 and ERα in dendrites (Figure 16B).

### 17αE2 Enhances Endolysosome Function and Protects Against HIV-1 gp120- and Tat-Induced Endolysosome Dysfunction

Given that ERα is present on endolysosomes, activating ERα with 17αE2 could affect endolysosome function. Thus, we wanted to investigate if 17αE2 could prevent the endolysosome impairment induced by HIV-1 proteins. We measured the pH with a pH-sensitive and pH-insensitive dextran and found that 17αE2 alone could acidify endolysosomes. When 17αE2 (10 nM) is used as a pretreatment for 10 mins followed by treatment with gp120 (0.5 nM, 30 mins), we discovered that 17αE2 was able to prevent the de-acidification induced by gp120

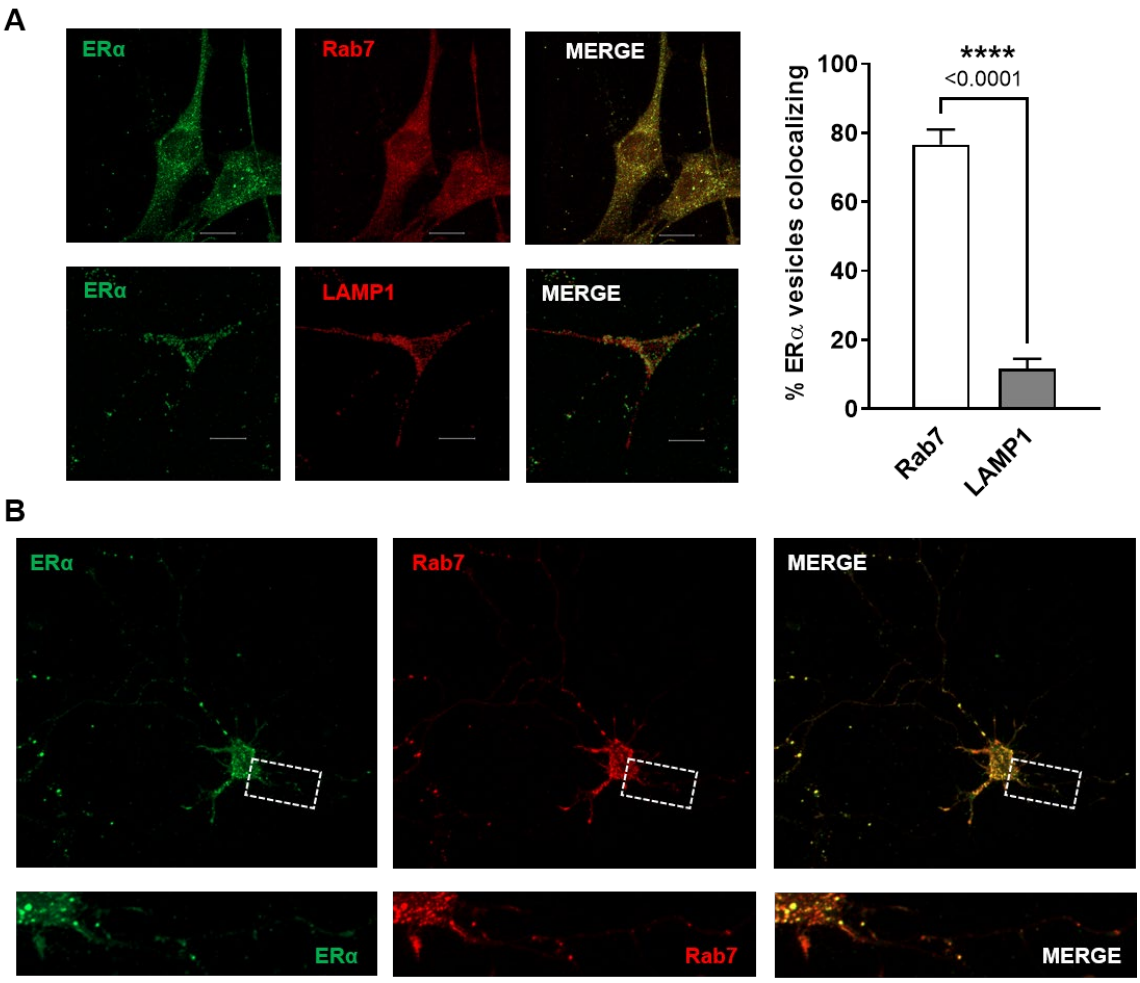



Figure 16. Localization of ERα on endolysosomes in neurons.
**(A)** Representative confocal images show the co-distribution of ERα (green) with Rab7 (red) or LAMP1 (red) in CLU199 cells. Scale bar-10 µm. Bar graph shows that ERα colocalized with Rab7 to a higher extent than with LAMP1. Data were presented as mean ± SEM (n= 3 replicates, ****p<0.0001, Student's t-test, two-tailed). **(B)** Representative images show the colocalization of ERα (green) and Rab7 (red)-positive endolysosomes in mouse hippocampal neurons (DIV 9). Scale bar-10 µm.

(Figure 17A). We also wanted to determine the effect 17αE2 has on lysosome hydrolase activity and found that 17αE2 alone significantly increased the percentage of Cathepsin D positive endolysosomes (Figure 17A). Pretreatment 17αE2 (10 nM) was able to protect against gp120-induced decrease of the percentage of CatD, therefore protecting the function of endolysosomes (Figure 17B). We repeated these experiments with HIV-1 Tat and found that when 17αE2 (10 nM) is used as a pretreatment followed by Tat (100 nM), 17αE2 was able to prevent the de-acidification that occurred with Tat (Figure 18A). Pretreatment with 17αE2 followed by Tat significantly increased the percentage of Cathepsin D positive endolysosomes, therefore, protecting the function of endolysosomes (Figure 18B). Our findings suggest that 17αE2 can prevent both gp120 and Tat from impairing the endolysosome pH and function.

**Effects of 17αE2 are Not Through Classical Genomic Pathway**

Estradiol is known to have two different mechanisms of action on neurons. The first is the classical genomic pathway which can take anywhere from hours to days, and the second is called the rapid membrane-associated pathway, which takes minutes to hours. These pathways both promote the formation of dendritic spines in the hippocampus and cortex (Hasegawa et al., 2015; C. Li et al., 2004; Mendez, Garcia-Segura, & Muller, 2011). We first confirmed that 10 nM 17αE2 for 30 minutes did not induce nuclear activation of ERα. CLU199 mouse hippocampal cell line was quantified with an ELISA (TransAM-ER). There was no significant nuclear ER activation with 17αE2 (10 nM) for 30 minutes (Figure 19B).

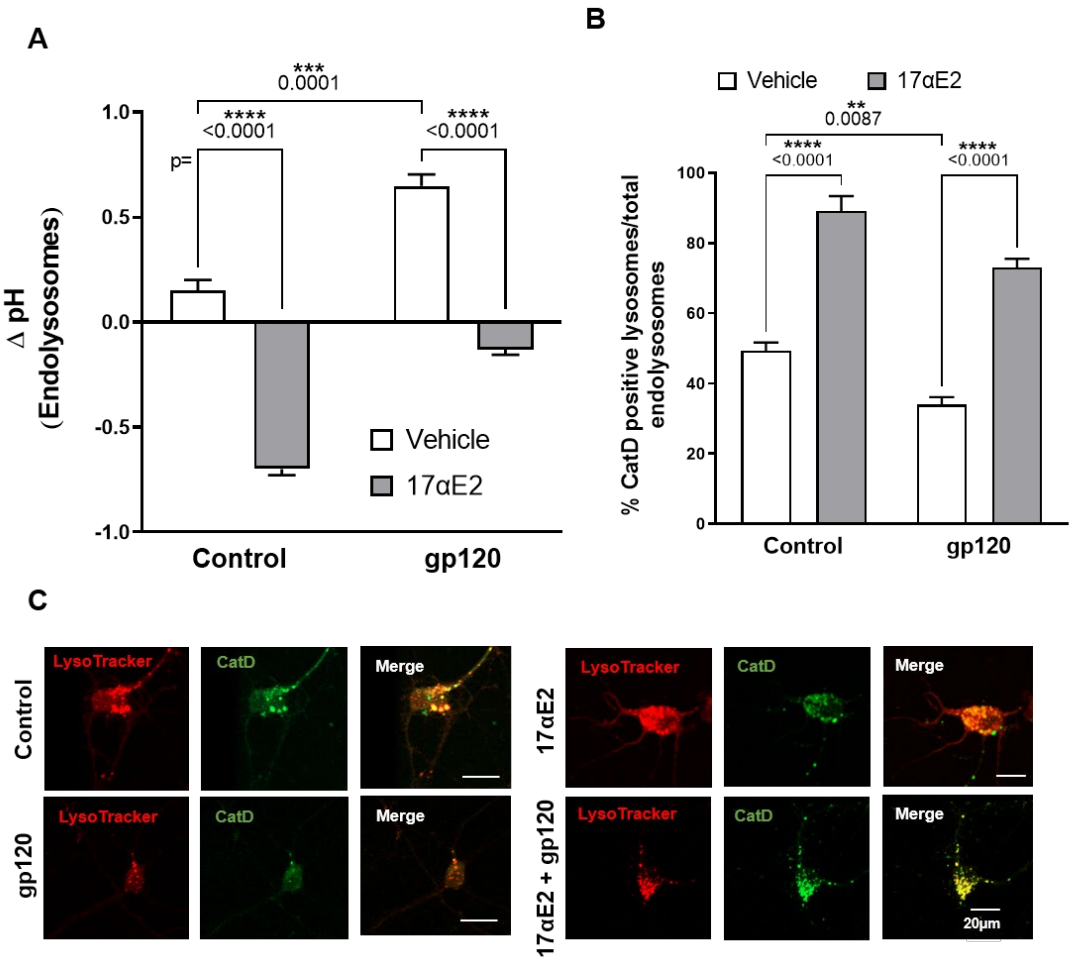



Figure 17. 17αE2 prevents HIV-1 gp120-induced endolysosome dysfunction. **(A)** Endolysosomal pH was measurement in primary hippocampal neurons using a ratio-metric method with a combination of pH-sensitive (pH rodo) and pH-insensitive (Texas Red) dextran. HIV-1 gp120 (0.5 nM, 10 min) de-acidified endolysosomes, while 17αE2 acidified endolysosomes. Pre-treatment with 17αE2 (10 nM, 10 min) prevented HIV-1 gp120-induced endolysosome de-acidification. Data were presented as mean ± SEM (n=3 replicates, ****p<0.0001, two-way ANOVA with Tukey's post-hoc comparison). **(B)** Bar graphs show changes in the percentage of active endolysosomes (active CatD-positive) to total endolysosomes. Pre-treatment with 17αE2 (10 nM, 10 min) increased the active CatD percentage in control neurons and prevented HIV-1 gp120-induced decreases in the active CatD percentage. Data were presented as mean ± SEM (n=3 replicates, **p<0.01, ****p<0.0001, two-way ANOVA). **(C)** Representative confocal images of hippocampal neurons (DIV9) show total endolysosomes as identified with LysoTracker Red (red) and active endolysosomes as identified with active CatD (green). Cathepsin D (CatD) was stained using BODIPY FL-Pepstatin A in hippocampal neurons for live imaging.

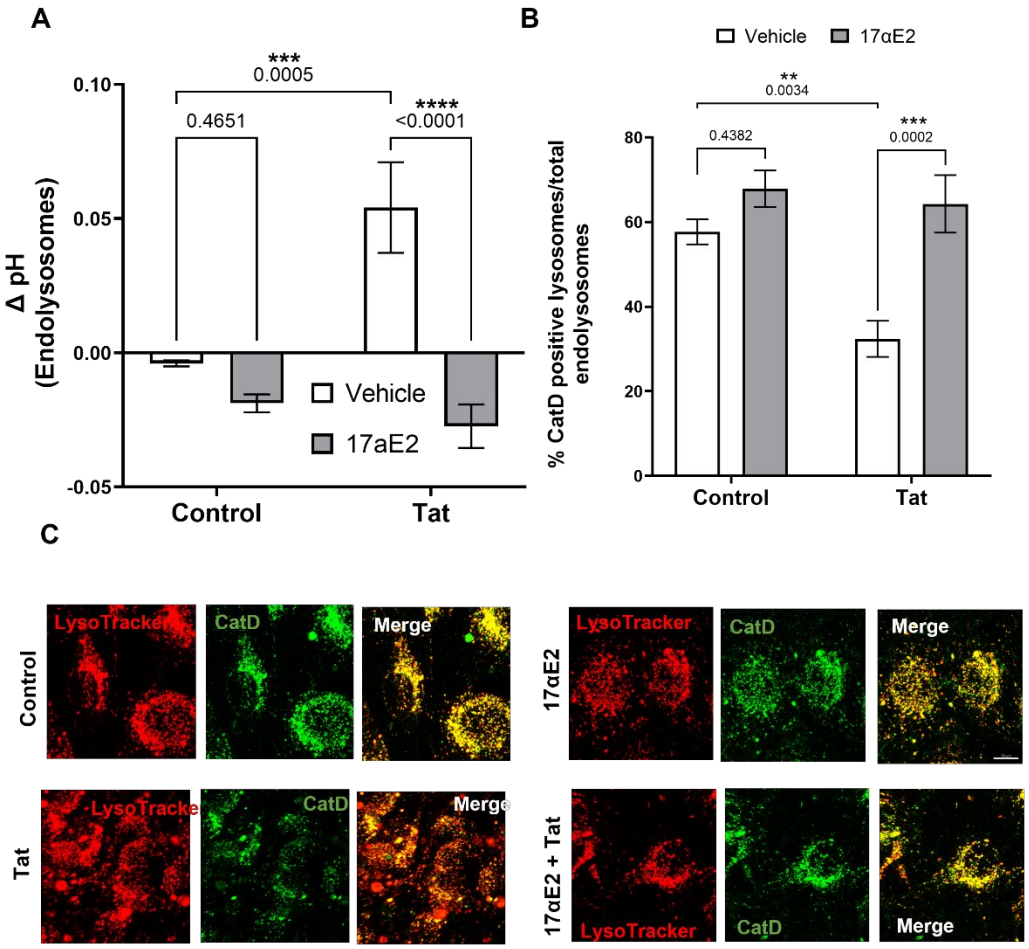



Figure 18. 17αE2 prevents HIV-1 Tat-induced endolysosome dysfunction. **(A)**. Endolysosomal pH was measured in primary hippocampal neurons with a ratio-metric dye LysoSensor Yellow/Blue DND-160. HIV-1 Tat (100 nM, 10 min) de-acidifed endolysosomes, while 17αE2 (10 nM, 10 min) acidified the pH. Pre-treatment with 17αE2 (10 nM, 10 min) prevented HIV-1 Tat induced endolysosome de-acidification. Data were presented as mean ± SEM (n= 2 replicates, **p<0.01, ****p<0.0001, two-way ANOVA with Tukey's post-hoc comparison). **(B)** Bar graphs show changes in the percentage of active endolysosomes (active CatD-positive) to total endolysosomes. Pre-treatment with 17αE2 (10 nM, 10 min) increased the active CatD percentage in control neurons and prevented HIV-1 Tat-induced decreases in the active CatD percentage. Data were presented as mean ± SEM (n=2 replicates, **p<0.01, ***p<0.001 ****p<0.0001, two-way ANOVA with Tukey's post-hoc comparison). **(C)** Representative confocal images of hippocampal neurons (DIV9) show total endolysosomes as identified with LysoTracker Red (red) and active endolysosomes as identified with active CatD (green). Cathepsin D (CatD) was stained using BODIPY FL-Pepstatin A in hippocampal neurons for live imaging.

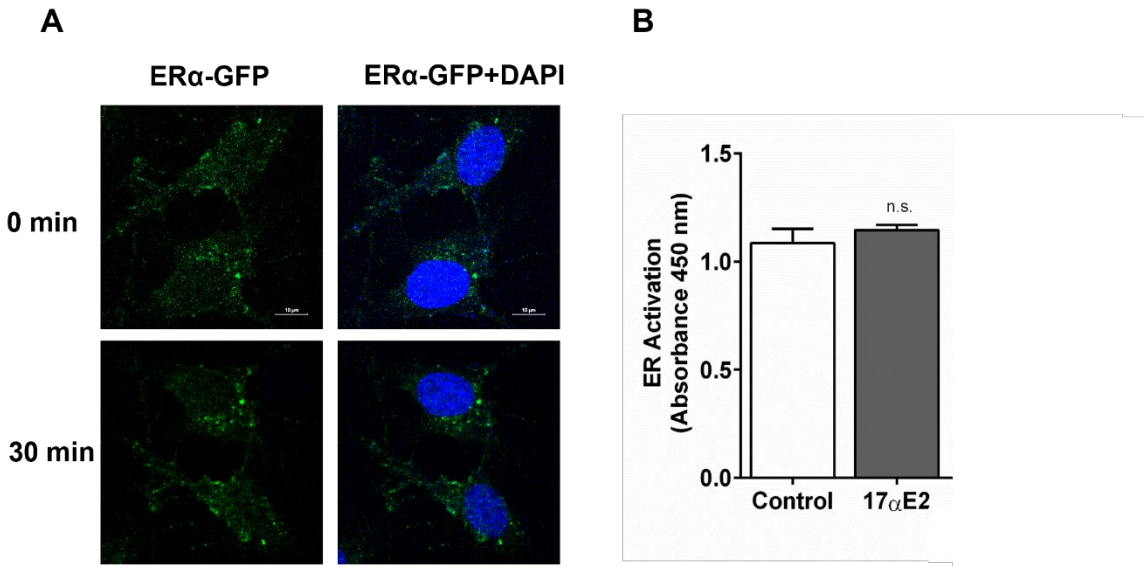



Figure 19. 17αE2 does not induce nuclear activation of ER over 30 min treatment. **(A)** Representative confocal images show the distribution of transduced ERα-GFP in mouse hippocampal cell line CLU199. The neurons were treated with 17αE2 (10 nM for 30 min) and co-stained with DAPI for nucleus. 17αE2 did not induce nuclear translocation of ERα. **(B)** Bar graph shows quantitative Estrogen Receptor Transcription Factor ELISA (TransAM-ER). There was no significant nuclear ER activation with 17αE2 (10 nM for 30 min). Data were presented as mean ± SEM (n=3 replicates, Student's t-test, two-tailed).

### 17αE2 Prevented HIV-1 Tat-Induced Endolysosome Enlargement

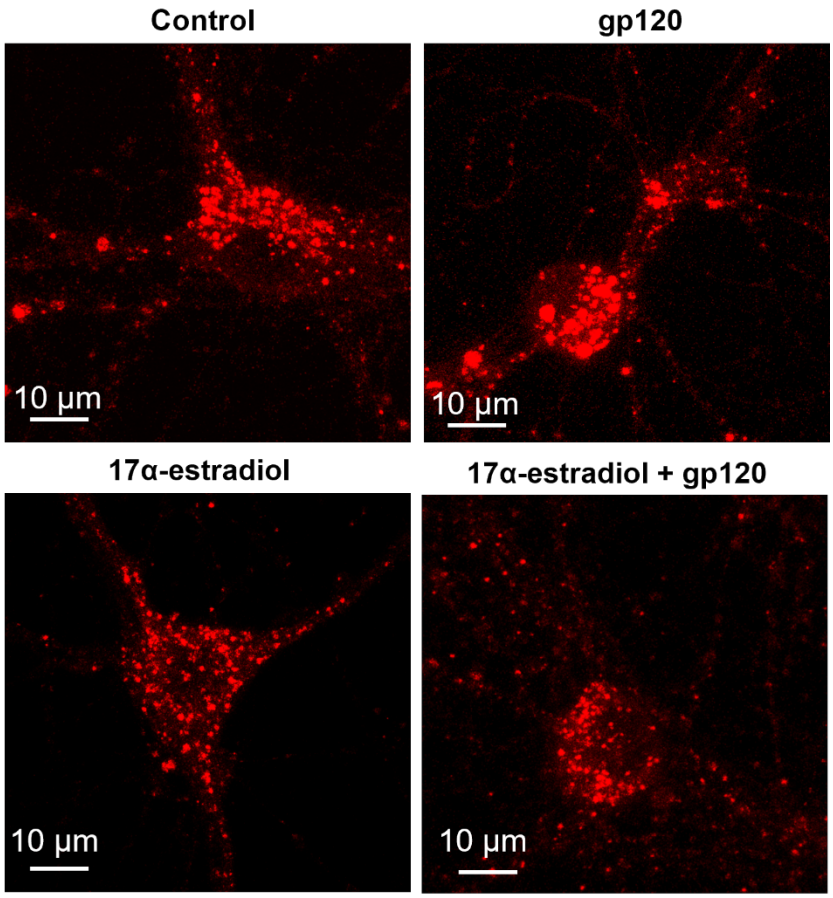
We confirmed that HIV-1 viral proteins gp120 and Tat increased the size of endolysosomes. We also determined if pretreatment with 17αE2 was able to protect against the HIV protein induced increases in size. Pretreatment with 17αE2 was able to unable to prevent the increase in size in gp120 treated cells (Figure 20B) but was able to prevent the size increase in Tat treated cells (Figure 21B). These findings suggest that 17αE2 is protective against Tat-induced changes in endolysosome sizes.

### 17αE2 Increases Dendritic Spines and Protects Against HIV-1 gp120- and Tat-Induced Reduction in Dendritic Spines

17αE2 is known to have neuroprotective effects (McClean & Nunez, 2008) and protects against gp120-induced neuronal cell death (Howard et al., 2001), and increases the formation of new spines in hippocampus (Sheppard, Choleris, & Galea, 2019). Before we investigated how the spine density was impacted with pretreatment of 17αE2 at different concentrations of 17αE2. 17αE2 slightly increased spine density at 10 nM but not 1 nM (Figure 22B). Primary hippocampal neurons (DIV 14-19) were stained for actin to visualize the dendritic spines. Pretreatment with 17αE2 (10 nM, 5 hours) significantly prevented the decrease in spine density due to Tat (100 nM) (Figure 23B).

Next, we determined the effects of 17αE2 dynamic changes of dendritic spines. Primary hippocampal neurons (DIV 9-14) were transduced with EGFP, and live

Figure 20. 17αE2 was unable to prevent HIV-1 gp120-induced enlargement of endolysosomes.
**(A)** Representative confocal images of rat hippocampal neurons (DIV14-19) show distribution and morphology of endolysosomes as identified with LysoTracker Red DND-99 (red) following treatment with HIV-1 gp120 for 48 hours in the absence and presence of 17αE2 (10 nM, pretreated for 5 hr). **(B)** Bar graph shows that HIV-1 gp120 increased the size of endolysosomes per neuron. Pretreatment of 17αE2 was unable to prevent HIV-1 gp120-induced enlargement of endolysosomes. Data were presented as mean ± SEM (n= 2 replicates, **p<0.01, ****p<0.0001, two-way ANOVA with Tukey's post-hoc comparison).


Control
gp120
10 µm
10 µm
17α-estradiol
17α-estradiol + gp120
10 µm
10 µm

**B**

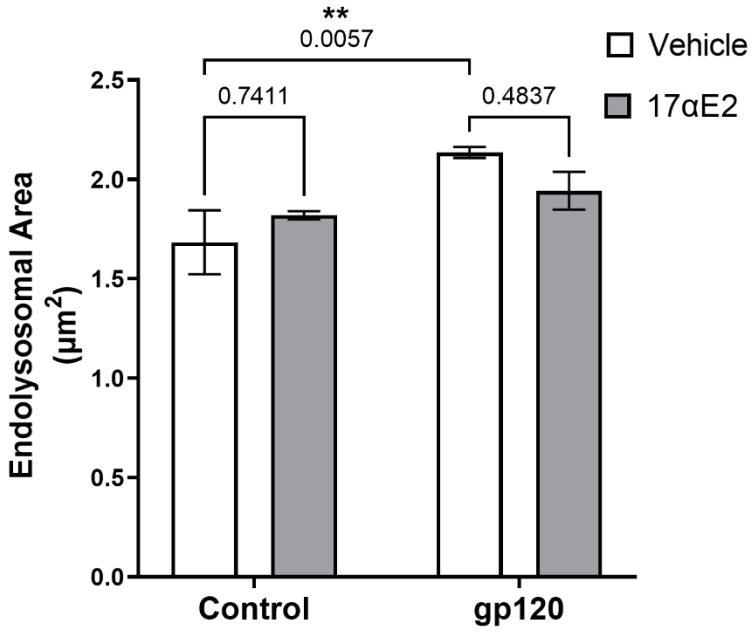
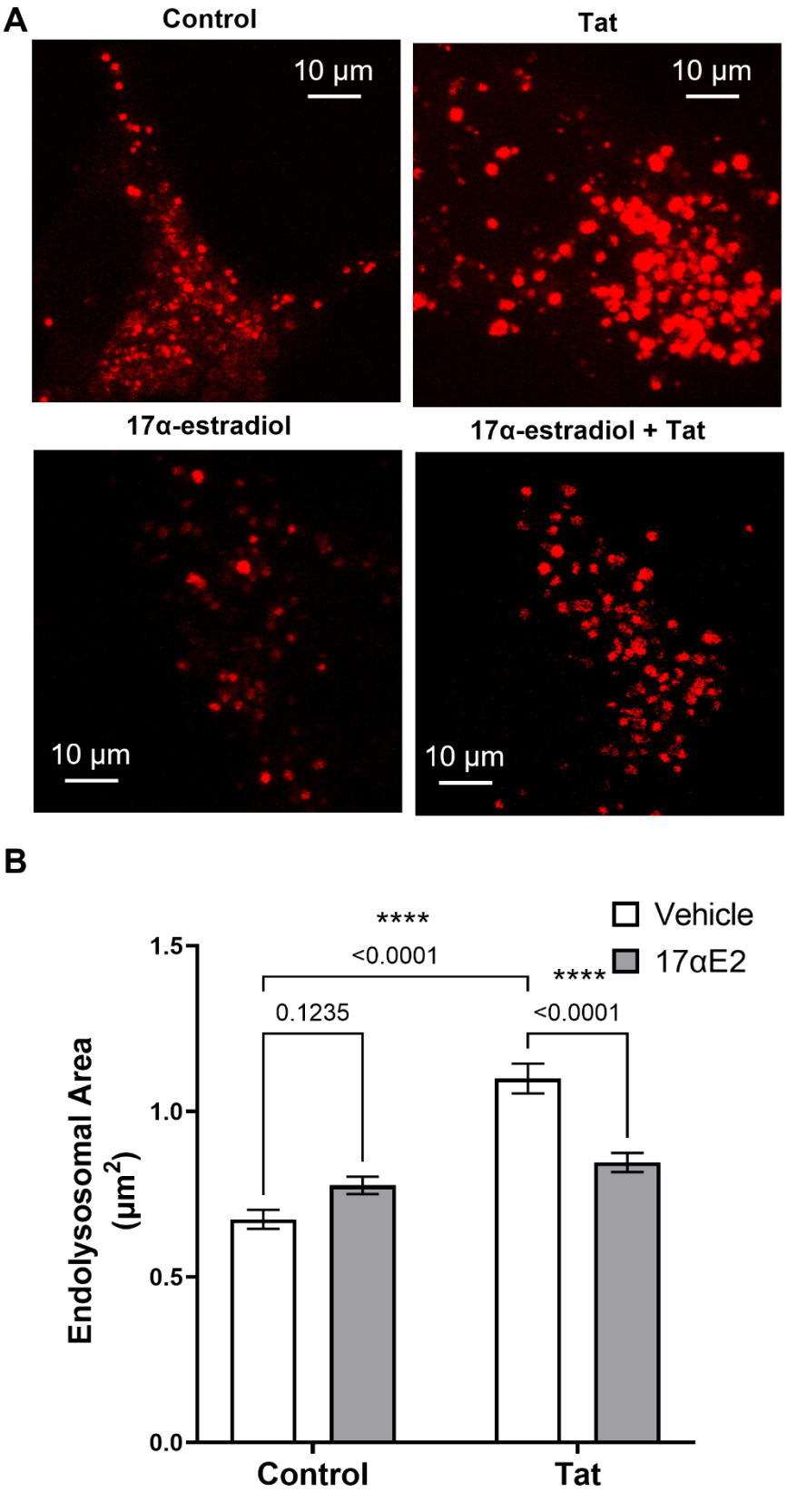

**
0.0057
0.7411
0.4837
Vehicle
17αE2
Endolysosomal Area (µm²)
2.5
2.0
1.5
1.0
0.5
0.0
Control
gp120

Figure 21. 17αE2 prevented HIV-1 Tat-induced enlargement of endolysosomes. **(A)** Representative confocal images of rat hippocampal neurons (DIV14-19) show distribution and morphology of endolysosomes as identified with LysoTracker Red DND-99 (red) following treatment with HIV-1 Tat for 48 hours in the absence and presence of 17αE2 (10 nM, pretreated for 5 hr). **(B)** Bar graph shows that HIV-1 Tat increased the size of endolysosomes per neuron. Pretreatment of 17αE2 prevented HIV-1 Tat-induced enlargement of endolysosomes. Data were presented as mean ± SEM (n=2 replicates, *****p<0.0001, two-way ANOVA with Tukey's post-hoc comparison).


A
Control
Tat
10 μm
10 μm
17α-estradiol
17α-estradiol + Tat
10 μm
10 μm
B
Vehicle
17αE2
****
<0.0001
****
0.1235
<0.0001
Endolysosomal Area (μm²)
1.5
1.0
0.5
0.0
Control
Tat

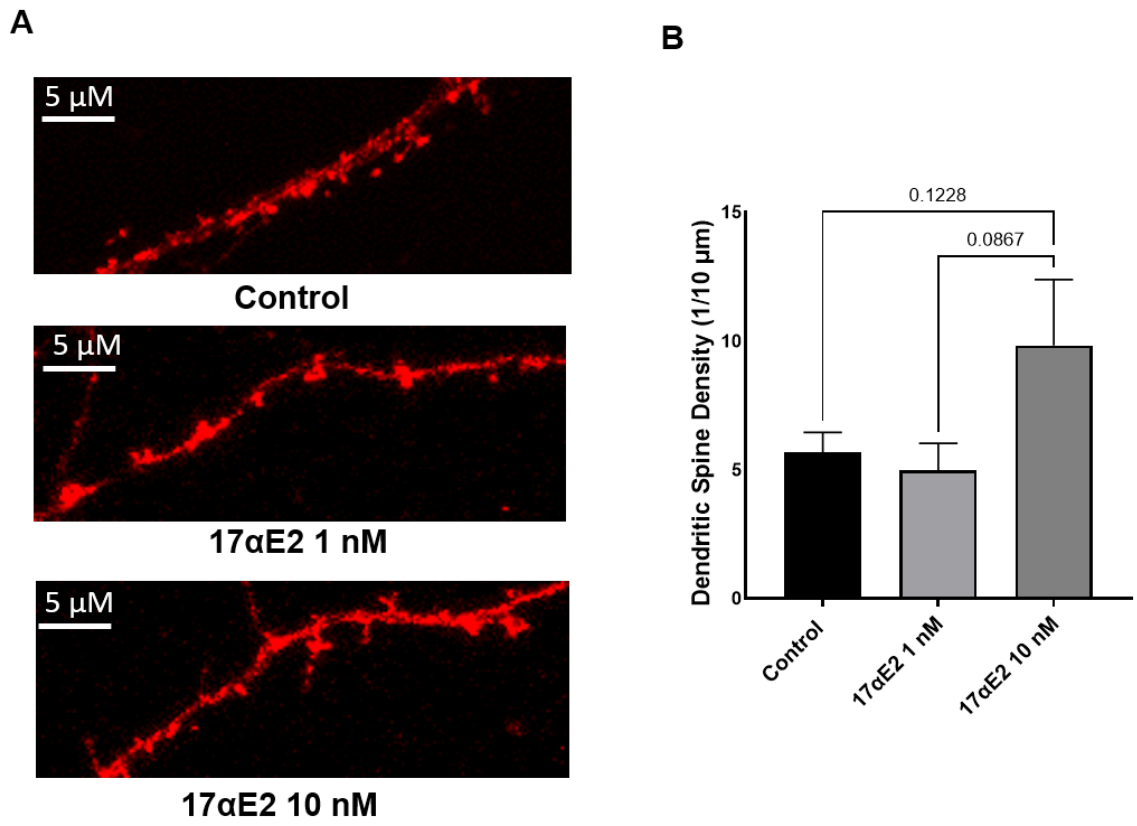



Figure 22. Concentration-dependent effects of 17αE2 on dendritic spines. **(A)** Representative confocal images of actin staining with phalloidin in primary mouse hippocampal neurons (DIV14-19) treated with 17αE2 (1-10 nM for 48 hrs). Scale bar-5 µm. **(B)** Bar graph shows that 17αE2 slightly increased dendritic spine density at a concentration of 10 nM. Data were presented as mean ± SEM (n=7-10 dendrites imaged per condition, *p<0.05, one-way ANOVA with Tukey's post-hoc comparison).

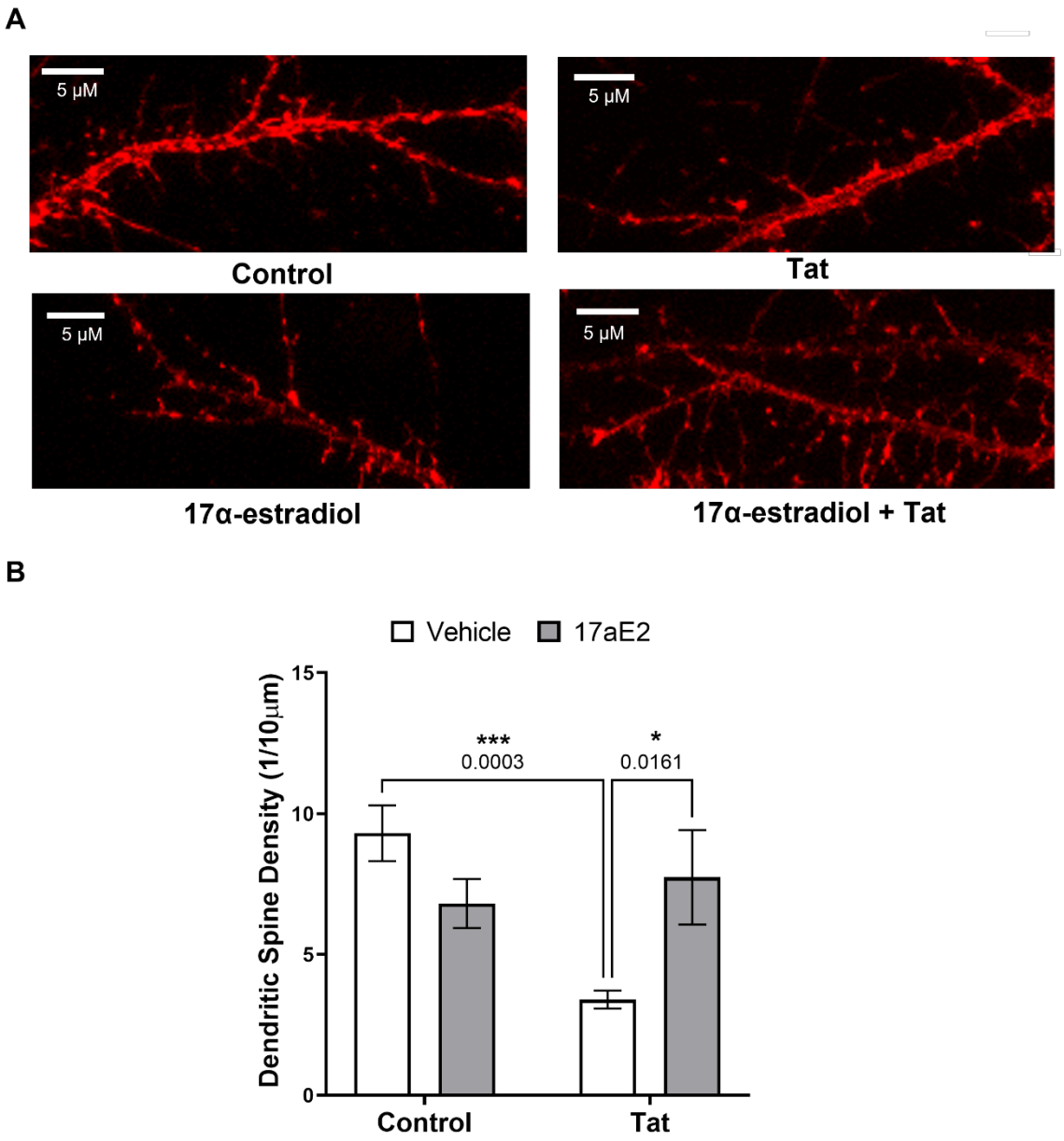



Figure 23. 17αE2 prevented HIV-1 Tat-induced reduction in dendritic spine density.
**(A)** Representative confocal images of actin staining with phalloidin in primary mouse hippocampal neurons (DIV14-19) treated HIV-1 Tat (100 nM for 48 hr) in the absence and presence of 17αE2 (10 nM, 6 hr pretreatment). Scale bar-5 μm. **(B)** Bar graph shows that HIV-1 Tat reduced dendritic spine density. Pretreatment of 17αE2 prevented HIV-1 Tat-induced reduction in dendritic spine density. Data were presented as mean ± SEM (n=2 replicates, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA with Tukey's post-hoc comparison).

images were taken of the same dendrite at 0 mins and then again after 10 mins of treatment. 17αE2 (10 nM) increased the formation of stubby and mushroom spines but decreased the production of long thin spines (Figure 24B). Pretreatment with 17αE2 (10 nM, 10 mins) followed by gp120 led to a protective effect in increasing the formation of stubby and mushroom spines and decreased long thin spines (Figure 25B). Next, we wanted to investigate if pretreatment with 17αE2 could also prevent the adverse effects of Tat. Pretreatment of 17αE2 (10 nM) significantly increased the amount of stubby, mushroom, and long thin spines (Figure 26B).

## Discussion

When HIV-1 gp120 and Tat are endocytosed (Fittipaldi et al., 2003; Richard et al., 2005; Vendeville et al., 2004; Wenzel et al., 2017), then induce endolysosome dysfunction as evidence in Chapter II by an increased pH, decreased percentage of CatD, and increased size of the endolysosome. Many studies have shown 17βE2 plays an important role in synaptic plasticity (Ooishi et al., 2012) and neuroprotection (Vierk, Brandt, & Rune, 2014). 17αE2 has similar effects as 17βE2 and 17αE2 has non-feminizing effects (Moos, Dykens, Nohynek, Rubinchik, & Howell, 2009; Michael B. Stout et al., 2016), thus 17αE2 was focused on in our studies. 17αE2 is present in adult mouse brains even after the removal of estrogen-producing organs, while 17βE2 is not detected (C. Dominique Toran-Allerand, Alexander A. Tinnikov, Ravinder J. Singh, & Imam S. Nethrapalli, 2005).

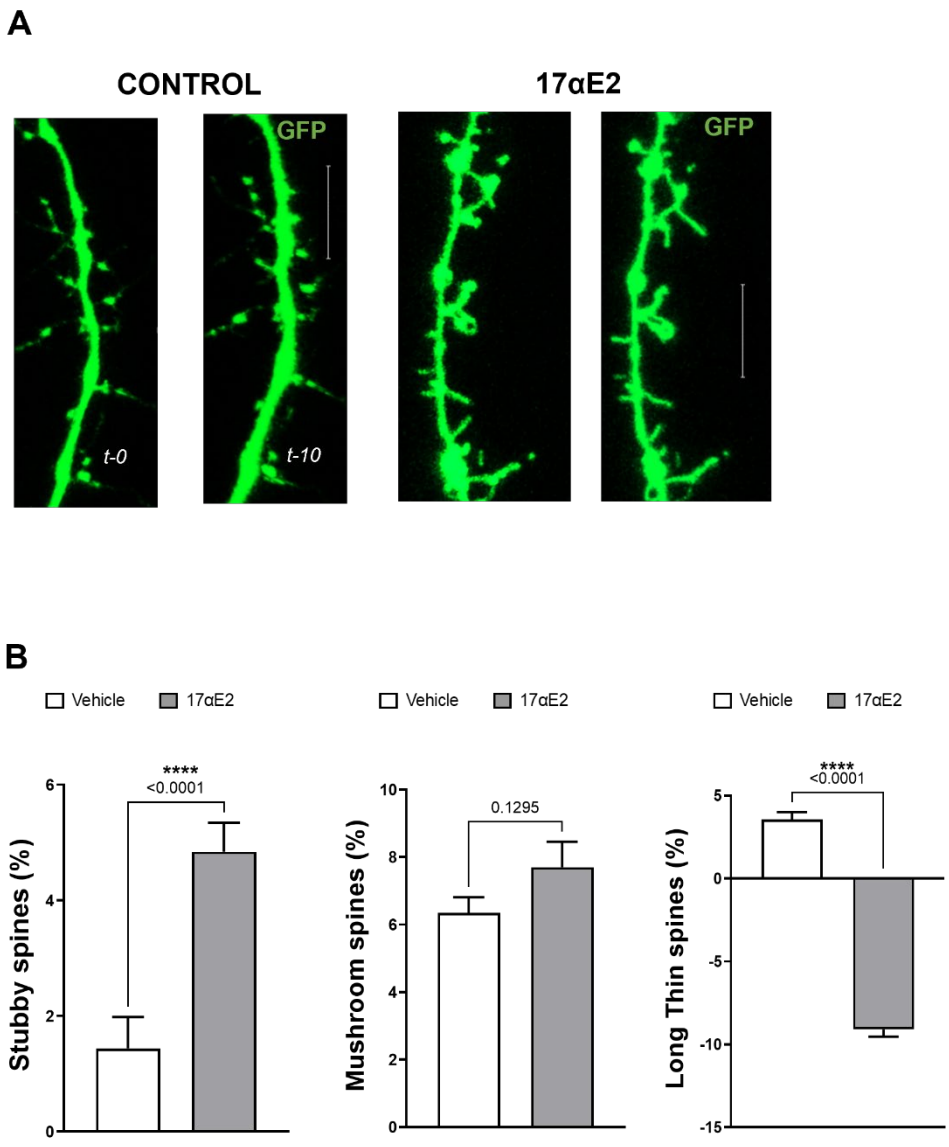



Figure 24. 17αE2 enhanced the formation of mature spines.
**(A)** Representative confocal images show the rapid change in the spine morphology of mouse hippocampal neurons (DIV 9-14) transduced with EGFP on the same dendrite imaged at 0 and 10 min with different treatments. Scale bar- 5 μm. **(B)** Bar graphs show changes in dendritic spine turnover over 10 min with different treatments. Positive values indicate spine formation, while negative values indicate spine elimination between 0- and 10-min. Control was used in Figure 12.17αE2 (10 nM) enhanced the formation of stubby and mushroom spines but decreased the formation of long thin spines. Data were presented as mean ± SEM (n=3 replicates, ***p<0.001, ****p<0.0001, two-way ANOVA with Tukey's post-hoc test).

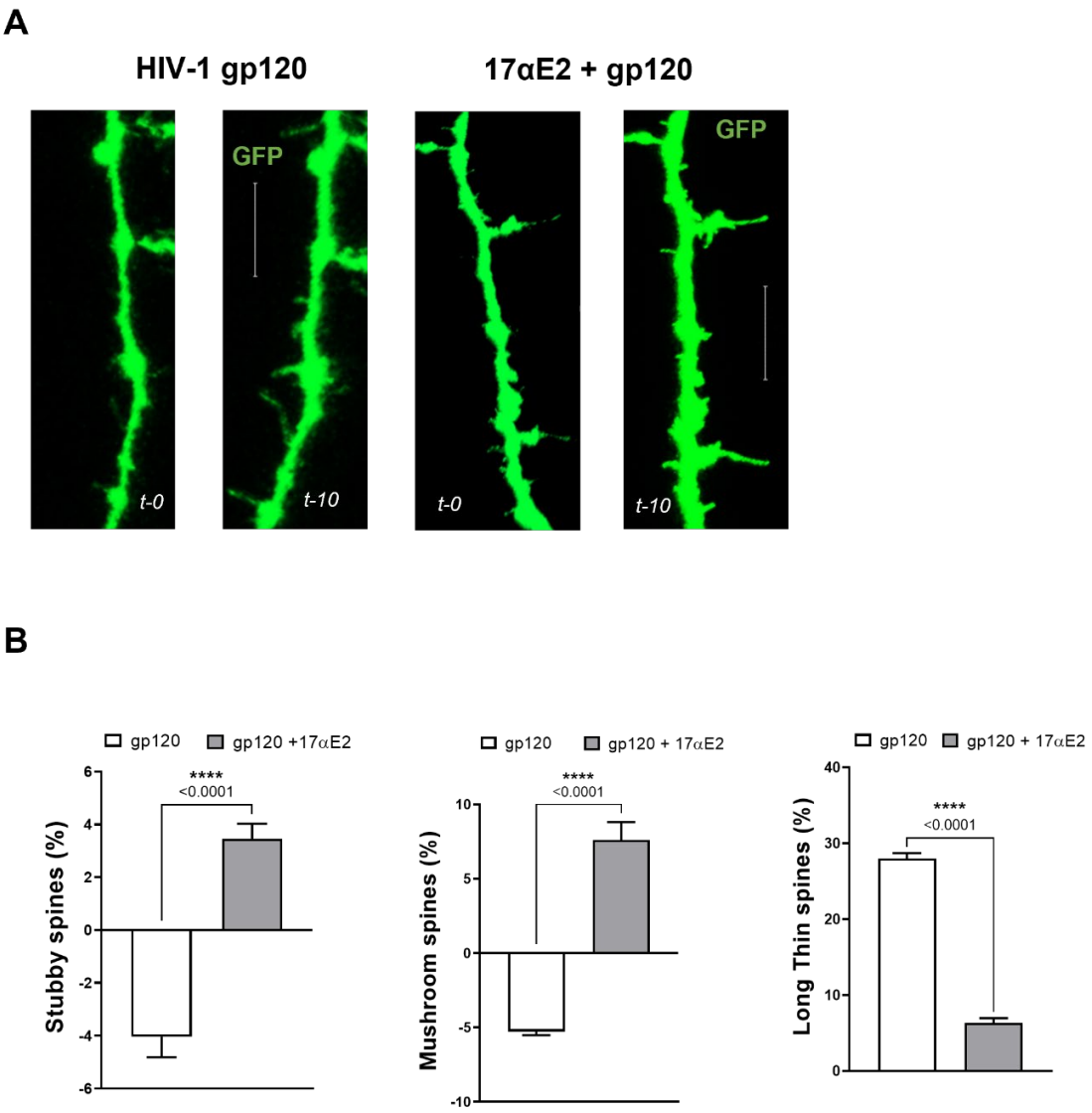



Figure 25. 17αE2 protected against HIV-1 gp120 induced dendritic damage. **(A)** Representative confocal images show the rapid change in the spine morphology of mouse hippocampal neurons (DIV 9-14) transduced with EGFP on the same dendrite imaged at 0 and 10 min with different treatments. Scale bar- 5 µm. **(B)** Bar graphs show changes in dendritic spine turnover over 10 min with different treatments. Positive values indicate spine formation, while negative values indicate spine elimination between 0- and 10-min. Pre-treatment with 17αE2 (10 nM, 10 min) prevented HIV-1 gp120-induced reduction in stubby and mushroom spines, but not long thin spines. Data were presented as mean ± SEM (n=3 replicates, *p<0.05, **p<0.01, ***p<0.001, Student's t-test, two-tailed).

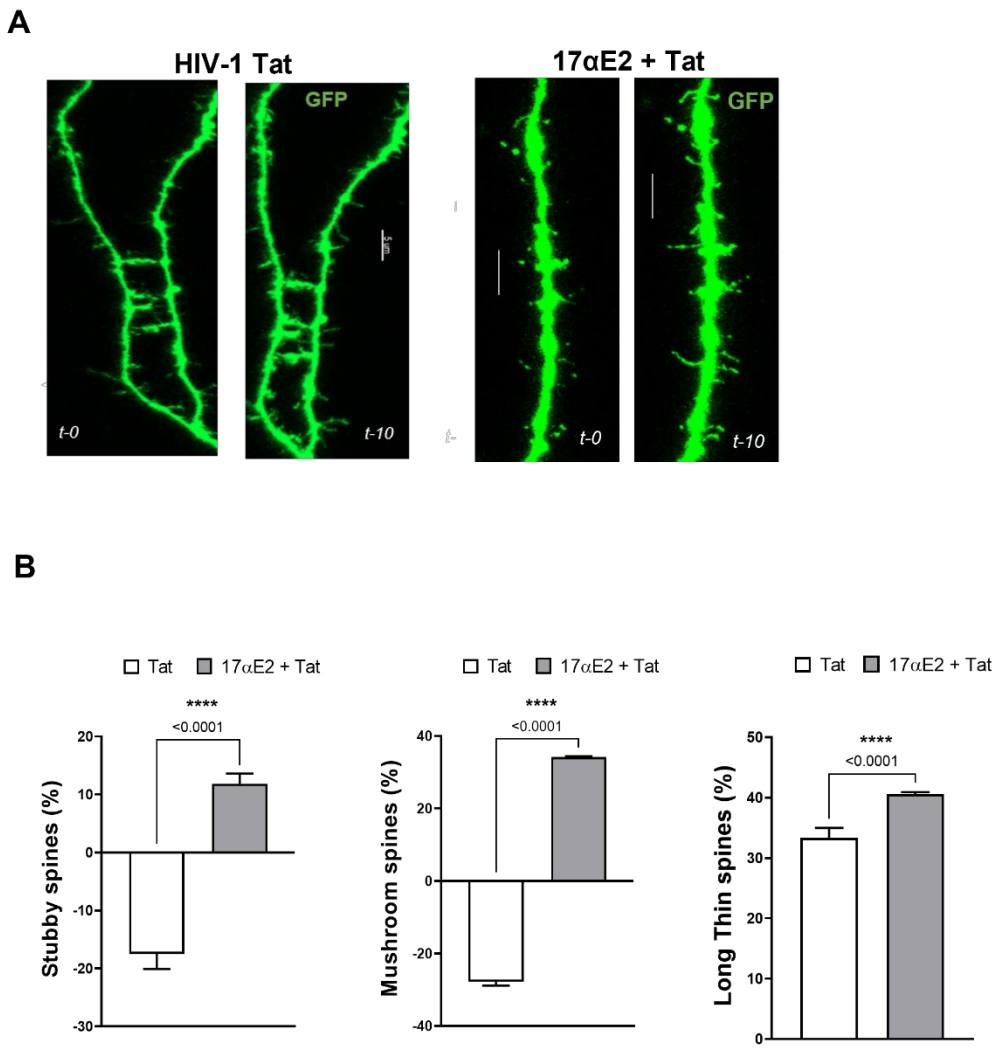



Figure 26. 17αE2 protected against HIV-1 Tat induced dendritic damage. **(A)** Representative confocal images show the rapid change in the spine morphology of mouse hippocampal neurons (DIV 9-14) transduced with EGFP on the same dendrite imaged at 0 and 10 min with different treatments. Scale bar- 5 µm. **(B)** Bar graphs show changes in dendritic spine turnover over 10 min with different treatments. Positive values indicate spine formation, while negative values indicate spine elimination between 0- and 10-min. Pre-treatment with 17αE2 (10 nM, 10 min) prevented HIV-1 Tat-induced reduction in stubby, mushroom, and long thin spines. Data were presented as mean ± SEM (n=2 repeats, *p<0.05, **p<0.01, ***p<0.001, Student's t-test, two-tailed).

ERα is present on the membrane of the endolysosome and is a receptor for 17αE2. We confirmed these data by double immunostaining ERα with either late endosomes or lysosomes. While ERα is present to a greater extent on Rab7 positive endolysosomes, they are also present on LAMP1 positive lysosomes. However, Rab7 and LAMP1 markers can overlap; hence ERα is located on endolysosomes. Activation of endolysosome localized ERα could directly affect endolysosome function. Indeed, we demonstrated that 17αE2 induces acidification of endolysosomes.

Active endolysosomes are essential for neuronal homeostasis and vesicle membrane trafficking along the dendrites to maintain the plasma membrane domains because neurons are polarized cells with extensive processes. When endolysosome dysfunction occurs, resulting from the internalization of gp120 or Tat, this may lead to abnormal neurite morphology and altered dendritic spines. When 17αE2 was used as a pretreatment, before treatment with either gp120 or Tat, it prevented the de-acidification of the endolysosome. 17αE2 prevented the effects of gp120 and Tat, which may be the first step in repairing the endolysosomal system. 17αE2 alone induced acidification of the endolysosomes, although the mechanism has yet to be discovered. 17αE2 restored the CatD percentage in the endolysosomes when used as a pretreatment, and alone it increased the percentage of CatD endolysosomes. Cathepsin activity is dependent upon pH (Claus et al., 1998). Therefore, when 17αE2 restores the pH, the percentage of cathepsin D positive endolysosomes is restored. 17αE2

restored the size of the endolysosomes when treated with gp120 or Tat, which may be indicative of preventing the accumulation of undigested cargo (Myers et al., 1991; Ohkuma & Poole, 1981; Yoshimori et al., 1991). 17αE2-induced enhancement of endolysosome function may facilitate the degradation of gp120 and Tat and may prevent Tat from escaping endolysosomes (Khan et al., 2020).17αE2 alone is an enhancing therapeutic agent on the endolysosome system. As a pretreatment to HIV-1 viral proteins, 17αE2 prevented the decrease in the percent of CatD endolysosomes, the increase in size, and the increased pH demonstrating a potential therapeutic drug that targets endolysosomes.

Neurons are post-mitotic cells that possess extensive processes that need constant upkeep and trafficking along the axonal and dendritic membranes. Lysosomes are found in dendritic spines and recycle and maintain plasma membranes of neurons, thereby regulating synaptic plasticity (Cheng et al., 2018; Marisa S. Goo et al., 2017; Padamsey et al., 2017; Yap et al., 2018). 17βE2 at 10 nM increases dendritic spine density (Srivastava & Penzes, 2011), and we found that pretreatment of 17αE2 (10 nM) prevented the Tat-induced decreases in dendritic spine density.

Dendritic morphology is altered in neurodegenerative diseases (Boros et al., 2019) and wanted to determine if 17αE2 could protect against these impairments. Morphologic changes in the spine types are associated with cognitive decline and memory impairment. 17αE2 alone increased the number of mature spines (stubby and mushroom) and decreased the amount of long thin

spines confirming its protective role in neurons. Pretreatment of 17αE2 was able to prevent the decrease in mature spines following gp120 and Tat treatment. However, 17αE2 prevented the increase in long thin spines with gp120 treatment, but long thin spines were increased with Tat treatment. The underlying mechanisms of 17αE2 need to be elucidated.

These results suggest that 17αE2 exerts neuroprotective effects, enhancing endolysosome function and increasing dendritic spines. Such protective effects may result from activation of ERα, which are localized to the membrane of endolysosomes and is present on neurons in the hippocampus (Totta et al., 2014; Warfvinge et al., 2020). Targeting the endolysosome system with 17αE2 protects against gp120 and Tat-induced endolysosome dysfunction and reduction in dendritic spines that occurs in HAND. Thus, 17αE2 represents a promising therapy for HAND.

# CHAPTER IV

## ENDOLYSOSOME LOCALIZATION OF ERα IS ESSENTIAL FOR THE PROTECTIVE EFFECTS OF 17α-ESTRADIOL (17αER) AGAINST TAT AND gp120-INDUCED NEURONAL INJURY

### Introduction

17αE2, is a natural non-feminizing estrogen, that we have shown protects against gp120 and Tat induced endolysosome dysfunction and synaptodendritic impairment which may contribute to the development of HAND. We know that 17αE2 interacts with estrogen receptors, particularly with ERα, which is expressed in the brain, (Warfvinge et al., 2020) hippocampal neurons (Heron et al., 2009) and located on endolysosomes (Levin, 2009; Totta et al., 2014). 17αE2 can interact with membrane bound ERα through cytosolic signaling involving second messengers and kinases with similar effects as the genomic pathway in modulating synaptic plasticity (Hasegawa et al., 2015; Mukai et al., 2007). ERα has been associated with enhancing cognition and synaptic function through estrogen (Frick, 2015; Hojo et al., 2008; Lai, Yu, Zhang, & Chen, 2017; Mukai et al., 2007; Sheppard et al., 2019; Srivastava et al., 2011). The endolysosomal pathway is important for transporting and degrading proteins in the dendritic spines, thus affecting synaptic plasticity (Marisa S. Goo et al., 2017; Nikoletopoulou & Tavernarakis, 2018; Padamsey et al., 2017; Yap et al., 2018). We have shown that ERα is present in endolysosomes and 17αE2 acidifies

endolysosomes, suggesting that endolysosome localization of ERα mediates the enhancing effect of 17αE2 on endolysosome function and dendritic spines.

Palmitoylation is a common lipid modification that can target proteins to the membrane. Palmitoylation involves a thioester bond between a cysteine thiol side chain and fatty acid. Many G-protein coupled receptors and synaptic vesicle proteins are palmitoylated. A study found that S-palmitoylation of the Cys447 was necessary for the ERα receptor to localize to the plasma membrane. Specifically, researchers mutated the Cys447 residue to Ala, which prevented the palmitoylation from localizing to the membrane. Cys447 seems to play a critical role in ER-induced non-genomic activities as ERα palmitoylation was needed for the activation of MAPK/ERK pathway. (Acconcia, Ascenzi, Fabozzi, Visca, & Marino, 2004). We hypothesize that 17αE2 protects against gp120 and Tat-induced endolysosome dysfunction and synaptodendritic injury via ERα present on endolysosomes.

## Methods

### Cell Culture

Mouse embryonic hippocampal E-18 cell line CLU199 (Cellutions Biosystems, Cedarlane, Ontario, Canada) were grown and maintained in 1x DMEM. Described in detail in Chapter II.

### Cultured Primary Rat Hippocampal Neurons

Primary mouse hippocampal neurons were cultured from embryonic day 18 rats. Described in detail in Chapter II.

### Cultured Primary Mouse Hippocampal Neurons

Primary mouse embryonic hippocampal neurons (C57EHP, Brainbits LLC, Springfield, IL) were obtained from E18 mouse cortex. Described in detail in Chapter II.

### Measurement of Endolysosome pH with LysoSensor

Endolysosome pH was measured using a ratio-metric lysosome pH indicator dye called LysoSensor (Yellow/Blue DND-160 from Invitrogen). Described in detail in Chapter II.

### Measurement of Endolysosome pH with Dual-Dextran Labeling

The pH of CLU199 cells were measured using a combination of dextran labelling as described (Nash el al., 2019). The treatments of HIV-1 gp120, HIV-1 Tat, HIV-1 heat-inactivated gp120, HIV-1 heat-inactivated Tat or 17$\alpha$E2 were added at mentioned concentrations. Described in detail in Chapter II.

### Immunofluorescence Staining in Fixed Cells

The following primary antibodies were used in immunofluorescence staining: ER$\alpha$ (1:50, sc-8002 Santa Cruz), LAMP1 (D2D11) (1:500, 9091S, Cell

Signaling), LAMP1 (1:200, ab21428, Abcam), Rab7 (1:500, ab137029, Abcam), MAP2 (1:500, ab32454, Abcam), MAP2 (1:500 ab92434, Abcam). Alexa Fluor 594 goat anti-rabbit, 488 goat anti-rabbit, 594 goat anti-mouse, 488 goat anti-mouse secondary antibodies were from Thermo Fisher. All secondary antibodies were used at 1:250 dilution. Described in detail in Chapter II.

**Live Imaging of Dendritic Spines**

Recombinant HIV-1 IIIB gp120 protein (Baculovirus expression) was obtained from ImmunoDx (1001, ImmunoDx LLC, Woburn, MA). Recombinant Tat HIV-1 IIIB (E. coli) was obtained from ImmunoDx (1002, ImmunoDx, Woburn, MA). The gp120 and Tat was heat-inactivated by heating at 95°C for 15 min. Described in detail in Chapter II.

**Active Cathepsin D Staining**

Active Cathepsin D in both CLU99 cells and primary neurons were identified using BODIPY-FL Pepstatin A (P12271, Thermo Fisher). BODIPY-FL was added at 1 μM for 30 min, along with LysoTracker Red DND-99 at 10 nM at 37°C. Described in detail in Chapter II.

**Plasmid Transfections, Transductions, and siRNA Knockdown**

The plasmids ERα C451A-GFP, ERα-GFP (MG227304), ERα-HA and ERα C451A-HA were ordered from Origene Technologies (Rockville, MD). The plasmids were cloned into pCMV6-AC-GFP, pCMV6-AC-RFP or pCMV6-AC-3HA

vectors. For the CLU199 cells, transient plasmid transfections were split and plated until the cells reached 50% confluency. The cells were then transfected with 2 µg of plasmid DNA and Lipofectamine 2000 transfection Reagent (11668019, ThermoFisher) in reduced serum media for 48-72 hours. The cells were then imaged. Primary mouse hippocampal neurons between div. 5-7 were transiently transfected with 1 µg of plasmid DNA and Lipofectamine 2000 for 48 hours. They were then imaged between div. 7-9.

On-Target plus mouse Esr1 (Entrez Gene ID-13982) siRNA-SMART pool to knockdown ERα was obtained from Dharmacon with the following target sequences: CCUACUACCUGGAGAACGA, GAAAGGCGGCAUACGGAAA, GUCCAGCAGUAACGAGAAA, and GGGCUAAAUCUUGGUAACA. All siRNA was dissolved in Accell1 transfection media (B-005000, Dharmacon) and DharmaFECT 1 (T-2001-02, Dharmacon). This was used for CLU199 cells and primary neurons with the final siRNA concentration was 50 nM. BacMam GFP Transduction Control (B10383, Thermo Fisher) was used to express GFP in primary neurons.

**Immunoblotting**

CLU199 cells or primary mouse hippocampal neurons were plated on Poly D-lysine coated 6-well plates at $1x10^6$/well or $0.5x10^6$/well. Cells were treated, harvested and lysed in RIPA lysis buffer (Thermo Fisher). The lysis buffer was supplemented with 10 mM NaF, 1 mM $Na_3VO_4$, and Protease Inhibitor Cocktail (Pierce). The cells were centrifuged (13,000×g) for 10 mins at 4°C and

supernatants were collected. DC protein assay (Bio-Rad) was used to determine estimated protein concentrations. Proteins (20 µg) were separated using SDS-PAGE (4-20% gel) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated overnight with primary antibody ERα (1:50, sc-8005, sc-8002 Santa Cruz). The membranes were washed with LiCor blocking solution (TBS) and secondary antibodies were added for an hour. The membranes were washed again in blocking solution and the blots were developed using chemiluminescence. LI-COR Odyssey Fc Imaging System was used to visual and analyze the bands.

### Statistical Analyses

All data was expressed as means and SEM. Statistical analyses was performed using GraphPad Prism 9.0 software (GraphPad Software, Inc.). Statistical significance was calculated by either Student's *t*-test between two groups, one-way analysis of variance or two-way analysis of variance between groups among multiple groups with Tukey *post-hoc* tests. A p value $< 0.05$ was considered to be significant.

## Results

### 17αE2 Increases Localization of ERα on Rab7-Positive Endolysosomes

ERα and ERβ are both estrogen receptors expressed in the brain, but Erα is expressed in distinct regions (Warfvinge et al., 2020). Estrogen receptors have

been found to localize on different organellar membranes. Consistent with others' finding (Levin, 2009; Sampayo et al., 2018; Totta et al., 2014), we have shown that ERα is located on endolysosomes. Activation of ERα with 17αE2 acidifies endolysosomes. Here, we wanted to determine if 17αE2 can increase the number of ERα present on endolysosomes. ERα was transduced with GFP and Rab7 with RFP. 17αE2 (10 nM, 30 min) increased the percent of colocalization between ERα and Rab7-positive endolysosome in CLU199 cells (Figure 27). This finding indicates that 17αE2 promotes the recruitment of ERα to endolysosome membrane.

**ERα Knockdown Attenuates 17αE2-Induced Acidifying Effects on Rab7-Positive Endolysosomes**

For 17αE2 to produce its neuroprotective effects on the endolysosome, we hypothesized that ERα would need to be present on the endolysosomes. Therefore, we used siRNA to knockdown the ERα receptor in the mouse hippocampal cell line CLU199 (Figure 28A). CLU199 cells were transfected with both scrambled siRNA (ERα scr) or siRNA against ERα (ERα KD) for 48 hours and transduced with EGFP. The resting pH of Rab7-positive vesicles from ERα KD transfected cells is significantly higher compared to control (ERα scr) in CLU199 cells (Figure 28B)—indicating that ERα directly or indirectly plays a role in maintaining the pH of endolysosomes. Because ERα is primarily located on Rab7 positive endosomes, we wanted to investigate the endolysosome pH in CLU199 cells to determine how the ERα KD would affect the pH of rab7-positive vesicles. We showed that 17αE2 acidified the Rab7 vesicles in

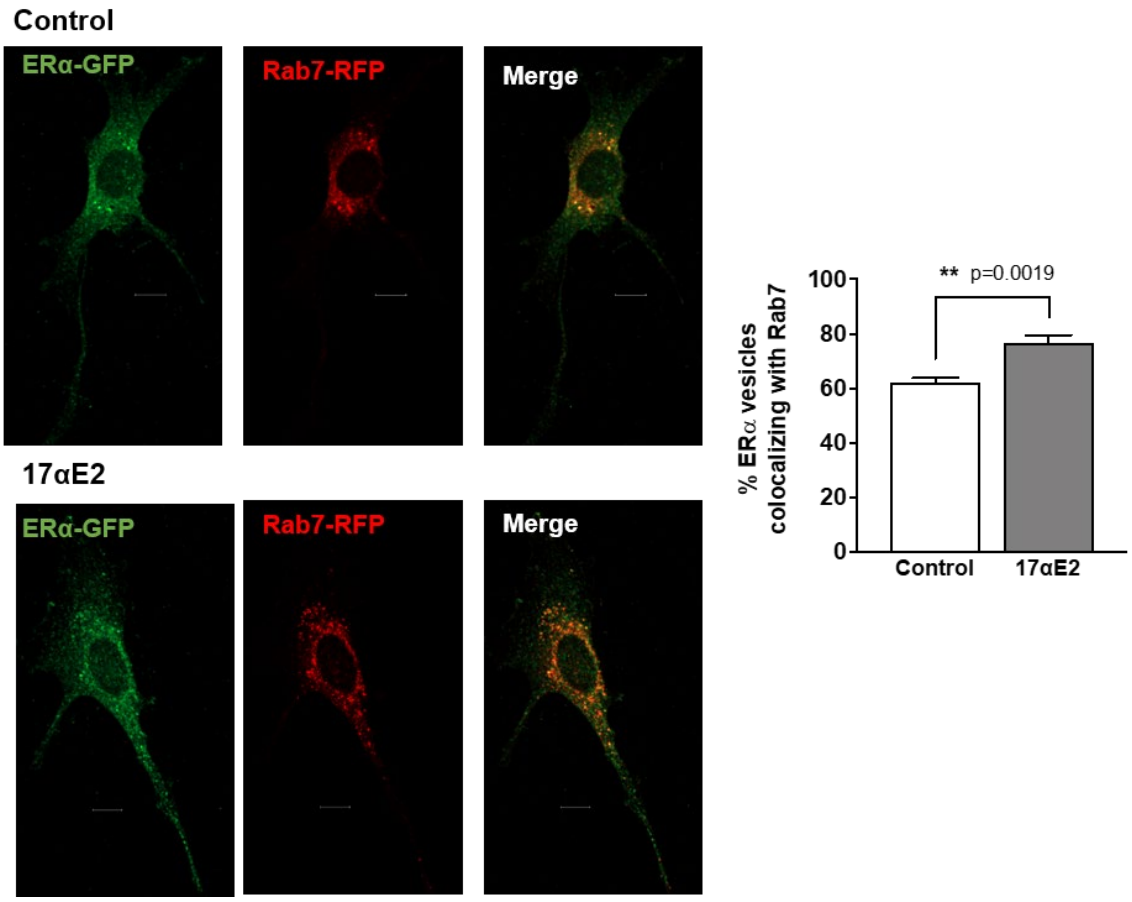



Figure 27. 17αE2 increased localization of ERα on Rab7-positive endolysosomes. Treatment with 17αE2 (10 nM for 30 min) increased the colocalization of ERα and Rab7-positive endolysosomes in CLU199 cells. The bar graph demonstrates percent of colocalization. Data were presented as mean ± SEM (n=3 replicates, *p<0.05, **p<0.01, ***p<0.001, Student's t-test, two-tailed).

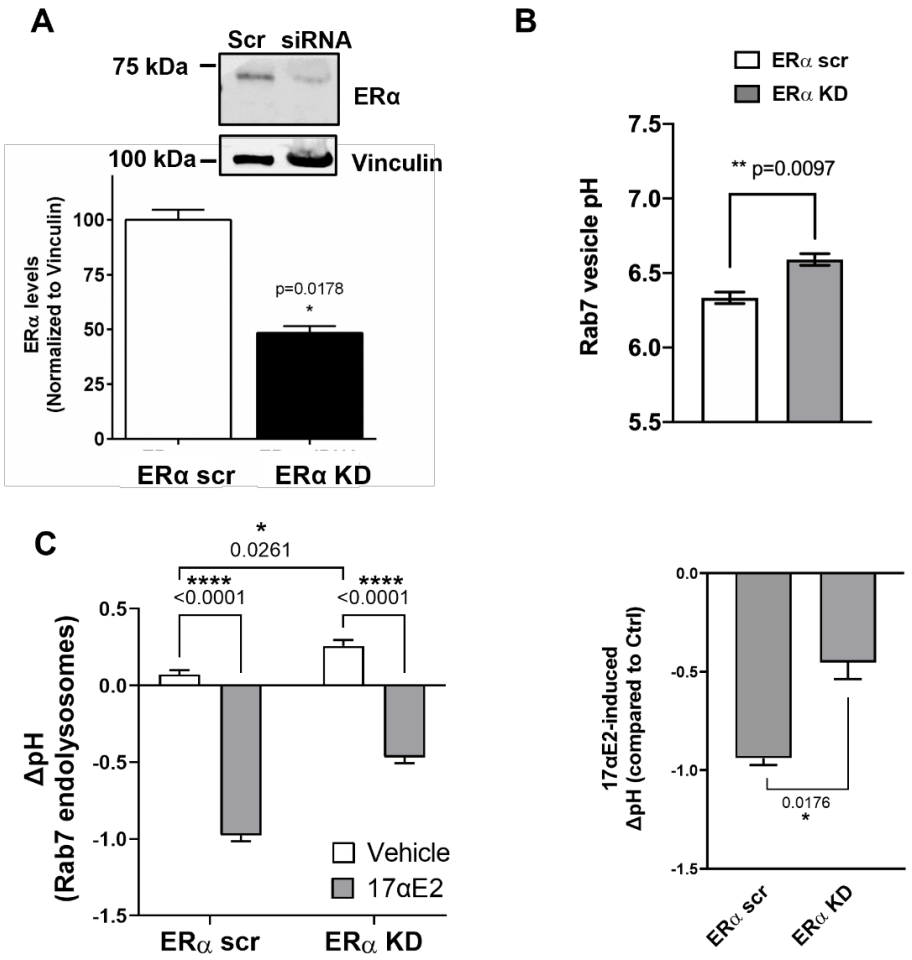



Figure 28. ERα knockdown attenuated 17αE2-induced acidifying effects on Rab7-positive endolysosomes.
**(A)** Representative immunoblots and bar graph show that siRNA directed against ERα (ERα KD) decreased ERα protein levels in mouse hippocampal cell line (CLU-199) compared with scrambled siRNA (ERα scr). Data were presented as mean ± SEM (n=3 replicates, *p<0.05, **p<0.01, Student's t-test, two-tailed). **(B)** The pH of Rab7-positive vesicles is significantly higher when ERα is knocked down with siRNA (ESR1 KD) than when a scrambled siRNA (ESR1 scr) is used. Data were presented as mean ± SEM (n=3 replicates, **p<0.01, Student's t-test, two-tailed.**(C)** Rab7 pH was measured in CLU199 cells using a ratio-metric method with a combination of pH-sensitive (pH rodo) and pH-insensitive (Texas Red) dextran. ERα KD de-acidifies Rab-7 positive endolysosome compared to ERα scr. 17αE2 treatment acidified the pH rab7 vesicles in the ERα scr; However, in the ERα KD 17αE2 did not acidify to the same extent. Data were presented as mean ± SEM (n=3 replicates, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA). Bottom bar graph shows the extent of 17αE2-induced decreases in luminal pH of Rab7-positive endolysosomes was significantly reduced in ERα KD cells. Data were presented as mean ± SEM (n=3 replicates, Student's t-test, two-tailed).

ERα scr cells, and that ERα KD impaired the ability to acidify Ra7 vesicles (Figure 28C). This provides strong evidence that ERα is responsible for the acidification of Rab7-positive vesicles.

### ERα Knockdown Prevented the Protective Effects of Against HIV-1 gp120 and Tat-Induced Endolysosome Impairment

Next, we wanted to determine if ERα knockdown alters the effects of 17αE2 on CatD activity in CLU199 cells. 17αE2 increased the percentage of CatD positive endolysosomes in ERα scr, but it was unable to significantly increase the CatD in ERα KD (Figure 29B). ERα KD impaired the ability of 17αE2 (10 nM, 10 min pretreatment) to restore gp120 (0.5 nM for 30 min)-induced decreases in the percentage of CatD positive endolysosomes (Figure 30B). ERα KD impaired the ability of 17αE2 (10 nM, 10 min pretreatment) to restore Tat (100 nM for 30 min)-induced decreases in the percentage of CatD positive endolysosomes (Figure 31B).

### ERα KD Prevented 17αE2-Induced Increases in Dendritic Spine Density

Next, we determined if ERα is involved in the enhancing effect of 17αE2 on dendritic spines. We used siRNA to knockdown ERα in primary mouse hippocampal neurons (DIV 9-14) (Figure 31A). Neurons were stained for actin to visualize dendritic spines (Figure 32B). Following ERα knockdown, we wanted to examine if 17αE2 (10 nM) increased the dendritic spine density as shown in the

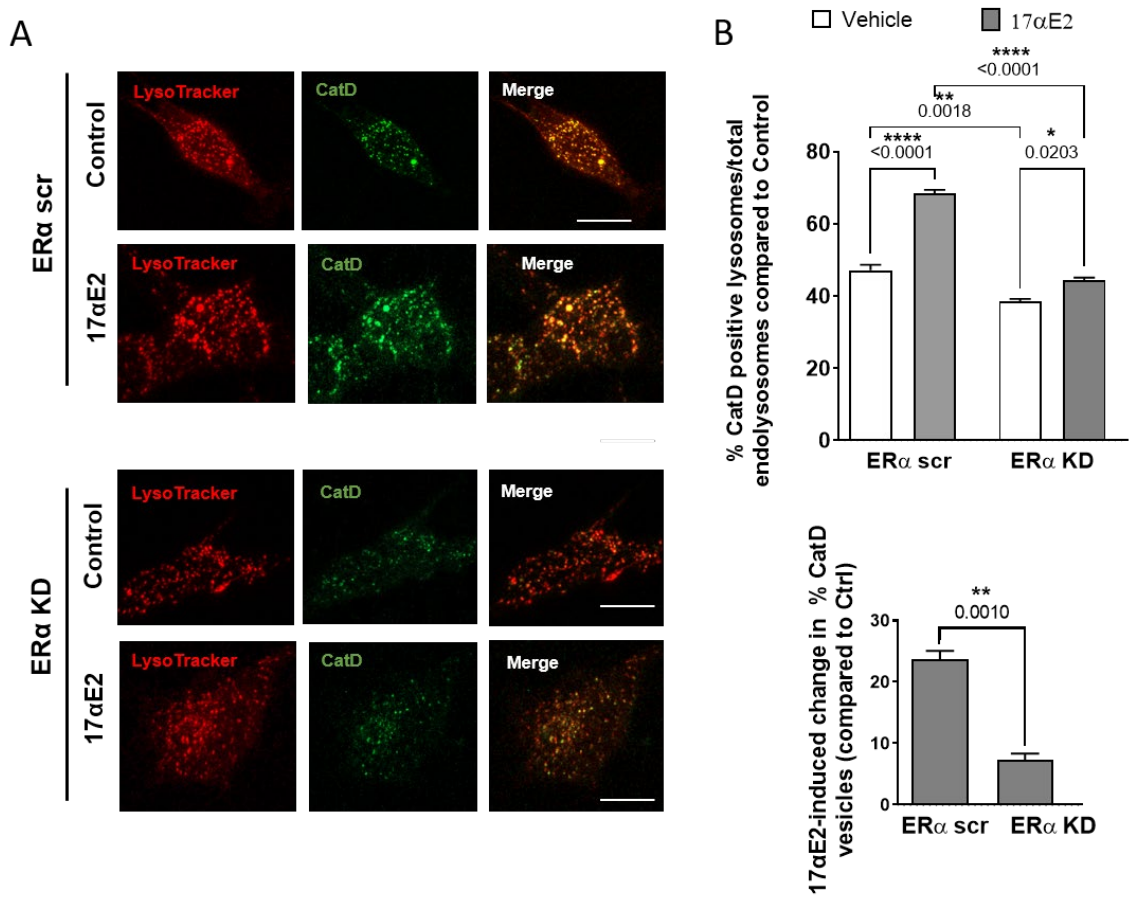



Figure 29. ERα knockdown prevented 17αE2-induced enhancing effects on endolysosome function.
**(A-B)** Representative confocal images and bar graph show changes in the percentage of active endolysosomes (as indicated by activate cathepsin D staining vs total LysoTracker-positive endolysosomes) in CLU199 cells, scale bar-10 μm. 17αE2 significantly increased the percentage of active cathepsin D positive endolysosomes in control cells. 17αE2 also significantly increased the percentage of active cathepsin D positive endolysosomes in ERα KD cells (n=3 replicates, $p<0.0001$, 2-way ANOVA). However, the bottom bar graphs shows the extent of 17αE2-induced increases in percentage of active cathepsin D positive endolysosomes was significantly reduced in ERα KD cells. Data were presented as mean ± SEM (n=3 replicates, Student's t-test, two-tailed).

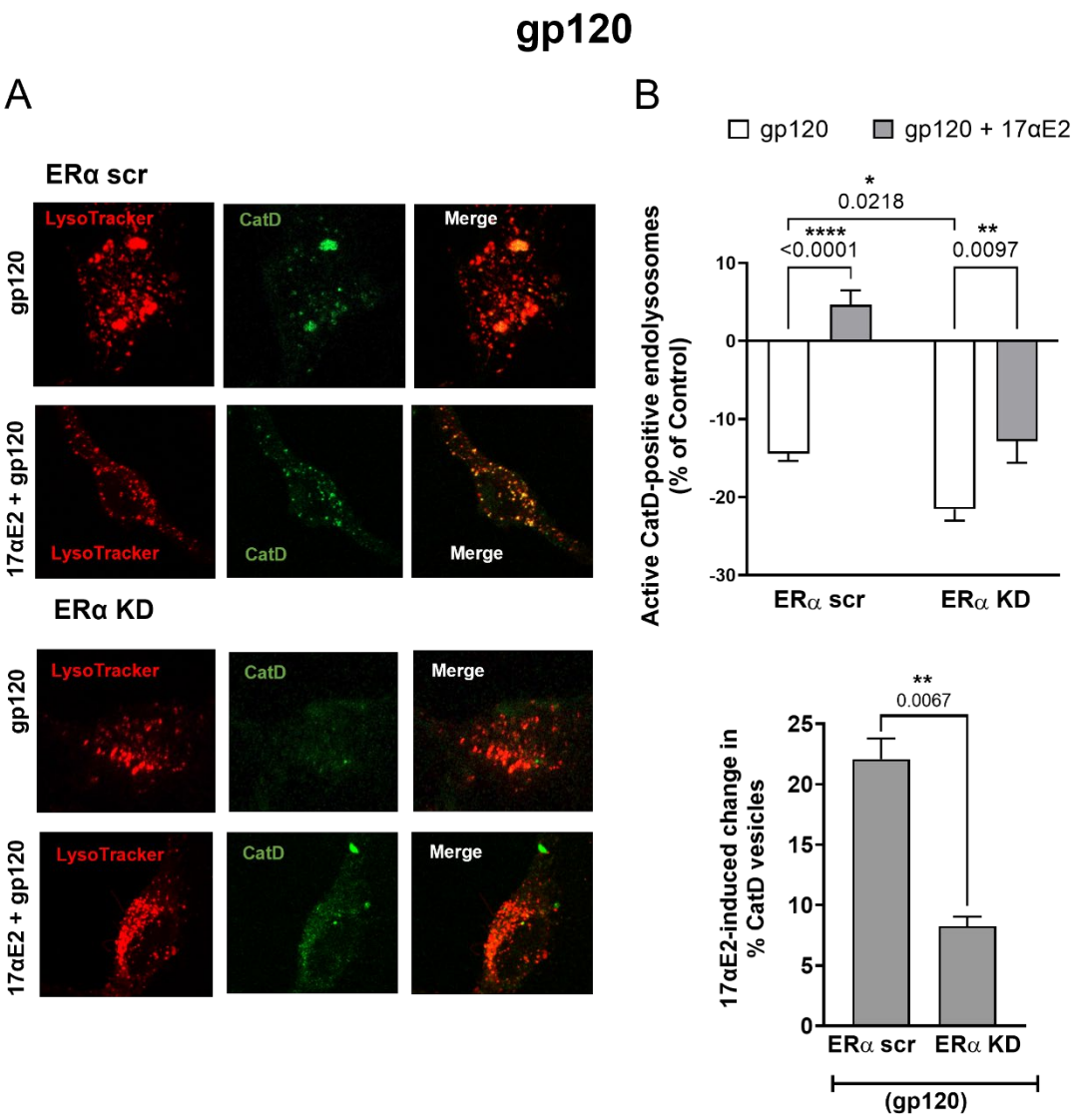



Figure 30. ERα knockdown prevented 17αE2's protective effect against HIV-1 gp120-induced endolysosome impairment.
**(A-B)** Representative confocal images and bar graph show 17αE2 pre-treatment (10 nM, 10 min) affects the percentage of active endolysosomes (as indicated by activate cathepsin D staining vs total LysoTracker-positive endolysosomes) in CLU199 cells treated with HIV-1 gp120 (0.5 nM, 10 min), scale bar-10 μm. 17αE2 significantly restored HIV-1 gp120-induced decreases in the percentage of cathepsin D positive endolysosomes in both control cells and ERα KD cells (n=3 replicates, two-way ANOVA). However, the bottom bar graph shows the extent of 17αE2-induced restoration in HIV-1 gp120-induced decreases in the percentage of active cathepsin D positive endolysosomes in ERα KD cells was significantly reduced (n=3 replicates, Student's t-test, two-tailed).

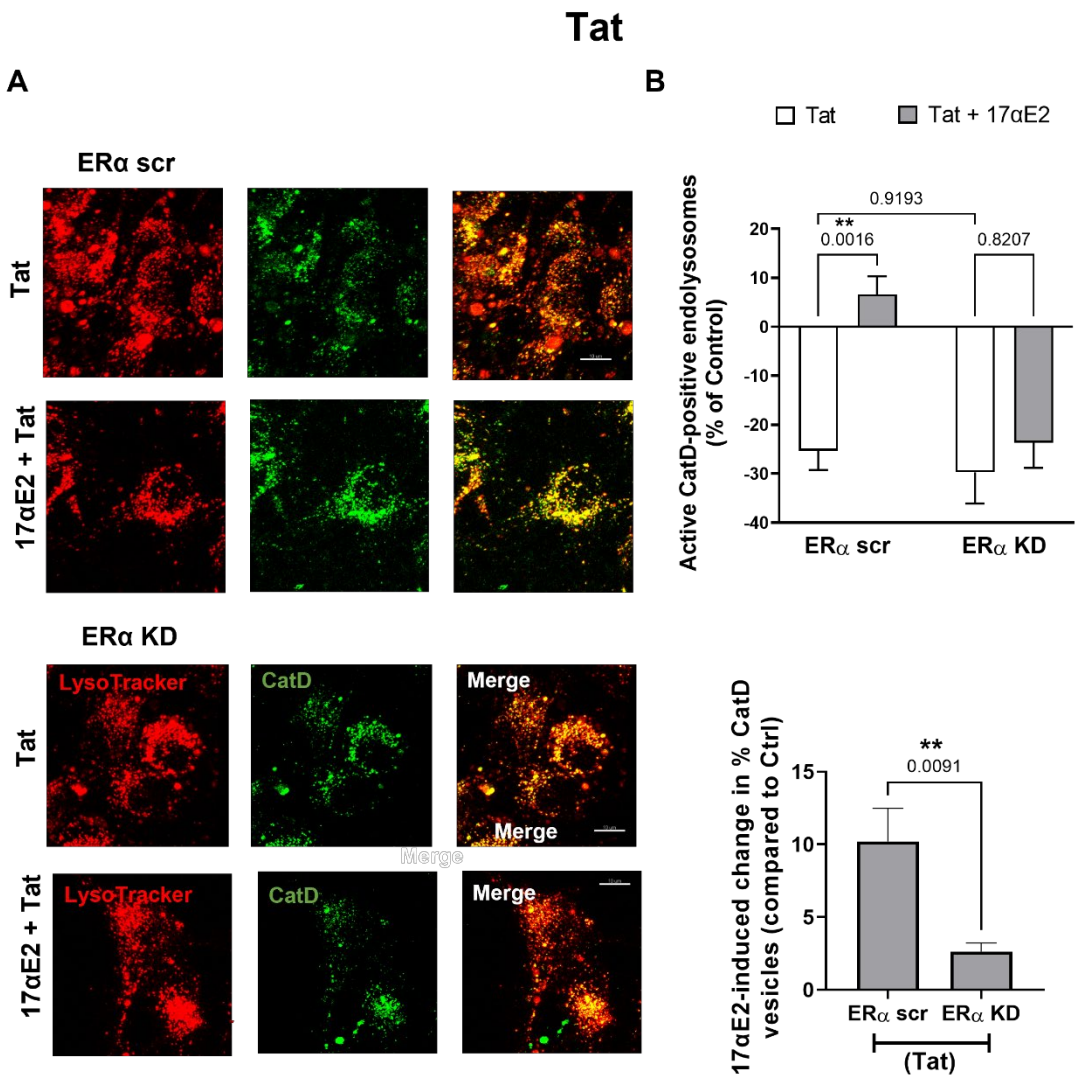



Figure 31. ERα knockdown prevented 17αE2's protective effects against HIV-1 Tat-induced endolysosome impairment.
**(A).** Representative confocal images and corresponding bar graphs of CLU199 cells with scrambled (scr) or targeted (KD) siRNA against ERα show distribution and ratio of CatD positive (green) to total (red) endolysosomes. **(B)** In cells with scr ERα siRNA, Tat treatment decreases the percentage of CatD vesicles, while 17αE2 increases the percentage. Pretreatment with 17αE2 prior to Tat addition partially restores the percentage of Cat D positive vesicles. In cells with ERα KD, the acidifying effect of 17αE2 is lower, and the pretreatment of 17αE2 with Tat was unable to restore the percentage of CatD positive vesicles. Data were presented as mean ± SEM (n=2 replicates, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA). However, the bottom bar graphs shows the extent of 17αE2-induced restoration in HIV-1 Tat-induced decreases in the percentage of active cathepsin D positive endolysosomes in ERα KD cells was significantly reduced (n=2 replicates, Student's t-test, two-tailed).

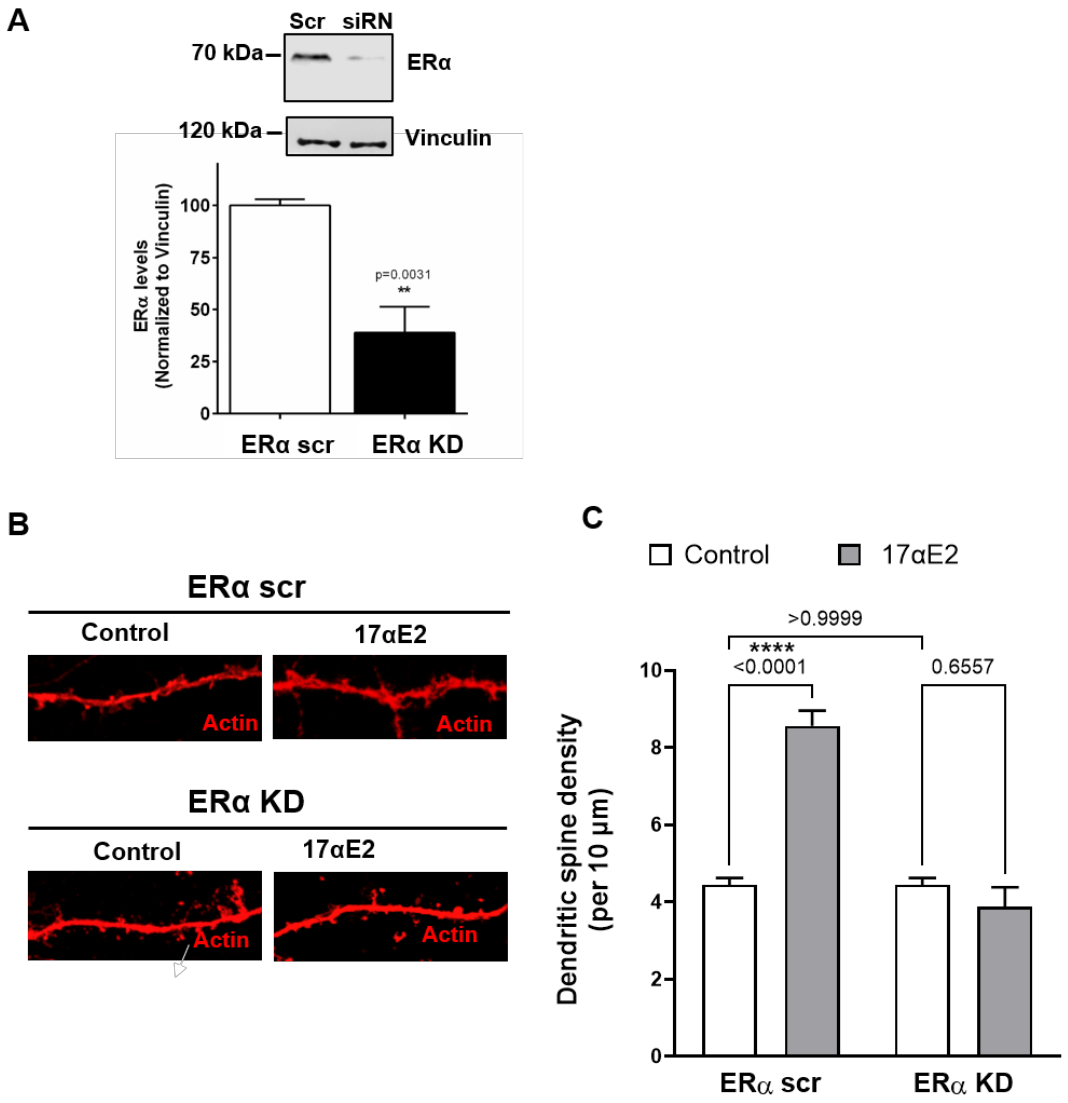



Figure 32. ERα KD prevented 17αE2-induced increases dendritic spine density. **(A)** Mouse hippocampal neurons (DIV 9-14) transfected simultaneously (48 h) with scrambled siRNA (scr) or siRNA against ERα (ERα KD). Immunoblotting data show that protein levels of ERα was significantly reduced with siRNA against ERα. Data were presented as mean ± SEM (n=3 replicates, **p<0.01, Student's T test, two-tail). **(B)** Representative confocal images of mouse hippocampal neurons (DIV 9-14) transfected Actin-RFP (48 h) in the presence of scrambled siRNA (scr) or siRNA against ERα (ERα KD). Scale bar 5 µm. **(C)** Bar graph quantification of above images show a significant decrease in dendritic spine density with ERα KD, and the treatment with 17αE2 (10 nM, 30 min) is unable to increase spine density unlike in ERα scr neurons. Data were presented as mean ± SEM (n= 3 replicates, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA).

ERα scr (Figure 32C). In the ERα KD cells, 17αE2 was unable to stimulate spine formation (Figure 32C).

### ERα Knockdown Prevented the Protective Effects of 17αE2 against HIV-1 gp120 and Tat-Induced Impairment in Dendritic Spines

We used siRNA to knockdown ERα in primary mouse hippocampal neurons (DIV 9-14). Neurons were transduced with EGFP to visualize dendritic spines (Figure 33A). Pretreatment with 17αE2 (10 nM, 10 mins) was able to protect against the gp120-induced decreases in spine density in ERα scr neurons, however, in the ERα KD neurons, it was not effective (Figure 33B). This experiment was repeated using Tat. Pretreatment with 17αE2 (10 nM, 10 mins) was able to protect against the Tat-induced decreases in spine density in ERα scr neurons, however, in the ERα KD neurons, it was not effective (Figure 34B).

### Palmitoylation of the ERα was Required for its Localization to Endolysosomes

For 17αE2 to produce its neuroprotective effects on the endolysosome, we hypothesized that ERα would need to be present on the endolysosomes. Studies have shown that Cys447 is essential for the ERα to localize to the endolysosome. When Cys447 was replaced with Ala, palmitoylation was prevented. Cys447 seems to play a critical role in ER-induced non-genomic pathways (Acconcia et al., 2004). Therefore, we wanted to determine if the palmitoylation of the ERα on the endolysosomes is required for 17αE2 effects.

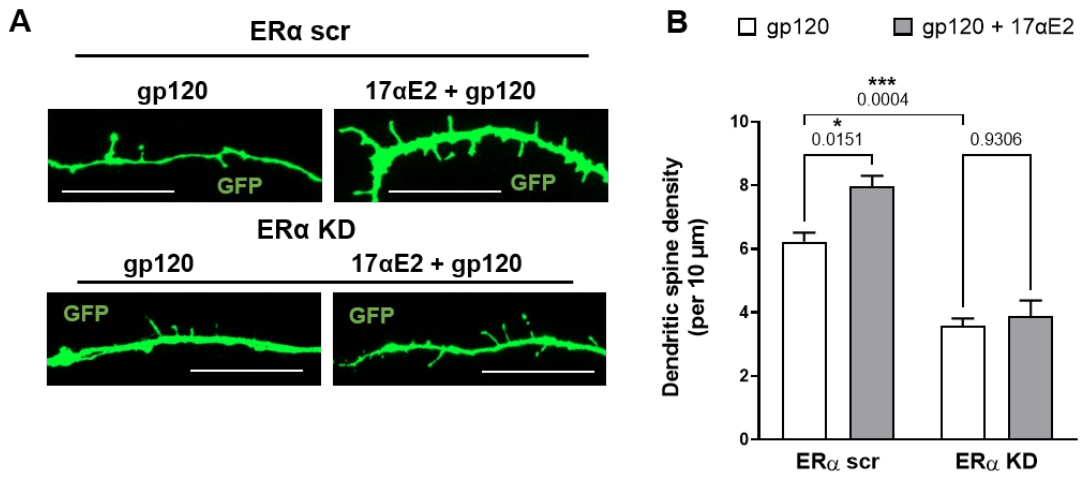



Figure 33. ERα knockdown prevented the protective effect of 17αE2 against HIV-1 gp120-induced impairment in dendritic spines.
**(A)** Representative confocal images of primary hippocampal neurons (DIV 9-10) transduced with EGFP (green) in the presence of scrambled siRNA (scr) or siRNA against ERα (ERα KD). **(B)** Bar graph shows that pretreating with 17αE2 (10 nM, 30 min) increased dendritic spine density in ERα scr neurons treated with gp120 (0.5 nM for 30 min); whereas pretreating with 17αE2 (10 nM, 30 min) failed to increase dendritic spine density in ERα scr neurons treated with gp120 (0.5 nM for 30 min). Data were presented as mean ± SEM (n=3 replicates, *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA).

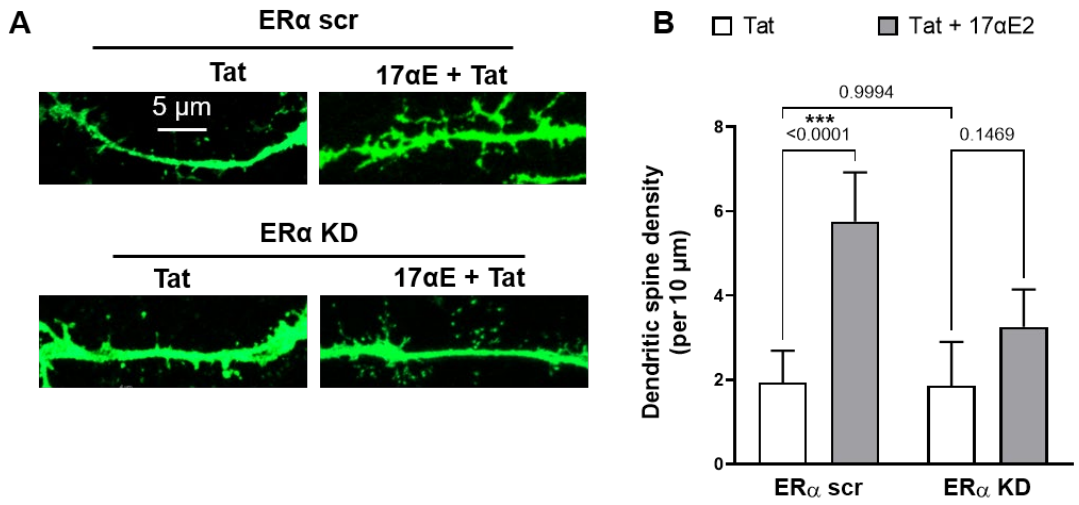



Figure 34. ERα knockdown prevented the protective effects of 17αE2 against HIV-1 Tat-induced impairment in dendritic spines. **(A)** Representative confocal images of primary hippocampal neurons (DIV 9-10) transduced with EGFP (green) in the presence of scrambled siRNA (scr) or siRNA against ERα (ERα KD). **(B)** Bar graph shows that pretreating with 17αE2 (10 nM, 30 min) increased dendritic spine density in ERα scr neurons treated with HIV-1 Tat (100 nM for 30 min); whereas pretreating with 17αE2 (10 nM, 30 min) failed to increase dendritic spine density in ERα scr neurons treated with HIV-1 Tat (100 nM for 30 min). Data were presented as mean ± SEM (n=5 dendrites per condition *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA).

In CLU199 cells, we transfected either ERα palmitoylation mutant (ERα C451A-GFP) and or ERα WT-GFP. In the ERα WT, there was a distinct punctum with ERα and Rab7 vesicles, while in the ERα C451A, there was a diffusion ERα throughout the cytoplasm (Figure 35). This evidence indicates that palmitoylation is needed for the ERα to localize on Rab7 positive vesicles.

### Endolysosome Localization of ERα is Responsible for the Endolysosome Enhancing Effects of 17αE2

Upon obtaining ERα C451A mutant that lacks endolysosome localization, we determined the effect of overexpressing this mutant on endolysosome functions. We wanted to know if 17αE2 alone would be able to increase the percentage of CatD active endolysosomes in CLU199 cells transfected with ERα C451A or ERα WT. CatD was significantly decreased in ERα C451A compared to ERα WT cells in the control treatment. 17αE2 was able to increase CatD in ERα WT, however, it was also able to increase the CatD ERα C451A but not to the same extent (Figure 36B). Thus, ERα C451A impairs the ability of 17αE2 to enhance endolysosome functions.

### Endolysosome Localization of ERα was Responsible for the Protective Effect of 17αE2 Against HIV-1 gp120-and Tat-Induced Endolysosome Dysfunction

We have shown that Tat and gp120 induce endolysosome dysfunction. Next, we repeated the experiment using 17αE2 (10 nM, 10 mins) as a pretreatment against Tat and gp120 effects. Pretreatment with 17αE2 (10 nM, 10 mins)

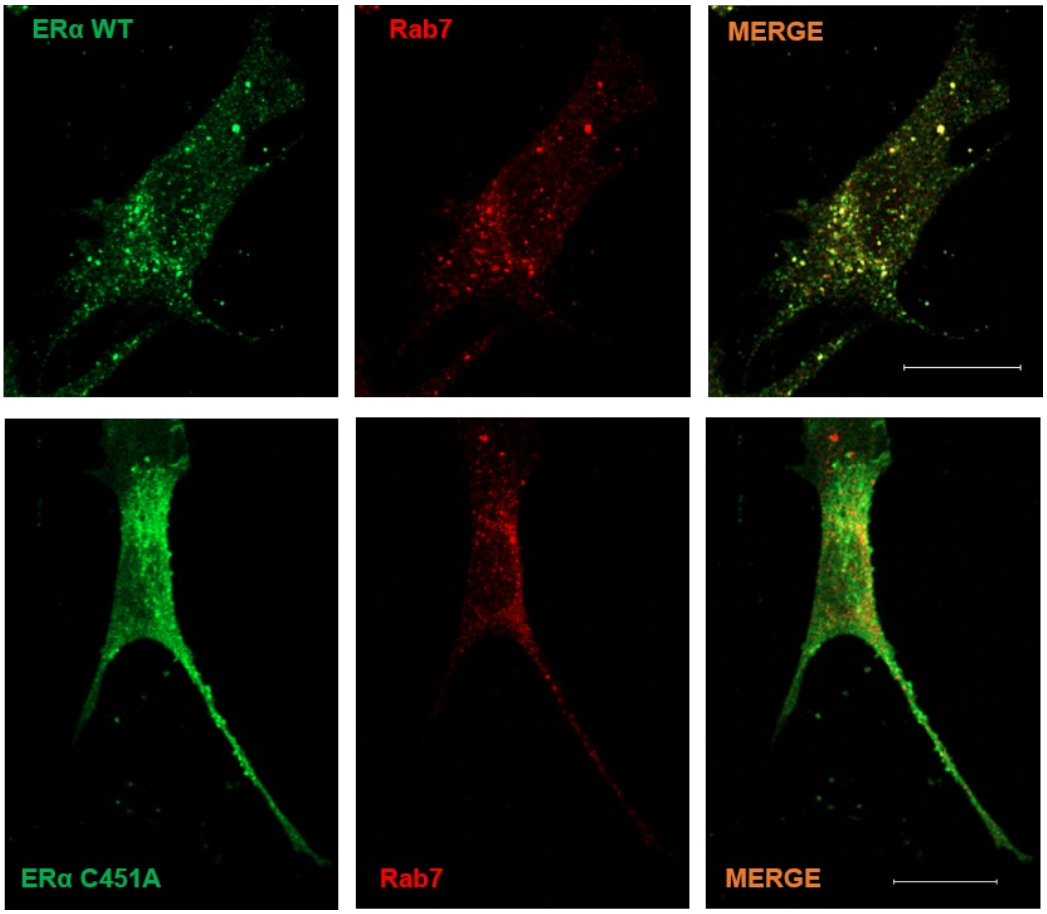



Figure 35. Palmitoylation of the ERα receptor was required for localization to Rab7 positive vesicles.
Colocalization of ERα WT-GFP or ERα C451A-GFP (green) and Rab7 (red) vesicles in mouse hippocampal CLU199 cells. Colocalized vesicles are shown in yellow. CLU199 cells transfected with ERα palmitoylation dead mutant (ERα C451A-GFP) show a diffusion throughout the cytoplasm, while ERα WT-GFP show distinct puncta.

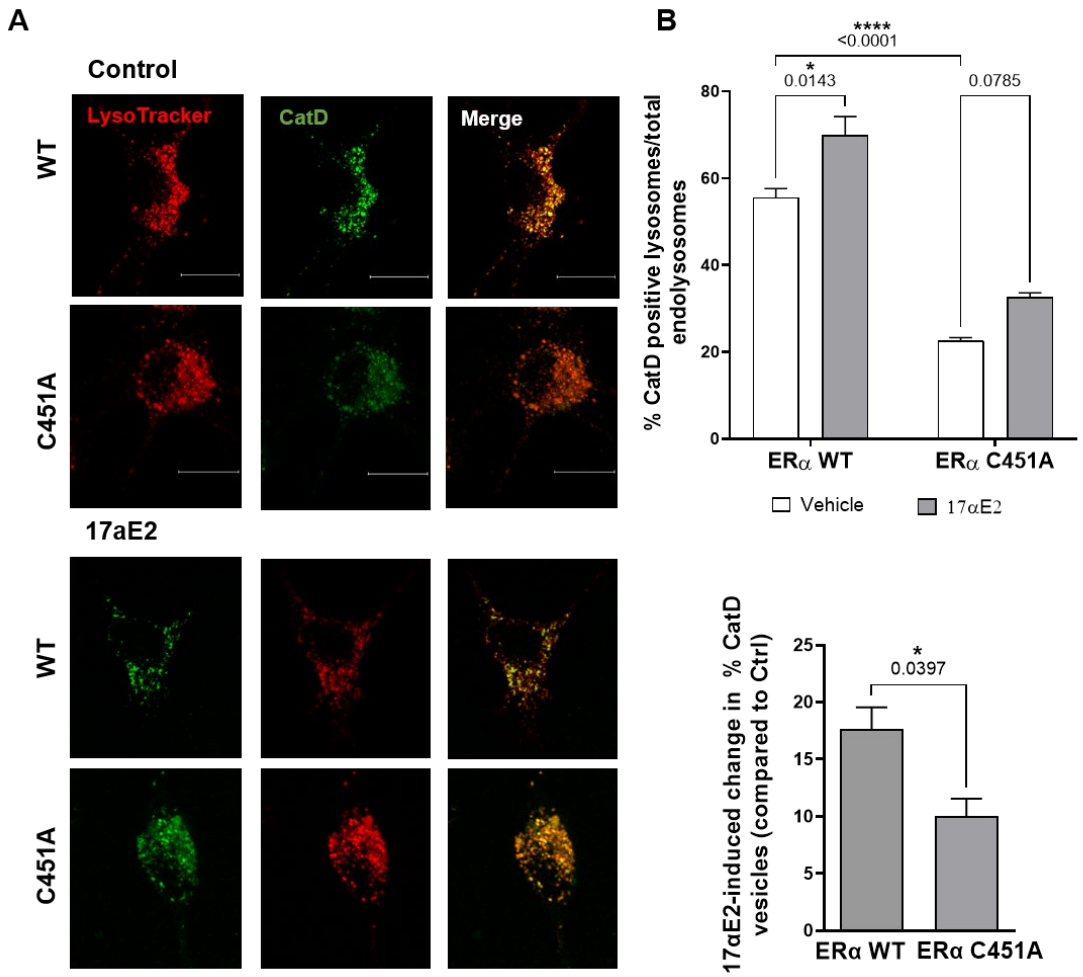



Figure 36. Endolysosome localization of ERα was responsible for the endolysosome enhancing effects of 17αE2.
**(A-B)** Representative confocal images and bar graph show changes in the percentage of active endolysosomes (as indicated by cathepsin D staining vs total LysoTracker-positive endolysosomes) in CLU199 cells expressing wild-type ERα (ERα WT) and in CLU199 cells expression ERα C451A mutant that lacks endolysosome localization (ERα C451A), scale bar-10 μm. 17αE2 significantly increased the percentage of cathepsin D positive endolysosomes in ERα WT cells; However, 17αE2 failed to increase the percentage of cathepsin D positive endolysosomes in ERα C451A cells (n=3 replicates, two-way ANOVA). Bottom bar graph shows over-expressing ERα C451A mutant significantly reduced 17αE2-induced changes in percentage of cathepsin D positive endolysosomes (n=3 replicates, Student's t-test, two-tailed).

followed by gp120 in ERα C451A was partially able to increase the percentage of CatD endolysosomes (Figure 37B). Pretreatment with 17αE2 (10 nM, 10 mins) followed by Tat in ERα C451A was unable to increase the percentage of CatD endolysosomes significantly, but it was able to in ERα WT cells (Figure 38B).

### Endolysosome Localization of ERα was Responsible for 17αE2's Protective Effects Against HIV-1 gp120-Induced Impairment in Dendritic Spines

Next, we transfected primary hippocampal neurons (DIV 10) with ERα WT-HA and the palmitoylation dead mutant C451A-HA for 48h. Neurons were transduced with EGFP to visualized dendritic spines. In ERα WT cells, pretreatment with 17αE2 rescued the effects of gp120. However, 17αE2 failed to rescue the effects of gp120 in neurons expressing ERα C451A mutants (Figure 39B).

## Discussion

The neuroprotective role of 17αE2 has been shown in cultured cells (Green et al., 1997) and in different animal models (Kaur et al., 2015; Lermontova et al., 2000; Levin-Allerhand, Lominska, Wang, & Smith, 2002; Manaye et al., 2011; McClean & Nunez, 2008; Ozacmak & Sayan, 2009). It is also known that 17αE2 increases the formation of dendritic spines (Luine & Frankfurt, 2012; MacLusky, Luine, Hajszan, & Leranth, 2005; Sengupta, Lantz, Rumi, & Quinlan, 2019). Estrogen receptors located on organellar membranes could be involved in estrogens'

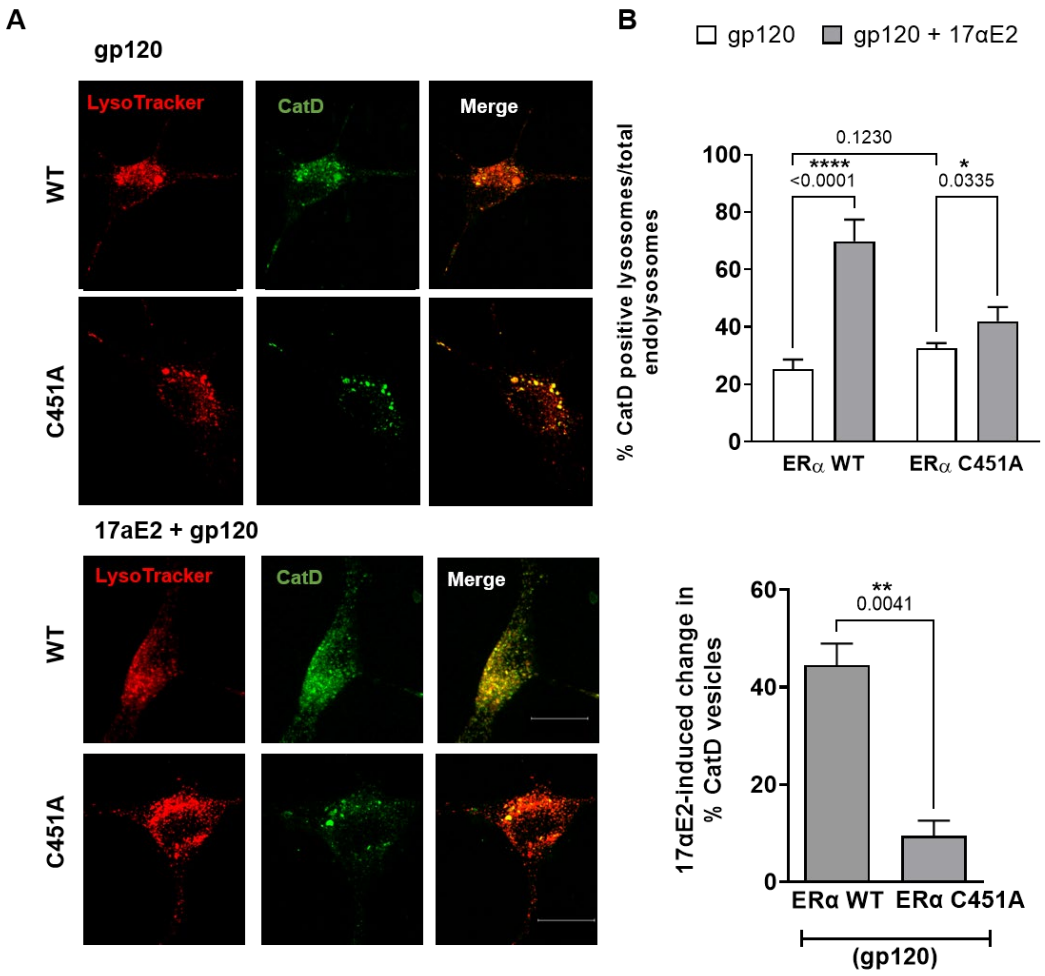



Figure 37. Endolysosome localization of ERα was responsible for the protective effect of 17αE2 against HIV-1 gp120-induced endolysosome dysfunction.
**(A-B)** Representative confocal images and bar graph show 17αE2 pre-treatment (10 nM, 10min) affects the percentage of active endolysosomes (as indicated by activate cathepsin D staining vs total LysoTracker-positive endolysosomes) in CLU199 cells treated with HIV-1 gp120 (0.5 nM, 10 min). Scale bar-10 μm. 17αE2 significantly increased the percentage of cathepsin D positive endolysosomes in ERα WT cells treated with HIV-1 gp120; 17αE2 partially increased the percentage of cathepsin D positive endolysosomes in ERα C451A cells treated with HIV-1 gp120, but not to the same extent as ERα WT cells (n=3 replicates, two way-ANOVA). Bottom bar graph shows over-expressing ERα C451A mutant significantly reduced 17αE2-induced changes in percentage of cathepsin D positive endolysosomes in CLU199 cells treated with HIV-1 gp120 (n=3 replicates, Student's t-test, two-tailed).

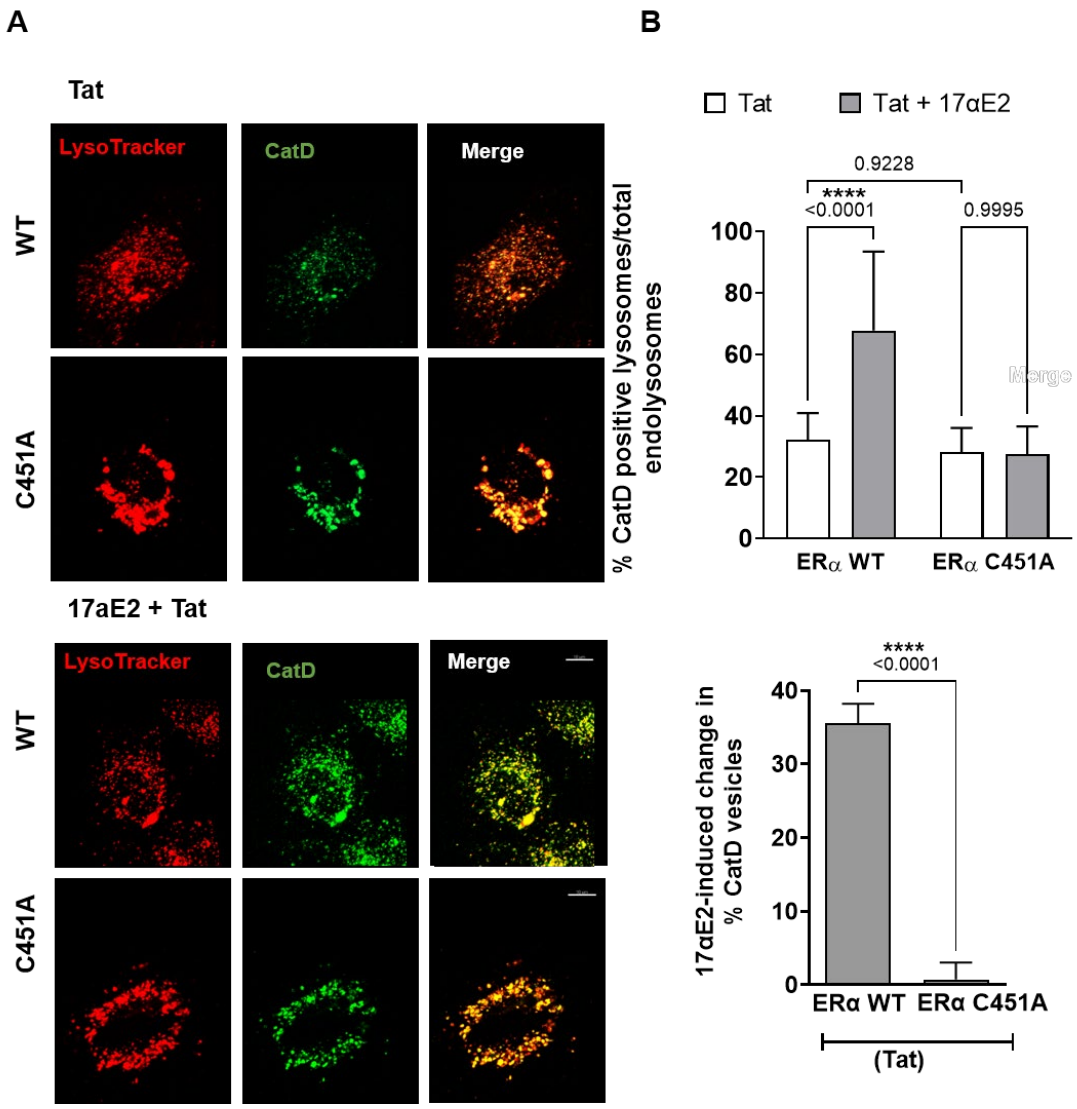



Figure 38. Endolysosome localization of ERα was responsible for the protective effect of 17αE2 against HIV-1 Tat-induced endolysosome dysfunction. **(A-B)** Representative confocal images and bar graph show 17αE2 pre-treatment (10 nM, 10min) affects the percentage of active endolysosomes (as indicated by activate cathepsin D staining vs total LysoTracker-positive endolysosomes) in CLU199 cells treated with HIV-1 Tat (100 nM, 10 min). Scale bar-10 μm. 17αE2 significantly increased the percentage of cathepsin D positive endolysosomes in ERα WT cells treated with HIV-1 Tat; However, 17αE2 failed to increase the percentage of cathepsin D positive endolysosomes in ERα C451A cells treated with HIV-1 Tat (n=3 replicates, two way-ANOVA). Bottom bar graph shows over-expressing ERα C451A mutant significantly reduced 17αE2-induced changes in percentage of cathepsin D positive endolysosomes in CLU199 cells treated with HIV-1 Tat (n=3 replicates, Student's t-test, two-tailed).

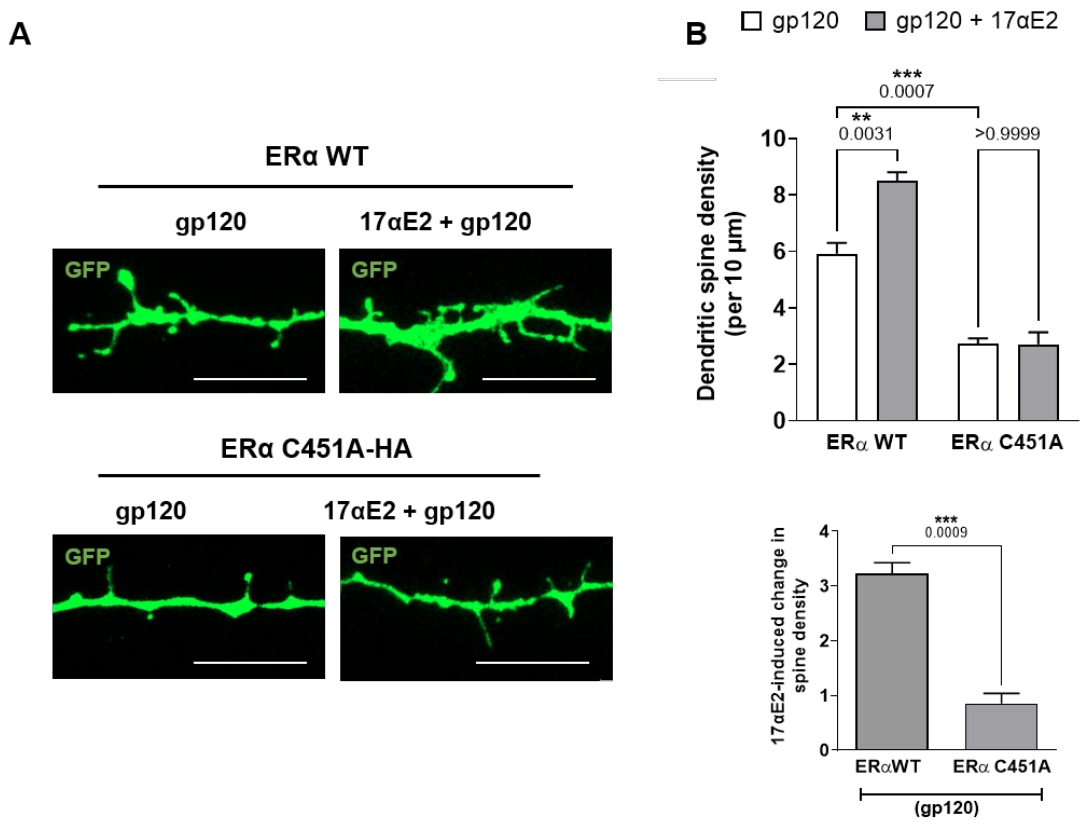



Figure 39. Endolysosome localization of ERα was responsible for 17αE2's protective effects against HIV-1 gp120-induced impairment in dendritic spines. **(A-B)** Representative images and bar graph show 17αE2 pre-treatment (10 nM, 10min) affects dendritic spine density (indicated by GFP-expressing) in ERα WT neurons and ERα C451A neurons treated with HIV-1 gp120 (0.5 nM, 10 min). Scale bar- 10µm. 17αE2 was able to increase dendritic spine density in ERα WT neurons treated with HIV-1 gp120; However, 17αE2 failed to increase dendritic spine density in ERα C451A neurons treated with HIV-1 gp120 (n=3 replicates *p<0.05, **p<0.01, ***p<0.001, two way-ANOVA). Bottom bar graph shows over-expressing ERα C451A mutant significantly reduced 17αE2-induced changes in dendritic spine density in neurons treated with HIV-1 gp120 (n=3 replicates, Student's t-test, two-tailed).

protective effects on cognition and synaptic function (Frick, 2015; Hojo et al., 2008; Lai et al., 2017; Mukai et al., 2007; Sheppard et al., 2019; Srivastava et al., 2011) ERα is found in brain regions responsible for learning and memory, such as the prefrontal cortex and hippocampus, and is also found on endolysosomes (Hara, Waters, McEwen, & Morrison, 2015; Levin, 2009; Sampayo et al., 2018; Totta et al., 2014) and in dendritic spines and at synapses (Hart, Snyder, Smejkalova, & Woolley, 2007; Heron et al., 2009; Lu et al., 2003; Milner et al., 2005; Milner et al., 2001; Mukai et al., 2007; A. C. Wang, Hara, Janssen, Rapp, & Morrison, 2010).

These membrane-bound ERα affect spine plasticity through multiple signaling pathways (Amateau & McCarthy, 2002; A. Christensen, Dewing, & Micevych, 2011; Dominguez & Micevych, 2010; Hasegawa et al., 2015). ERα is a soluble cytosolic protein (Schlegel et al., 1999); therefore, ERα localizes on the endolysosome membrane facing the cytosol and allowing interaction with 17αE2.

We observed that 17αE2 treatment increased the colocalization of ERα present on endolysosomes. This may have been a time-dependent increase in the distribution of ERα as cells were only treated for 30 mins with 17αE2. A more extended treatment would need to be investigated to determine if this effect diminished over time. This may result from less degradation of endolysosome ERα, increased endocytosis from the plasma membrane, or more ERα from the cytoplasm gets attached to endolysosomes. Nonetheless, endolysosome localization of ERα could mediate 17αE2's enhancing effect on endolysosomes.

We also wanted to determine if ERα is involved in 17αE2's effect on endolysosome pH and function. Because ERα is primarily located on Rab7 positive vesicles, we determined luminal pH of Rab7 positive vesicles. ERα knockdown increased endolysosome pH and prevented 17αE2 from acidifying endolysosomes. ERα knockdown prevented 17αE2 from increasing the percentage of CatD positive endolysosomes compared to ERα scr. Lastly, ERα knockdown impaired the ability of 17αE2 to restore decreased CatD positive endolysosomes induced by gp120 or Tat. These findings indicate that ERα was essential for 17αE2's enhancing effect on endolysosomes.

We also wanted to determine if endolysosome localization of ERα is involved in 17αE2's enhancing effect on endolysosome. An ERα mutant (C451A) was generated that prevents the palmitoylation site (Adlanmerini et al., 2014; Pedram, Razandi, Lewis, Hammes, & Levin, 2014), thereby preventing the localization of ERα on endolysosomes; expressing such ERα mutant resulted in a diffusion of ERα throughout the cytosol instead of distinct puncta. First, over-expressing the ERα mutant prevented 17αE2 from enhancing the percentage of CatD positive endolysosomes. Over-expressing ERα mutant impaired the ability of 17αE2 to restore decreased CatD positive endolysosomes treated with gp120 or Tat. These findings suggest that endolysosome localization of ERα was essential for 17αE2's enhancing effect on endolysosomes.

Neurons require constant membrane trafficking to maintain plasma membrane domains as neurons have highly extensive processes and are post-mitotic cells. Neuronal endolysosomes have been observed throughout the dendrites, axons, and dendritic spines and can modulate synaptic plasticity (Cheng et al., 2018; Marisa S. Goo et al., 2017; Padamsey et al., 2017; Yap et al., 2018).

Our findings indicate that 17αE2 enhanced endolysosome function via the ERα localization on endolysosomes and increased the density of dendritic spines. Next, we wanted to determine if ERα localization on endolysosomes was necessary for the protective effects of 17αE2 on dendritic spines. ERα knockdown prevented 17αE2 enhancing effects on dendritic spines. ERα knockdown prevented the protective effects of 17αE2 against gp120 and Tat-induced synaptodendritic impairment. Over-expressing the ERα mutant (C451A) prevented 17αE2 from protecting dendritic spine density against gp120-induced synaptodendritic impairment. Thus, endolysosome localization of ERα plays an important role in the enhancing effect of 17αE2.

The mechanism for how endolysosome localization of ERα affects dendritic spines is unknown. However, localization could affect the mobility of endolysosomes to travel along the dendrites, thus, preventing the endolysosomes from maintaining the dendritic spines. Further research needs to be completed to understand the mechanisms of how endolysosome localization ERα modulates dendritic spines.

Overall, our findings demonstrate that ERα localization on endolysosomes is necessary for enhancing the effects of 17αE2 on endolysosome acidification, increasing the percentage of CatD positive endolysosomes, and increasing in dendritic spines. Additionally, we have shown that endolysosome localization of ERα is responsible for 17α-E2's protective effect against HIV-1 gp120 and Tat-induced endolysosome dysfunction and decreases in dendritic spines.

# CHAPTER V

# 17αE2 IS PROTECTIVE IN HIV-1 TRANSGENIC RATS

## Introduction

Hormonal changes such as menopause, result in a substantial decrease in estrogen, which has been linked to cognitive deficits and could impact women's development of HAND (Quinn & Overbaugh, 2005; Weber et al., 2012). Estrogen is protective against HIV-1 infection and HIV-associated neuropathology. 17βE2 is neuroprotective and has been shown in many studies to protect against Tat and gp120 induced damage, such as Tat-activated transcription, preventing inflammation, and protecting against oxidative stress and cell death (Wilson, Dimayuga, et al., 2006). However, 17βE2 has feminizing effects along with other side effects, making it a poor candidate for treatment. 17αE2 has been shown to exert neuroprotective effects, although it has not been studied to the extent that 17βE2.

17βE2 and 17αE2 were shown to protect at the same level against gp120 neuronal cell death (Howard et al., 2001). Thus, 17αE2 is a promising alternative to 17βE2 as it is a natural non-feminizing estrogen (Kaur et al., 2015). In our studies presented previously, we show 17αE2 is protective against gp120 and Tat-induced endolysosome dysfunction and synaptodendritic impairment, making

this a potential treatment for HAND. Several estrogen replacements therapies (ERT) and hormone replacement therapy (HRT) studies have been conducted to determine the effectiveness of hormone therapy in the CNS.

## Hormone Therapy and CNS Effects

Unfortunately, no studies have been conducted looking at the effects on cognition and memory with individuals infected with HAND or HIV on the effects of ERT or HRT in the post-cART era. HAND shares similar features with neurodegenerative diseases such as PD and AD. Neurodegenerative diseases have microglial activation, neuroinflammation, and different levels of cognitive impairment. Therefore, looking into the studies done with AD and PD may give us an idea of how these treatments could protect or prevent the development of HAND.

Observational studies of women taking estrogen replacement treatment have been reported and suggest that estrogen may delay the cognitive decline associated with aging and delay AD. Three different studies showed that postmenopausal women taking ERT and were associated with a decreased risk in AD. In these studies, the researchers found that the longer the use of ERT, the lower the risk for AD and cognitive decline (Dye, Miller, Singer, & Levine, 2012; A. K. Jacobs, 2000; D. M. Jacobs et al., 1998; Paganini-Hill & Henderson, 1994; Tang, Abplanalp, Ayres, & Subbiah, 1996; Yaffe, Haan, Byers, Tangen, & Kuller, 2000; Yaffe, Lui, et al., 2000). Similar results found that the risk for AD was decreased in the Baltimore Longitudinal Study of Aging (Kawas et al., 1997). The

last observational study looked at women who had unilateral or bilateral ovaries removed compared to women who did not have their ovaries removed. Women whose ovaries were removed were at a higher risk for cognitive impairment compared to women with ovaries (Dye et al., 2012). In clinical trials, women who took ERT that were younger than 65 years old showed an increase in episodic verbal memory than women older than 65 (Joffe et al., 2006; Phillips & Sherwin, 1992; Sherwin, 1988), suggesting that timing is a factor in the protection of ERT.

Several randomized clinical trials have been completed using HRT to determine the cognitive risks. Women were given estrogen at 2 mg with progestins or a placebo for 2 years to determine the cognitive outcomes. Women who received HRT have a 64% decrease in risk for cognitive impairment compared to the placebo group (Bagger et al., 2005). Another study used estrogen at 0.5 mg compared to the placebo group for 12 months and found that the women taking estrogen had less mistakes during verbal recall and remembered more information (Dye et al., 2012; Joffe et al., 2006).

Estradiol was given at 2 mg versus placebo for 2 months, and the women taking estrogen showed no difference in cognition (LeBlanc, Neiss, Carello, Samuels, & Janowsky, 2007). Estradiol was given at 1 mg versus placebo for 2 years, and women on estrogen showed less delay in verbal memory than those on placebo (Tierney et al., 2009). A study found that ERT reduced the risk of developing dementia in women with Parkinson's disease without dementia (Dye et al., 2012).

The risk for developing devastating conditions such as osteoporosis, heart attack, and atherosclerosis increases when estradiol decreases. Estradiol decreases in women who have their ovaries removed, a hysterectomy, or women going through menopause (Kato, Kase, Okada, Tohma, & Ishiwata, 1998; Rocca, Grossardt, & Shuster, 2011; Shuster, Rhodes, Gostout, Grossardt, & Rocca, 2010). Premature menopausal women (<40 years of age) who received no estrogen treatment compared with women who did not go through premature menopause were five times more likely to die from neurological disorders (PD and dementia), stroke, and coronary disease (Henderson & Sherwin, 2007; Rivera et al., 2009; Rocca et al., 2007, 2008; Shuster et al., 2010).

Low estrogen levels are present in postmenopausal HIV-infected women, which is associated with a higher risk, compared to men, of developing neurocognitive impairments. Studies have indicated that estrogen is protective against Tat and may slow the progression of HIV into HAND (Wallace, 2006).

## Animal Models for HIV/HAND

Animal studies need to be completed to elucidate HAND pathogenesis but choosing the appropriate model needs to be considered as there are limitations to each animal model.

## Non-Human Primate Models

Primate models of HIV include both SHIV and SIV-infected macaque models, and these models are the most similar to human HIV infection. SIV-infected macaque can also be treated with cART (Zink et al., 2010). The CNS, plasma, and CSF samples can be studied throughout the infection of HIV and at the different stages of HAND. The neuropathology in primate models resembles that of humans and helps study the pathogenesis of HAND (Sharer et al., 1988). The main limitations of the primate model are the cost, availability, and longevity of the primates.

## Rodent Models

GFAP, doxycycline- inducible HIV-1 Tat transgenic mouse model has been found to have disrupted synaptic proteins, synaptotagmin2, and gephyrin and reduced the number of dendritic spines in pyramidal CA1 hippocampal neurons, which was accompanied by disruptions in learning and memory (Fitting et al., 2013). HIV gp120 transgenic mice are used as an animal model for the study of HIV and HAND, displaying decreased synaptic and dendritic density, neuronal loss along with increased microgliosis and astrogliosis. This model expresses gp120 under control of the promoter of glial fibrillary acidic protein (Thaney et al., 2018). Mice and rats can also carry the human CD4 and CCR5 transgenes. This model is best used to observe natural HIV infection as these a viral receptor needed for HIV-1 replication (Goffinet, Allespach, & Keppler, 2007; Goffinet, Michel, et al.,

2007; Seay et al., 2013). However, there is no development of the disease or HIV brain entry in this model (Hatziioannou & Evans, 2012).

A mouse model infected with the chimeric virus called EcoHIV allows the infection to enter through murine cells by the cationic amino acid transporter-1 (mCAT) (Deves & Boyd, 1998).EcoHIV is an engineered virus that removes the surface protein gp120 with the gp80 gene; the gp80 gene is from a mouse leukemia virus. The infection is in the brain, lymphoid tissue, but not in specific tissues where HIV is found in humans such as the liver, gastrointestinal tract, skin, adipose tissue (Deves & Boyd, 1998; Kelschenbach et al., 2012; Potash et al., 2005; Wong & Yukl, 2016). A limitation on this model is that these animals do not have immunodeficiency and do not progress to AIDS (Hadas et al., 2013; Kelschenbach et al., 2012; Potash et al., 2005).

The transgenic rat model is a model for HAND as they display gene expression profiles, synaptodendritic damage, and behavioral deficits similar to individuals with HAND (J. A. Fields et al., 2016; M. D. Li et al., 2013; Moran, Booze, Webb, & Mactutus, 2013). Memory was negatively impacted in the transgenic HIV rats compared to control rats (Lashomb, Vigorito, & Chang, 2009). Cross-sectional studies have provided evidence that HAND affects the neurocognitive functions of individuals, such as attention, executive function, and working memory. Therefore, having a model to study the progression of HAND and the long-term effects is essential. The HIV-1 Tg rat contains 7 out of the nine genes in the HIV-1 virus and contains a *gag-pol* deleted provirus (Vigorito, Connaghan, & Chang,

2015). This model looks at how the viral gene products can lead to the progression of HAND. These rats experience clinical signs including wasting, skin lesions, neurological impairments, respiratory problems, and opaque cataracts. The neuropathology in HIV-1 Tg rats consists of neuronal cell loss, reactive gliosis, and the blood-brain barrier integrity breakdown (Reid et al., 2001b).

HIV-1 Tg rats produce viral proteins that model HIV-1 infected individuals on cART (Abbondanzo & Chang, 2014). A study found that the progression of neurocognitive impairments was significantly affected in HIV-1 Tg rats compared to controls. Female HIV-1 Tg rats displayed significantly worse defects in temporal processing and long-term episodic memory compared to the male HIV-1 Tg rats from 3 months to 9 months (K. A. McLaurin, Booze, & Mactutus, 2018; K. A. McLaurin, A. K. Cook, et al., 2018; Moran, Fitting, Booze, Webb, & Mactutus, 2014). HIV-1 Tg rats had deficiencies in attention while using the signal detection task. They also have difficulty learning a novel stimulus, and in discrimination learning, they had a slower acquisition rate (Moran et al., 2014). The water maze was used to determine the HIV Tg rat's psychomotor functions and cognition. Compared to control rats, HIV-1 Tg rats showed significant cognitive impairment when locating the platform (Lashomb et al., 2009). These responses are comparable to the neurocognitive decline that HIV-positive individuals experience. In female HIV-1 Tg animals, dendritic branching and neuronal arbor complexity were altered. Males showed no difference in branch order distribution. However, males had decreased dendritic spine volume in the

nucleus accumbens (K. A. McLaurin, A. K. Cook, et al., 2018). The limitation to this model is that they do not have the whole HIV genome and are not infected by the HIV-1 virus as humans are; therefore, they cannot be used for evidence in the infection of HIV or the progression of AIDS.

Here, we chose the HIV-1 Tg rat as the HAND animal model to assess the effect of 17αE2 on endolysosome and dendritic spines. HAND individuals and HIV-1 Tg rats present with inflammatory and neuropathological features (Lashomb et al., 2009; Vigorito et al., 2015). HIV-1 Tg rats has been shown to exhibit HAND-like gene expression profiles, synaptodendritic damage in various brain regions (hippocampus, striatum, and pre-frontal cortex), and behavioral deficits (Reid et al. 2001, Lashomb et al. 2009, Li et al. 2013, Moran et al. 2013, Roscoe et al. 2014, Festa et al. 2015, Vigorito et al. 2015, Reid et al. 2016, McLaurin et al. 2018, McLaurin et al. 2018). Furthermore, the non-infectious HIV-1 Tg model display immune dysfunction and controlled viral replication similar to cART-treated HIV patients (Peng et al., 2010). The effects of 17αE2 on endolysosome size and dendritic spines in HIV-Tg rats was investigated.

## Methods

### Animals

Male and female non-infectious HIV transgenic rats and their controls (F344) were obtained from Envigo RMS. A total of 55 rats were used: 14 male F344 rats, 13 male HIV-1 Tg rats, 14 female F344, and 14 female HIV-1 Tg. The rats

were group-housed in an animal care facility and kept at 23°C with a 12 h light/dark cycle. All animal procedures were performed in accordance with approved protocols from Seton Hall University (IACUC protocol # SC1701). Animals were trained to eat pure chocolate-flavored hazelnut cream with sesame oil with and without 17αE2 (30 ug 17α-estradiol/ 5 ul sesame oil/1 g Nutella cream/kg body weight/day). The feeding duration was 21 days. On day 22, 24 hours after the last feeding, animals were then sacrificed. The rats were 13 months old when they were sacrificed.

**Immunohistochemistry**

Rat brains were removed from perfused rats (4% PFA), placed overnight in 4% PFA at 4 °C, placed in 15% sucrose until equilibrated, and replaced in 20% sucrose. The tissue was cut into 10 μm sagittal sections on a Leica CM1520, where they were stored at -80°C until stained. The slides were washed in PBS and blocked for an hour in blocking solution (3% BSA, 1% normal goat serum, and 0.05 Triton X-100). Primary antibodies were added and left at 4 °C overnight. The following primary antibodies were used: MAP2 (1:100, Abcam 92434), LAMP1 (1:200, Abcam 24170), Rab7 (1:100, Abcam ab137029), ERα (1:50, sc-8005, sc-8002 Santa Cruz). The antibodies were washed with PBS-T (Tween-0.01%) for 5 mins and washed with PBS twice. Secondary antibodies (Alexa Fluor 488, Alexa Fluor 594) were added for 1 hour, then washed with PBS. The tissue was mounted with Vectashield Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories). Images were taken on a confocal Zeiss LSM800

microscope using ZEN acquisition software, and z-stacks at intervals of 0.5 μm were acquired. Imaris 9.6 (Bitplane, Oxford Instruments, MA, USA) was used to calculate colocalization and lysosome volume. Endolysosome volume was calculated by using the Imaris spot module to recreate the LAMP1 vesicles. Pearson's colocalization coefficient of ERα with Rab7 or ERα with LAMP1 were calculated using the colocalization module in Imaris. Images of different hippocampi regions (CA1-CA3, DG) from at least 2 animals per group were acquired for analysis.

**Golgi-Cox Dendritic Spines**

To stain for dendritic spines, the Golgi-Coz method was used by adapting the method from rats that were anesthetized and perfused transcardially with PBS, decapitated, and the brain was removed. Half of the brain was placed in Golgi-Cox solution for 24 hours at RT in the dark. The solution was replaced after 24 hours and left for 10 days at RT. The brains were placed in a protectant solution for 24 hours at 4°C, and then the protectant solution was replaced for 7 days. The brains were placed in molds and cut using a vibratome (Lecia) into 100 μm sagittal sections. The sections were washed twice with distilled water, 50% ethanol for 5 mins, and incubated in ammonia for 8 mins. This was followed by 2 washes of distilled water for 5 mins each and then incubated in 5% sodium thiosulfate for 10 min in the dark, and then washed with distilled water. The sections were dehydrated with progressively higher levels of ethanol; 50%, 75%, 90%, and 100%. The tissue was then cleared in xylene twice, mounted onto

gelatin-coated slides, and kept in the dark before the staining process with a permount mounting medium (SP15-100, Fisher Scientific). Images were acquired using an Olympus BX-63 upright microscope with 100X, 63X, and 2X objectives with 0.2 1 μm z-stacks. The images were analyzed using Neurolucida 360 software (MBF Bioscience, Williston, VT). Dendrites were detected using the user-guided tracing mode, and spines were detected both automatically and then observed manually for adjustments. The spine classifications were the same as used in IMARIS to ensure all experiments follow the same parameters. The data was saved and opened in Neurolucida Explorer, and the analysis was performed and exported into Excel. GraphPad Prism 9.0 was used for statistical analyses. Data from 2 animals per treatment group were imaged from the hippocampi region.

**Statistical Analyses**

All data was expressed as means and SEM. Statistical analyses was performed using GraphPad Prism 9.0 software (GraphPad Software, Inc.). Statistical significance was calculated by either Student's *t*-test between two groups, one-way analysis of variance or two-way analysis of variance between groups among multiple groups with Tukey *post-hoc* tests. Pearson's correlation coefficient was used to determine the colocalization between ERα and LAMP1 or ERα and Rab7. In vivo experiments, 'n' refers to the number of images acquired from each hippocampus area from each animal per group. A p value of $< 0.05$ was considered to be significant.

## Results

### Co-Distribution of ERα with Endolysosomes in Rat Hippocampus

ERα has been shown to be expressed in the brain (Warfvinge et al., 2020) in the human hippocampus in neurons (Heron et al., 2009) on plasma membranes and endolysosomes (Levin, 2009; Totta et al., 2014). We have shown the presence of ERα on endolysosomes in primary cultured neurons. Here, we first confirmed the presence of ERα on endolysosomes in rat hippocampus. Male and female controls (F344) were double immunostained for ERα and Rab7 or LAMP1. The degree of colocalization was determined using Pearson's correlation coefficient in CA3, CA3, and DG (dentate gyrus) in the hippocampus. Consistent with our in vitro findings that ERα is primarily localized on Rab7 positive endosomes, we demonstrated that ERα and Rab7 colocalize to a greater extent than ERα and LAMP1 in all hippocampal brain regions (Figure 40 A&B). Therefore, ERα is located on endolysosomes in the hippocampus of rats.

### 17αE2 Protected Against Endolysosome Enlargement in the Hippocampus of HIV-1 Tg Rats

Previously, morphological increases in the size of lysosomes have been recorded in people with AIDS (Gelman et al., 2005) and in primary hippocampal neurons treated with HIV-1 Tat (Hui et al., 2012). We have shown that 17αE2 prevents gp120- and Tat-induced endolysosome enlargement in cultured neuron and wanted to see 17αE2 effects in vivo. Male HIV Tg rats and their controls

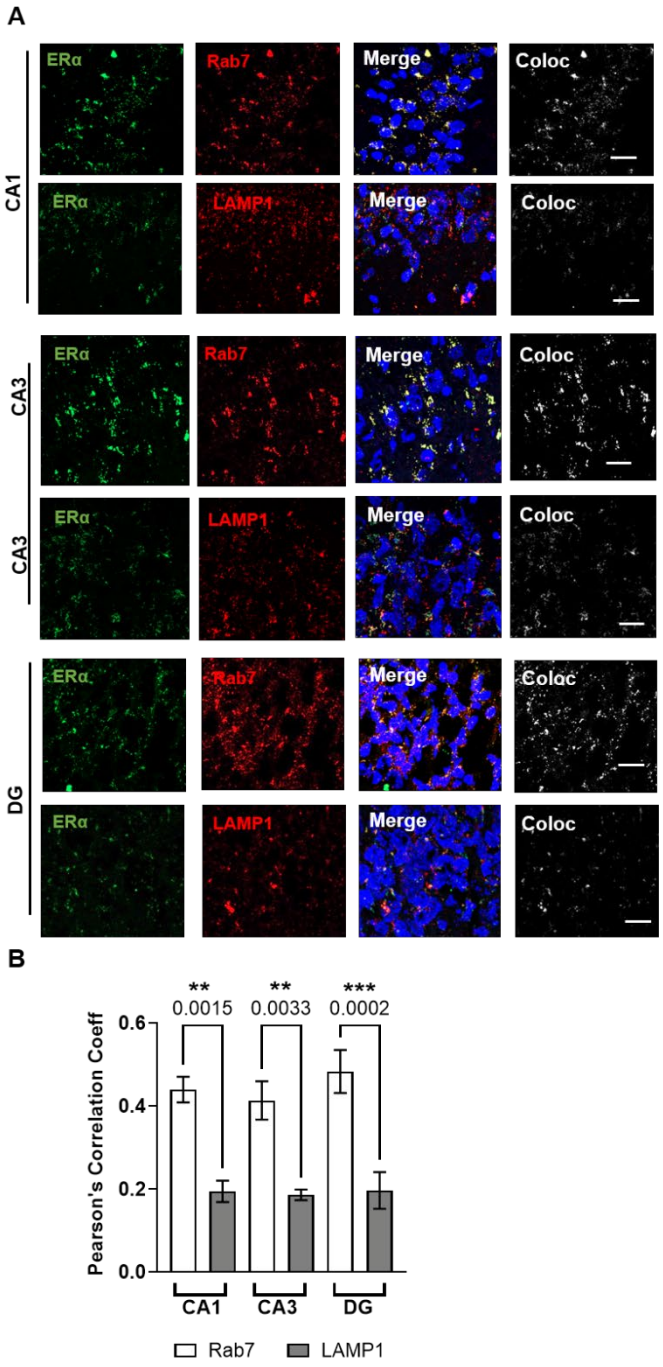



Figure 40. Co-distribution of ERα with endolysosomes in the rat hippocampus. **(A)** Representative confocal images shows the co-distribution of ERα (green) with Rab7-positive (red) or LAMP1-positive endolysosomes in CA1, CA3, and GD regions of hippocampus from control (F344) rats (male and female). DAPI was used to stain the nucleus. Scale bar-20 µm. **(B)** Bar graph shows a higher colocalization of ERα with Rab7 than that with LAMP1 in all regions. Data were presented as mean ± SEM (n=4 animals, **p<0.01, ***p<0.001, one-way ANOVA).

(F344) were double immunostained for DAPI, and LAMP1, and images of the CA1, CA3, and DG hippocampal sections were taken. The mean lysosome volume was calculated and found that HIV-1 Tg rats had a significantly higher volume than control rats. HIV-1 Tg rats treated with 17αE2 were also shown to significantly decrease the mean volume compared to HIV-1 Tg rats with no treatment (Figure 41B).

**17αE2 Protected Against Dendritic Impairments in the Hippocampus of Male HIV-1 Tg Rats.**

Earlier evidence has shown that Tat and gp120 can induce synaptic impairment in primary hippocampal neurons. HIV-1 Tg rats display neuropathology that includes neuronal loss, BBB breakdown, synaptodendritic damage in the hippocampus, striatum, and the prefrontal cortex deficits (Reid et al. 2001, Lashomb et al. 2009, Li et al. 2013, Moran et al. 2013, Roscoe et al. 2014, Festa et al. 2015, Vigorito et al. 2015, Reid et al. 2016, McLaurin et al. 2018, McLaurin et al. 2018). 17αE2 increases spine density by the formation of new spines (Luine & Frankfurt, 2012; MacLusky, Hajszan, & Leranth, 2005; Sengupta et al., 2019). Therefore, we wanted to determine the protective effects of 17αE2 in HIV-1 Tg rats. Dendritic spines were visualized with Golgi-Cox staining in male HIV Tg rats and their controls (F344). Neurolucida 360 software was used to identify the dendritic spines in 3D following published protocols (Ruszczycki et al., 2012). The dendritic length decreased in HIV-1 Tg without treatment. Pretreatment with 17αE2 prevented the dendritic length decreases in HIV-1 Tg rats (Figure 42A).

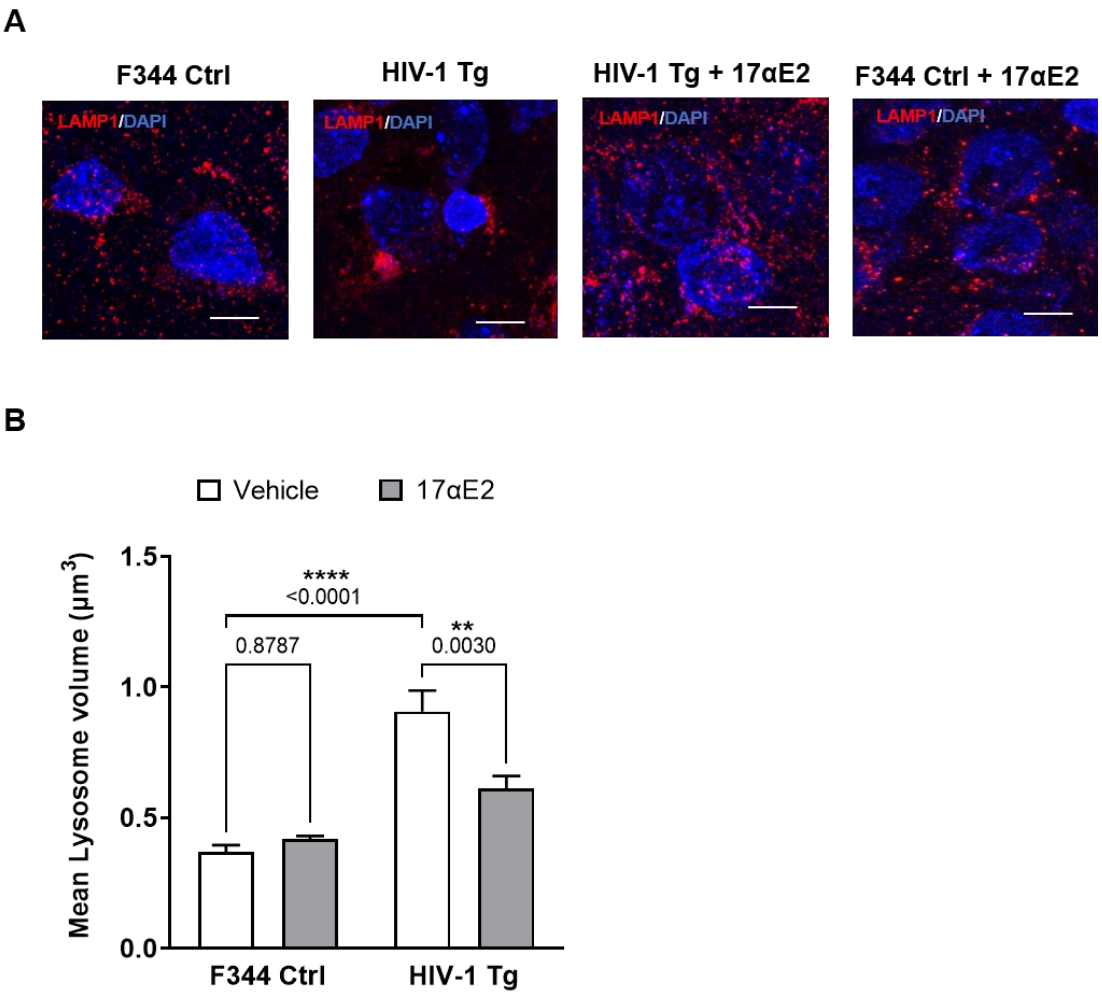



Figure 41. 17αE2 protected against endolysosome enlargement in hippocampus of HIV-1 Tg rats.
**(A)** Representative confocal images show LAMP1 (red) positive endolysosomes and the nucleus (DAPI)in hippocampal (CA1 and CA3) brain regions. Scale bar-20 µm. **(B)** Bar graph shows that LAMP1 positive endolysosomes (red) in CA1 and CA3 regions of HIV transgenic rats were enlarged compared to controls. Scale bar-20 µm. Data were presented as mean ± SEM, (n=10 images from 2 animals, $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, two-way ANOVA).

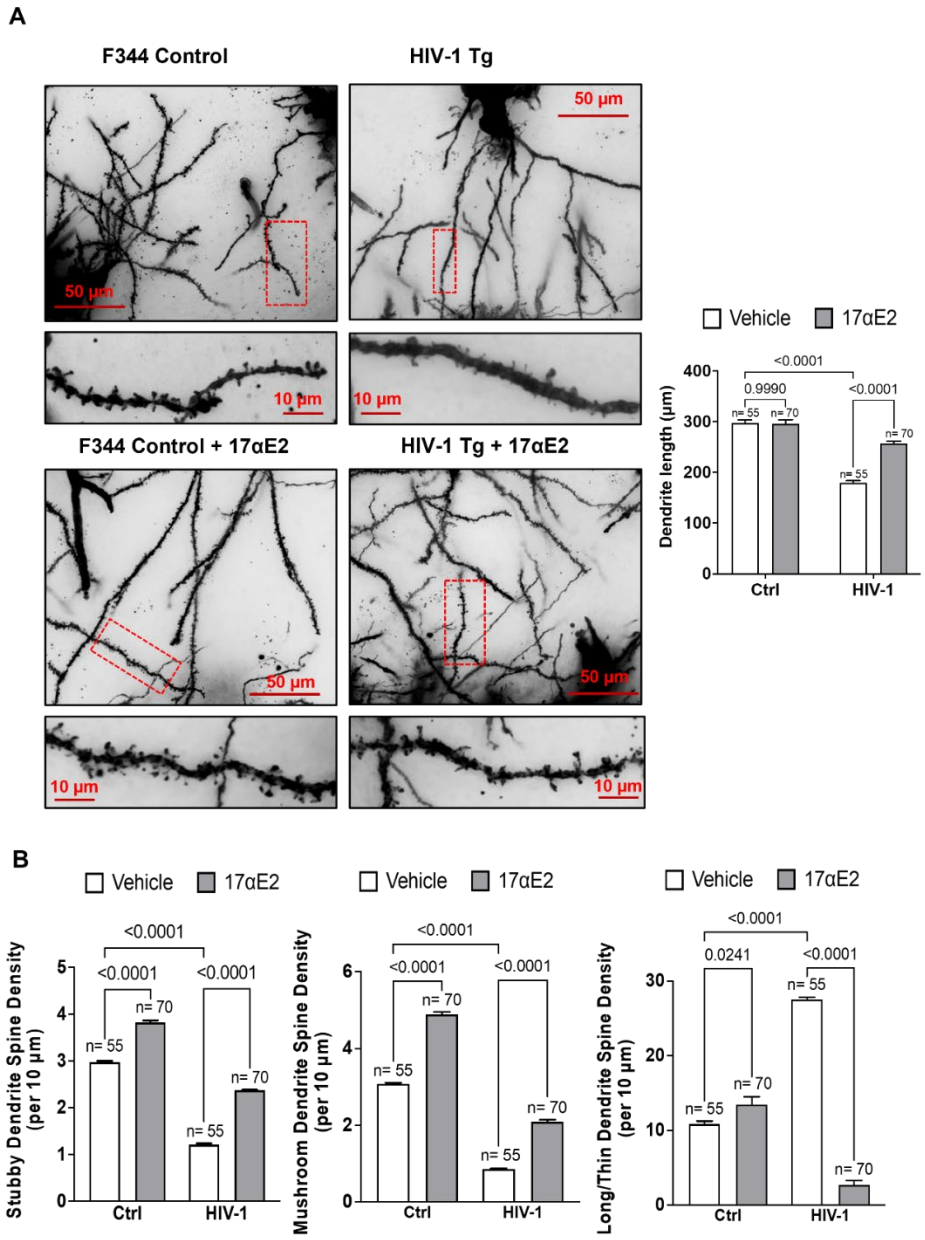



Figure 42. 17αE2 protected against dendritic impairments in the hippocampus of male HIV-1 transgenic rats.
**(A)** Golgi-Cox staining of the neuronal dendritic spines are shown as representative images. The bottom panel is enlarged to show the specific dendritic spines of each group. HIV-1 Tg rats demonstrated decreases in the dendritic length while pretreatment with 17αE2 treatment prevented the decrease in dendritic length. **(B)** Bar graphs showed that in HIV-1 Tg rats without 17αE2 treatment, has a lower density of all spine classes. HIV-1 Tg rats with 17αE2 treatment experienced an increase in stubby and mushroom spines and a decrease in long thin spines. Data were presented as mean ± SEM. (n= number of neurons from 2 male animals, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001,n.s.- not significant, two-way ANOVA).

Given that HIV-1 Tg rats have been shown to exhibit impairments in subtypes of dendritic spines (Kristen A. McLaurin, Li, Booze, Fairchild, & Mactutus, 2018), we further explored the possibility that 17αE2 could attenuate impairments in subtypes of dendritic spines in HIV-1 Tg rats. Control rats with the 17αE2 treatment had increased stubby, mushroom, and long thin than control rats without treatment. In HIV-1 Tg rats without 17αE2 treatment, demonstrated a decrease in the stubby and mushroom spines but an increase in long thin. In HIV-1 Tg rats with 17αE2 treatment, the stubby and mushroom spines were significantly increased compared to HIV-1 rats without treatment. In HIV-1 Tg rats with 17αE2 treatment, the long thin spines significantly decreased compared to HIV-1 rats without treatment (Figure 41B).

## Discussion

HIV-1 associated neurocognitive impairments show sex-specific progression (Rubin et al., 2019; Sundermann et al., 2018). Cognitive decline is associated with decreased estrogen levels once HIV-positive women go through menopause (Maki et al., 2021).

Estrogen protects against neuronal injury (Adams, Aksenova, Aksenov, Mactutus, & Booze, 2010; Baker, Brautigam, & Watters, 2004; Corasaniti et al., 2005; Kendall et al., 2005; Kristen A. McLaurin, Moran, Booze, & Mactutus, 2020), and suppresses HIV-1 viral replication (Bick & Hapgood, 2018; Bourinbaiar, Nagorny, & Tan, 1992; B. Das et al., 2018; Heron et al., 2009; Rodriguez-Garcia et al., 2013; Szotek et al., 2013; Wilson, Allred, et al., 2006).

However, most of published studies have used 17βE2. Here we have treated HIV-Tg rats with 17αE2 to determine the morphology of endolysosomes, changes in dendritic spine density, and altered morphology of dendritic spines.

First, we double immunostained for ERα with Rab7 or LAMP1 on rat brains to determine if ERα colocalizes with endolysosomes. Similar to cell culture results, ERα colocalizes to a greater extent on Rab7 positive vesicles over LAMP1 positive vesicles. Therefore, confirming that ERα is localized to endolysosomes in the hippocampus allowing 17αE2 to interact with ERα.

HIV-1 infected individuals with HAND have documented endolysosome dysfunction (Cysique et al., 2015; Gelman et al., 2005; Spector & Zhou, 2008; Zhou & Spector, 2008). HIV-1 viral protein, including Tat and gp120, as well as ART drugs, have shown to induce endolysosome dysfunction (Bae et al., 2014; Chen et al., 2013; Datta et al., 2019; Fan & He, 2016; J. Fields et al., 2015; Peter W. Halcrow et al., 2021; Hui et al., 2012; Hui et al., 2021). Consistently, we demonstrated that endolysosomes are enlarged in the hippocampal brain regions of HIV-1 Tg rats. This could be indicative of endolysosome dysfunction possibly due to the deacidification leading to impaired endolysosome function. Furthermore, we demonstrated that HIV-1 Tg rats treated with 17αE2 prevent the enlargement of endolysosomes, which could be from 17αE2 re-acidifying the endolysosome pH.

In the postmortem prefrontal cortexes of individuals with HAND, neurons were beaded. Golgi-Cox staining was also completed and the dendritic spines were substantially diminished compared to HIV-negative individuals (R. Ellis et al., 2007). HIV-1 Tg rats are a model for HAND in terms of neurocognitive impairments and alterations in spine density (Reid et al., 2001a; Royal et al., 2012).

Consistently we show that HIV-1 Tg rats had decreased dendritic length and treatment with 17αE2 significantly prevented the decreases in dendritic length in HIV-1 Tg rats. HIV-1 Tg rats develop alterations in spine morphologies and subtypes of dendritic spines (Kristen A. McLaurin et al., 2018). In our research, we find that HIV-1 Tg rats without treatment, have increased immature spines and decreased percentage of mature spines.17αE2 was able to produce more mature spines and lead to a decrease in the immature spines in HIV-1 Tg rats. Thus, 17αE2 is able to protect against alterations in the spine morphology developed in HIV-1 Tg rats.

Given the complexity of dendritic spine plasticity, along with the damage induced by the HIV-1 virus, viral proteins, ART drugs, and sex differences demonstrate that multiple factors are at play. Thus, more studies need to be completed to determine the underlying mechanism of 17αE2 and if 17αE2 can improve cognition and memory in HIV-1 Tg rats. Nonetheless, 17αE2 represents as a promising novel therapeutic strategy against HAND.

# CHAPTER VI

# DISCUSSION

## Summary of Findings, Conclusion, Limitations, and Future Directions

### Summary of Findings

During my graduate career, I investigated the role of endolysosome dysfunction in the development of HAND and explored new therapeutic strategies. Specifically, I investigated the effects of Tat and gp120-induced effects on primary cells determine if these proteins lead to endolysosome dysfunction and synaptodendritic impairment, both of which are observed in HAND individuals (Cysique et al., 2015; Gelman et al., 2005; Spector & Zhou, 2008; Zhou & Spector, 2008) (Kristen A. McLaurin et al., 2018). While researching potential therapeutic strategies, I found that HIV-1 infected women have more neurocognitive issues, especially after menopause (Maki et al., 2021). This data led me to investigate if hormonal imbalances could lead to the progression of HAND. From here, I investigated ERT and HRT studies in women with neurodegenerative diseases in which estrogen is associated with better cognitive outcomes. Literature also suggests that 17βE2 could serve as a potential therapy; however, the feminizing effects and side effects of 17βE2 limits its potential use as a therapy in a larger population. 17$\alpha$E2 is a non-feminizing

estrogen with the same neuroprotective effect as 17βE2. Therefore, I investigated 17αE2 as a therapeutic drug.

In Chapter II, I demonstrated that HIV-1 viral proteins gp120 and Tat led to endolysosome dysfunction as evidenced by de-acidifying endolysosome pH, decreasing the percentage of CatD, and enlargement of endolysosomes. HIV-1 viral proteins gp120 and Tat also lead to decreased dendritic spine density and alterations in the morphology of dendritic spines. Next, we decided to determine if 17αE2 could prevent gp120 and Tat-induced damage.

In Chapter III, 17αE2 was shown to enhance the function of the endolysosome and 17αE2 prevented the effects of HIV-1 gp120 and Tat-induced endolysosome dysfunction. 17αE2 also enhanced the percentage of mature spines and prevented HIV-1 gp120 and Tat-induced reductions in spine density. This research demonstrated the possibility of 17αE2 being a therapeutic drug in the protection of viral protein-induced damage. From here, we wanted to determine if the ERα was responsible for these protective effects.

In Chapter IV, ERα presence on endolysosomes was shown to be necessary for the neuroprotective effects of 17αE2 against HIV-1 gp120 and Tat. ERα colocalized mostly to Rab7 endolysosomes in primary hippocampal neurons. The knockdown of ERα attenuated 17αE2-induced acidifying effects on Rab7-positive endolysosomes and prevented 17αE2-induced enhancing effects on endolysosome function. Knocking down ERα also prevented 17αE2-induced

increases in dendritic spine density and in mitigating the reduction in spine density against HIV-1 Tat and gp120. Given that ERα palmitoylation was necessary for targeting it to endolysosomes, and we demonstrated that over-expressing the palmitoylation deficient mutant ERα was able to block the enhancing effect of 17αE2 on endolysosomes and dendritic spines as well as the ability of 17αE2 to protect against gp120 and Tat-induced endolysosome dysfunction and alteration in dendritic spines, therefore concluding that endolysosomal ERα is important in the neuroprotective effects of 17αE2. Finally, we wanted to determine if 17αE2 is protective in HIV-1 Tg rats.

In chapter V, ERα was shown to be localized on endolysosomes in the neurons of the hippocampus of rat brains. 17αE2 protected against endolysosome enlargement in the hippocampus of HIV-1 Tg rats. 17αE2 was unable to increase total spine density of HIV- Tg rats. However, 17αE2 increased the percentage of mature spines while decreasing the percentage of immature spines, concluding that 17αE2 may prevent endolysosome dysfunction in vivo and prevent altered morphology of dendritic spines in HIV-1 Tg rats.

**Conclusions**

Here, we demonstrated, in primary cultured neurons and in HIV-1 Tg rats, that endolysosome dysfunction, as induced by HIV-1 viral proteins (gp120 and Tat), plays an important role in the development of impairments in dendric spines. Further we demonstrated that 17αE2 is protective against gp120 and Tat-induced endolysosome dysfunction and synaptodendritic impairment, and such protective

effect of 17αE2 is mediated via ERα that is localized on endolysosomes. Considering the complexity of dendritic spine plasticity along with the functional compartmentalization of different neuronal compartments, and not without caveats, these findings suggest strongly that endolysosome dysfunction leads to dendritic spine impairment in HAND. Our findings provide novel mechanistic insight on the neuroprotective effects of 17αE2, which may serve a potential treatment for preventing the development of HAND.

**Limitations**

Findings from studies conducted in cultured neurons showed that endolysosome localization of ERα was critical for the enhancing effects of 17αE2 on endolysosomes and dendritic spines and for the protective effects of 17αE2 against gp120- and Tat-induced endolysosome dysfunction and alterations in dendritic spines. However, we did not provide such strong mechanistic evidence in HIV-1 Tg rats. This could be overcome by using ERα knockout animals or transgenic animals that over-express an ERα mutation that lacks endolysosome localization.

In the animal studies, we primarily focused on male rats to minimize the role of endogenous 17αE2. The male rats, however, could have been gonadectomized and adrenalectomized to eliminate endogenous 17αE2 levels. Sex differences in the neuroprotective role of 17αE2 against HIV-1 induced synaptodendritic damage were not determined in this study, as only the data from male rats have been analyzed independently of female rats. Comparing the data from the male

rats with that from ovariectomized female rats would have given a more accurate picture of possible sex differences in 17αE2 induced neuroprotection. Brain-specific delivery techniques would have to be investigated either by intranasal administration or delivery with nanoparticles to ensure 17αE2 reaches site of action at pharmacologically relevant doses. Ideally, the serum levels and brain levels of 17αE2 would be measured.

Another limitation is that we focus only on direct neurotoxic effect of HIV-1 viral proteins (gp120 and Tat). But both gp120 and Tat can lead to neuronal injury via indirect mechanisms like neuroinflammation. Similarly, 17αE2 could also exert neuroprotective effects by inhibiting neuroinflammation.

While 17αE2 is a promising treatment for both men and women infected with HIV, more studies need to be developed to determine the effects of 17αE2, along with potential side effects. Sex differences, ART interactions, the active virus, and viral proteins all need to be investigated before 17αE2 can move forward into a potential treatment for HIV-infected individuals.

**Future Studies**

HIV-1 Tg rats and controls with and without 17αE2 would need to be repeated to obtain a higher number of animals to determine if memory and learning are impacted with 17αE2, along with producing ERα knockout or genetically modified ERα animals to determine if ERα is responsible for the protection of 17αE2. For example, gp120 transgenic mice could be crossed with mice with knockout ERα

to potentially study the combined effects of 17αE2 induced neuroprotection against HIV-1 induced synaptodendritic damage. Animals would need to have endogenous estrogen removed; therefore, males would have their adrenal glands and testes removed. Females would have their adrenal glands and ovaries removed to ensure the only major source of estrogen is brain de novo synthesis and the treatment. In addition, involvement of neuroinflammation could be explored. Furthermore, it would also be interesting to see if 17αE2 could potentially restore (to a clinically significant extent) the neurocognitive deficits observed in late-stage MNI or HAND patients.

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# APPENDIX A

## ABBREVIATIONS

| | |
|---|---|
| 17αE2 | 17α-estradiol |
| 17βE2 | 17β-estradiol |
| HSD17B | 17β-hydroxysteroid-dehydrogenases |
| AIDS | Acquired Immunodeficiency Syndrome |
| AD | Alzheimer's disease |
| AβPPswe | Amyloid-β protein precursor |
| ANI | Asymptomatic neurocognitive impairment |
| AZT | Azidothymidine |
| BBB | Blood-brain barrier |
| CatD | Cathepsin D |
| CDC | Center for Disease Control |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| cART | Combined antiretroviral therapy |
| DG | DG |
| EFV | Efavirenz |
| Endolysosomes | Endosomes and Lysosomes |
| C451A | ERα mutant |
| ERα | Estrogen receptor alpha |
| ERβ | Estrogen receptor beta |
| ERT | Estrogen replacement therapy |
| FBS | Fetal bovine serum |
| HAD | HIV acquired dementia |
| HAND | HIV-associated neurocognitive disorder |
| HRT | Hormone replacement therapy |
| HD | Huntington's disease |
| LMPs | Lysosomal membrane proteins |
| MMP-9 | Matrix metalloproteinase 9 |
| MND | Mild neurocognitive disorder |
| NMDA | N-methyl-D-aspartate |
| NNRTI | Non-nucleoside reverse transcriptase inhibitor |
| NRTI | Nucleoside reverse transcriptase inhibitors |
| PD | Parkinson's disease |
| PSD | Postsynaptic density |

| | |
|---|---|
| PS1ΔE9 | Presenilin 1 |
| PI | Protease inhibitor |
| ERα scr | Scrambled siRNA |
| SHBG | Sex hormone-binding globulin |
| ERα KD | siRNA against ERα |
| TAR | Transactivator response element |
| Tg | Transgenic |
| TRPML1 | Transient receptor potential mucolipin 1 |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |

# APPENDIX B

| Reagents and Drugs | Company |
|---|---|
| CLU199 Mouse hippocampal cell line | Cedarlane, Ontario, Canada |
| 10x tris/glycine buffer | Bio-Rad Laboratories, Hercules, CA |
| 17αE2 | Tocris Biosciences Minneapolis, MN |
| 2-Methylbutane | Sigma-Aldrich, Saint Louis, MO |
| Accell1 transfection media | Dharmacon, Lafayette, CO |
| Alexa Flour Phalloidin 594 | Invitrogen, Waltham, MA |
| Ammonia Hydroxide | Fisher Scientific, Waltham, MA |
| B27 supplement | GIBCO BRL, Grand Island, NY |
| BacMam GFP Transduction Control | Invitrogen, Waltham, MA |
| Beta-mercaptoethanol | Sigma-Aldrich, Saint Louis, MO |
| BODIPY-FL Pepstatin A | Invitrogen, Waltham, MA |
| Bovine Albumin | Sigma-Aldrich, Saint Louis, MO |
| CellLight™ Late Endosomes-RFP | Thermo Fisher Scientific, Waltham, MA |
| CellLight™ Lysosome-RFP | Thermo Fisher Scientific, Waltham, MA |
| DC protein assay | Bio-Rad Laboratories, Hercules, CA |
| DharmaFECT 1 | Dharmacon, Lafayette, CO |
| DMEM | GIBCO BRL, Grand Island, NY |
| DMSO | Sigma-Aldrich, Saint Louis, MO |
| Fetal Bovine serum | Altana Biologicals, Atlanta GA |
| Normal Goat Serum | Invitrogen, Waltham, MA |
| Hibernate E Low Fluorescence Medium | Brainbits LLC, Springfield, IL |
| HIV Tg rats | Envigo RMS, Indianapolis, IN |
| HIV-1 gp120 | ImmunoDx LLC, Woburn, MA |
| HIV-1 Tat | ImmunoDx LLC, Woburn, MA |
| L-glutamine | GIBCO BRL, Grand Island, NY |
| LiCor blocking solution (TBS) | LI-COR Biosciences, Lincoln, NE |
| Lipofectamine 2000 transfection Reagent | Invitrogen, Waltham, MA |
| Lysis buffer with NP-40 | Sigma-Aldrich, Saint Louis, MO |
| LysoSensor (Yellow/Blue DND-160) | LysoSensor (Yellow/Blue DND-160) |
| LysoTracker Red DND-99 | Invitrogen, Waltham, MA |
| NbActiv1 media | Brainbits LLC, Springfield, IL |
| Opti-MEM Reduced Serum media | GIBCO BRL, Grand Island, NY |
| PageRuler™ Prestained Protein Ladder | Thermo Fisher Scientific, Waltham, MA |
| Paraformaldehyde | Sigma-Aldrich, Saint Louis, MO |
| Penicillin/streptomyocin | GIBCO BRL, Grand Island, NY |
| Permount mounting medium | Fisher Scientific, Waltham, MA |
| pH calibration kit | Thermo Fisher Scientific, Waltham, MA |
| pH insensitive Texas Red Dextran 10 kDa | Invitrogen, Waltham, MA |
| pH sensitive pHrodo Green Dextran10 kDa | Invitrogen, Waltham, MA |
| Phosphate-Buffered Saline Tablets | Invitrogen, Waltham, MA |
| Plasmids for Transfection | Origene Technologies, Rockville, MD |
| Polyvinylidene difluoride membranes | Millipore-Sigma, Burlington, MA |

| | |
|---|---|
| Precision Red Advanced Protein Assay | Cytoskeleton Inc., Denver, CO |
| Primary mouse hippocampal neurons | Brainbits LLC, Springfield, IL |
| Precast 4-16% gels, Tris-Glycine | Bio-Rad Laboratories, Hercules, CA |
| ProLong Gold Antifade | Thermo Fisher Scientific, Waltham, MA |
| Protease Inhibitor Cocktail | Pierce Biotechnology, Rockford, IL |
| RIPA lysis buffer | Thermo Fisher Scientific, Waltham, MA |
| Sodium thiosulfate | Thermo Fisher Scientific, Waltham, MA |
| Sucrose | J.T. Baker, Radnor, Pennsylvania |
| TransAM ER assay Kit | Active Motif, Carlsbad, CA |
| Triton X-100 | Invitrogen, Waltham, MA |
| Tween-20 | Sigma-Aldrich, Saint Louis, MO |
| Vectashield Antifade Mounting Medium with DAPI | Vector Laboratories, Burlingame, CA |
| Xylene | Sigma-Aldrich, Saint Louis, MO |