HIV-1 TAT AFFECTS INTERORGANELLE COMMUNICATION IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS (HAND)

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ABSTRACT

Among Human Immunodeficiency viruses (HIV), type-1 (HIV-1) is the most common worldwide and has the highest virulence and infectivity. Though the virus only infects a few cell types, the infection affects almost every organ system causing multiple comorbidities. One of the comorbidities is HIV-associated Neurocognitive Disorders (HAND). Interorganelle communication regulates many cellular functions including calcium exchange, lipid exchange, intracellular trafficking, and mitochondrial biogenesis. Interestingly, all these processes have been implicated in HAND suggesting that dysregulation of interorganelle communication plays a role in the progression of HAND. Using neuronal cell cultures, I show that mitochondrial-associated ER membranes (MAM)-associated protein and MAM-tethering protein expression and interactions are altered in the presence of the HIV-1 protein Tat. I also show, PTPIP51 and VAPB, two MAM-tethering proteins, expression is altered in the MAM fraction but not the whole cell fraction, indicating a localization problem. Phosphorylation of PTPIP51 has been shown to regulate the subcellular localization and I show that tyrosine phosphorylation is upregulated with Tat. In addition, I show that PTPIP51 binding with VAPB can be rescued with the addition of kinase inhibitors blocking PTPIP51 phosphorylation suggesting that Tat is altering the phosphorylation of PTPIP51 affecting its subcellular localization and binding to VAPB. Furthermore, I show that ER-Golgi communication is altered in the presence of Tat where there is an increase in the interactions between YIF1A and VAPB, two ER-Golgi tethering proteins. The altered

interactions between MAM and ER-Golgi tethering proteins in the presence of Tat lead

to the disruption of cellular pathways associated with dysfunctional interorganelle

communication that can lead to neuronal dysfunction and can contribute to HAND.

DEDICATION

To my friends and family, I hope I make you proud.

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LIST OF ABBREVIATIONS

43S PIC	43S Pre-Initiation Complex
aa	Amino Acid
ACAT1	Acetyl-CoA Acetyltransferase
AD	Alzheimer's Disease
AD	Alzheimer's Disease
AIDS	Acquired Immunodeficiency Syndrome
ALS	Amyotrophic Lateral Sclerosis
ANI	Asymptomatic Neurocognitive Impairment
APOBEC3G	Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3G
APP	Amyloid Precursor Protein
ART	Antiretroviral Therapy
$A\beta$	Amyloid β
Bap31	B-cell Receptor-Associated Protein 31
BBB	Blood Brain Barrier
CA	Capsid Protein
Ca^{2+}	Calcium
cART	Combination Antiretroviral Therapy
CCR5	C-C Motif Chemokine Receptor 5
CDC	Center for Disease Control and Prevention

CDK9	Cyclin Dependent Kinase 9
CERT	Ceramide Transfer Protein
CL	Cardiolipin
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
CXCR4	C-X-C Motif Chemokine Receptor 4
Drp1	Dynamin-Related Protein 1
EAAT2	Excitatory Amino Acid Transporter-2
ER	Endoplasmic Reticulum
FACL4	Fatty Acid-CoA Ligase 4
Fis1	Mitochondrial Fission 1
FTD	Fronto-Temporal Dementia
Grp75	Glucose-Regulated Protein 75
GSK-3β	Glycogen Synthase Kinase-3β
HAD	HIV-Associated Neurocognitive Dementia
HAND	HIV-Associated Neurocognitive Disorder
HIV	Human Immunodeficiency Virus
HIV-1	HIV Type 1
HIV-2	HIV Type 2
IHDS	International HIV Dementia Scale
IL-1β	Interleukin 1β
IL-6	Interleukin 6

IL-8	Interleukin 8
IN	Integrase
IP$_3$	Inositol Trisphosphate
IP$_3$R	Inositol Trisphosphate Receptor
LC3	Microtubule Associated Protein 1 Light Chain 3 B
LDL	Low-Density Lipoprotein
MA	Matrix Protein
MAMs	Mitochondrial-Associated ER Membranes
MAO-B	Monoamine Oxidase B
MAPK	Mitogen Activated Protein Kinase
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MMSE	MiniMental State Exam
MND	Mild Neurocognitive Disorder
MoCA	Montreal Cognitive Assessment
NC	Nucleocapsid
Nef	Negative Regulatory Factor
NLRP3	NLR Family Pyrin Domain Containing 3
NMDA	N-Methyl-D-Aspartate
OCR	Oxygen Consumption Rate
ORP10	OSBP-Related Protein 10
ORP4L	OSBP-Related Protein 4L

ORP5/8	OSBP-Related Protein 5/8
ORP9	OSBP-Related Protein 9
OSBP1	Oxysterol Binding Protein 1
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PD	Parkinson's Disease
PD	Parkinson's Disease
PE	Phosphatidylethanolamine
PI4P	Phosphatidylinositol 4-Phosphate
PIC	Pre-Integration Complex
PLEKHA8/FAPP2	Pleckstrin Homology Domain Containing A8
PolII	RNA Polymerase II
PR	Protease
PS	Phosphatidylserine
PSS1	Phosphatidylserine Synthase 1
PSS2	Phosphatidylserine Synthase 2
P-TEFb	Positive Transcription Elongation Factor B
PTPNM1/Nir2	Phosphatidylinositol Transfer Protein Membrane Associated 1
pTyr	Phosphorylated Tyrosine
RIPA	Radio-Immunoprecipitation Assay Buffer

RMND3/PTPIP51	Regulator of Microtubule Dynamics 3/Protein Tyrosine Phosphatase Interacting Protein 51
ROS	Reactive Oxygen Species
RRE	Rev Response Element
RT	Reverse Transcriptase
Sig-1R	Sigma-1 Receptor
SU	Surface Subunit
TAR	Trans-Activation Response Element
Tat	Trans-Activator of Transcription
TDP-43	TAR DNA Binding Protein 43
TLR	Toll-like Receptor
Tomm40	Translocase of Outer Mitochondrial Membrane 40
TNF-α	Tumor Necrosis Factor α
TU	Transmembrane Subunit
Tyr176	Tyrosine 176
UPR	Unfolded Protein Response
VAMP	Vesicle-Associated Membrane Potential
VAPA	VAMP-Associated Protein A
VAPB	VAMP-Associated Protein B
VDAC	Voltage-Dependent Anion Channel
Vif	Virion Infectivity Factor
YIF1A	Yip1-Interacting Factor Homologue A

CHAPTER 1

AN INTRODUCTION TO HIV-1

1.1 HIV-1 Prevalence

The Center for Disease Control and Prevention (CDC) keeps an updated HIV Surveillance Report on Human Immunodeficiency Virus (HIV) diagnoses every few years (HIV Statistics Center, Centers for Disease Control and Prevention, 2019). According to the CDC's 2019 report, around 1.2 million people in the United States have been diagnosed with HIV. In the same year, it was estimated that around 38 million people worldwide were living with HIV. The CDC also estimates that 33 million people have died from acquired immunodeficiency syndrome (AIDS)-related illness since the beginning of the epidemic. These numbers incorporate infections of both HIV type 1 (HIV-1) and type 2 (HIV-2). Of the two types of HIV, HIV-1 is more prevalent globally than HIV-2 (Campbell-Yesufu & Ganhdi, 2011). HIV-2 is primarily predominant in West Africa but there is evidence of infections in other countries including the United States (Campbell-Yesufu & Ganhdi, 2011). Therefore, for the remainder of this dissertation, I will focus on HIV-1.

1.2 HIV-1 Structure and Life Cycle

HIV-1 is a retrovirus in the *Lentivirus* genus (Ferguson, Rojo, von Lindern, & O'Brian, 2002) that carries its genomic material as RNA packaged in a viral particle (Frankel & Young, 1998). HIV-1 contains genes that are common to all retroviruses as well as some HIV-1 specific genes (Frankel & Young, 1998).

1.2.1 HIV-1 Structure

HIV-1 particles are comprised of structural proteins and packaged material (Kalinichenko, Komkov, & Mazurov, 2022). They are spherical particles made up of a lipid bilayer membrane that contains the structural glycoprotein Env (gp160) encoded by the *env* gene (Figure 1). The Env protein is made up of two subunits, the surface subunit (SU; gp120) and the transmembrane subunit (TU; gp41). The virion also contains the structural matrix protein (MA) and the capsid protein (CA), both encoded by the *gag* gene (Frankel & Young, 1998) (Figure 1). Packaged inside the capsid are the nucleocapsid proteins (NC), also encoded by the *gag* gene, which surround two copies of genomic single-stranded RNA (Kalinichenko, Komkov, & Mazurov, 2022) (Figure 1). Also inside the capsid are the proteins reverse transcriptase (RT), protease (PR), and integrase (IN) all of which are encoded by the *pol* gene (Figure 1). The proteins p6 (encoded by *gag*), virion infectivity factor (Vif), viral protein R (Vpr), and negative regulatory factor (Nef) are also packaged inside the capsid (Figure 1). All of these proteins play a role in viral entry and replication.
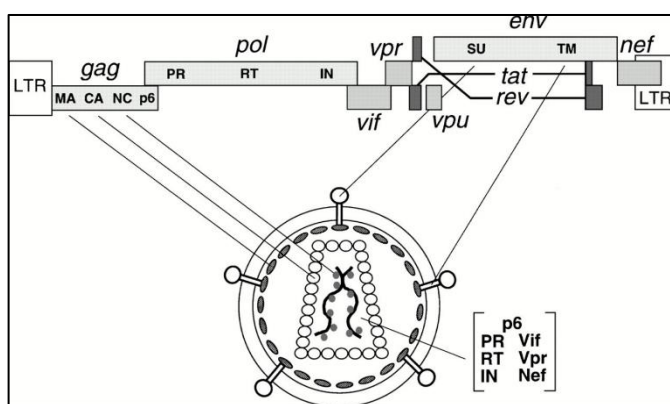
Figure 1. HIV-1 Viral RNA Genome and Structure.
Schematic of the viral genome of HIV-1 and the structure
of a single HIV-1 viral particle. (Frankel & Young, 1998).

1.2.2 HIV-1 Life Cycle

The mechanisms involved in viral replication from a single virion are called the HIV-1 life cycle. This life cycle can be broken into seven different steps: Binding to the host cell receptors, fusion of the viral membrane to the host membrane, reverse transcription of the viral RNA genome, integration of the viral DNA into the host genome, transcription and translation of viral RNA and proteins, assembly of viral proteins and new genomic RNA, and budding of mature virions.
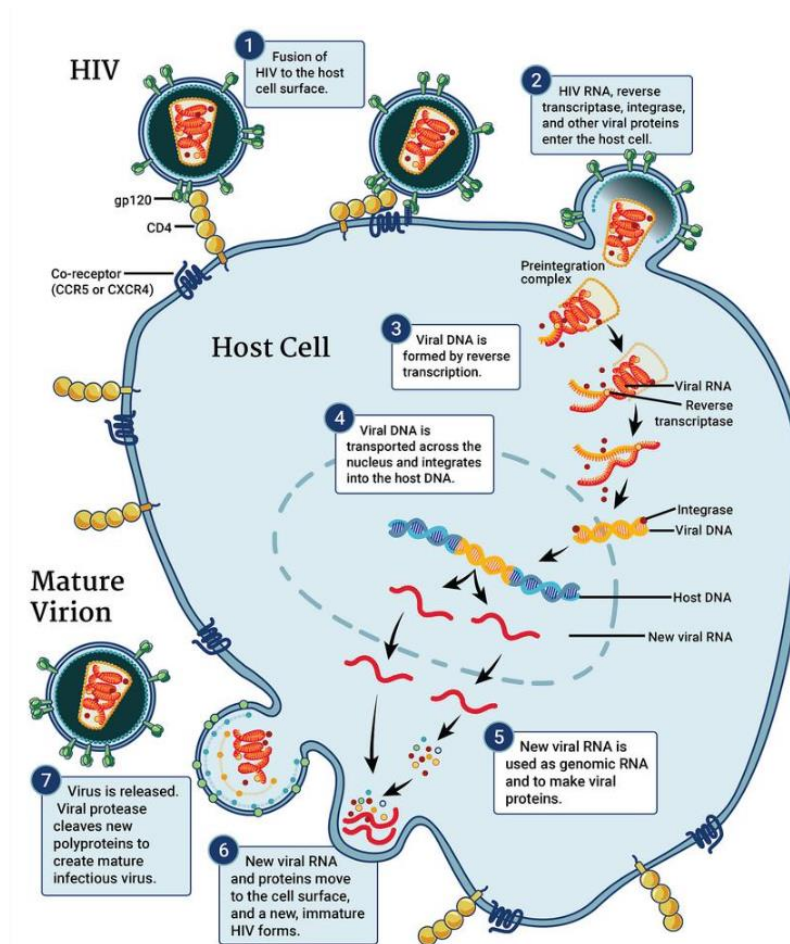
Figure 2. HIV-1 Life Cycle.
Schematic showing the viral life cycle of HIV-1 starting with 1) fusion and ending in 7) release of newly formed virions. (National Institute of Allergy and Infectious Diseases, Last reviewed 2018).

1.2.2.1 Binding to Host Receptors

An HIV-1 virion must first bind to the CD4 receptor on the surface of the host cell it is infecting before entering and replicating its genome (Fanales-Belasio, Raimondo, Suligoi, & Butto, 2010) (Figure 2). This binding is facilitated by the binding of a co-receptor, C-C motif chemokine receptor 5 (CCR5) or C-X-C motif chemokine receptor 4 (CXCR4). The HIV-1 SU (gp120) subunit of the Env (gp160) protein binds to CD4 and undergoes a conformational change that allows it to bind to a co-receptor. This binding triggers a conformational change in the TM (gp41) subunit of Env (Chen, 2019). The two subunits are cleaved from one another by host proteases (e.g. furin), and gp41 inserts into the host cell's membrane to begin fusion (Chen, 2019).

1.2.2.2 Fusion to Host Membrane

Once the gp120 and gp41 subunits of gp160 are cleaved, they remain associated with one another (Harrison, 2005). Because they no longer have a tight bond, the gp120 subunit can dissociate to circulate in the extracellular space (Harrison, 2005). The gp41 subunit then undergoes a conformational change that exposes an N-terminal fusion peptide that inserts into the cell membrane (Fanales-Belasio, Raimondo, Suligoi, & Butto, 2010). Gp41 then forms a hairpin structure that causes the viral membrane to come in close contact with the host membrane. Thus, membrane fusion can occur (Figure 2).

1.2.2.3 Reverse Transcription

Once the host cell membrane and the virion fuse, the capsid enters the cell and un-coats, releasing the internal contents (viral RNA, RT, etc.) into the cytoplasm

(Fanales-Belasio, Raimondo, Suligoi, & Butto, 2010). RT can then begin to process viral RNA (Figure 2). RT binds to viral RNA and begins at the primer-binding site to polymerize minus-strand DNA (Fanales-Belasio, Raimondo, Suligoi, & Butto, 2010). The RNA/DNA hybrid strands fold into a double helix. RT contains an RNase H domain at the C terminus (Schultz & Champoux, 2008), which then unwraps and cleaves the viral RNA from the RNA/DNA hybrid molecule. RT then binds to the minus-strand DNA to synthesize the plus-strand and form a double helix DNA molecule (Schultz & Champoux, 2008) (Frankel & Young, 1998). Integration into the host genome can then begin with the formation of the pre-integration complex (PIC) consisting of the new viral DNA, IN, and CA proteins.

Human host cells possess the antiretroviral protein, apolipoprotein B mRNA editing enzyme catalytic subunit 3G (APOBEC3G) that mutates viral DNA (Borzooee, Joris, Grant, & Larijani, 2019). These mutations can lead to mid-sequence stop codons. In addition, APOBEC3G can prevent the initiation of reverse transcription (Borzooee, Joris, Grant, & Larijani, 2019). Therefore, HIV-1 reverse transcription must overcome this immune response to create new virions. The HIV-1 protein, Vif encoded by the *vif* gene, is utilized by HIV-1 to downregulate APOBEC3G (Takaori-Kondo & Shindo, 2013). Vif has been shown to recruit E3 ligase to ubiquitinate APOBEC3G and mark it for degradation (Takaori-Kondo & Shindo, 2013). Vif is especially important in non-permissive cells such as CD4 T cells and macrophages which have high APOBEC3G expression.

1.2.2.4 Integration into Host Genome

Integration of viral DNA into the host genome is essential for the transcription and translation of viral proteins to be packaged as newly formed virions. In this sense, integration allows the virus to utilize the host cell's replication machinery to infect more efficiently. Integration is also important in HIV-1 latency and contributes to immune evasion (Lusic & Siliciano, 2017).

Vpr, encoded by the *vpr* gene, facilitates the transportation of the PIC to the host cell nucleus (Frankel & Young, 1998). In dividing cells, these viral components can enter the nucleus during prometaphase of mitosis, where the nuclear envelope is broken down (Frankel & Young, 1998). Vpr is thus necessary for nuclear entry in non-dividing cells, such as macrophages where Vpr localizes and interacts with the nuclear pore complex (NPC) (Lusic & Siliciano, 2017). Along with Vpr, some host proteins have been shown to be involved in nuclear transport as well.

Integration occurs anywhere in the host cell's genome but is mainly localized to regions that are transcribed at a high rate (Lusic & Siliciano, 2017). Within the PIC, IN tetramerizes onto the newly formed DNA molecule and removes two nucleotides from the 3' ends (Lusic & Siliciano, 2017). Removal of these nucleotides causes the 3' ends to become highly reactive with major grooves of the host DNA. Viral DNA 3' ends are then combined with host DNA 5' ends, and viral DNA is integrated into the host's genome (Figure 2).

1.2.2.5 Transcription and Translation of New Viral Components

After integration into the host cell's genome, HIV-1 viral DNA can become transcribed to produce viral mRNA (Figure 2). Transcription occurs using host cell machinery, including host RNA polymerase II (Pol II) (Liu, Wu, Shao, & Xue, 2014). Pol II begins transcription but only constructs short transcripts and is not efficient in viral RNA elongation alone. Therefore, Pol II must first transcribe accessory proteins in order to facilitate elongation (Liu, Wu, Shao, & Xue, 2014).

Trans-activator of transcription (Tat) is a protein that is encoded by the *tat* gene (Figure 1) (Clark, Nava, & Caputi, 2017). Tat aids in elongation by recruiting positive transcription elongation factor b (P-TEFb). P-TEFb is a complex comprised of cyclin-dependent kinase 9 (CDK9), cyclin T1, and other proteins (Liu, Wu, Shao, & Xue, 2014). Tat binds to P-TEFb and then binds to the transactivation response RNA (TAR) element, a stem-loop structure on viral RNA newly synthesized by Pol II (Liu, Wu, Shao, & Xue, 2014). Regulation of Tat binding to these protein complexes occurs via phosphorylation (Rice, 2017). Tat has been shown to upregulate phosphorylation of Pol II and may increase elongation efficiency via this mechanism (Frankel & Young, 1998).

Once the viral mRNA is newly synthesized, it is transported out of the nucleus and into the cytoplasm (Guerrero, et al., 2015). mRNA export is mediated by adaptor molecules and NXF1/NXT1 dimers that interact with nucleoporins. Newly synthesized mRNA is present as full-length transcripts. These full-length transcripts are later packaged into new virions as the genomic RNA (Emery & Swanstrom, 2021). Other mRNAs, however, must be spliced into smaller transcripts. Newly synthesized mRNA

contains multiple splice sites where host spliceosomes can bind (Tazi, et al., 2010). Three of these sites are employed to produce multi-spliced *tat, rev,* and *nef* mRNAs. Rev protein is then translated outside the nucleus and can bind to the rev response element (RRE) on the *env* gene (Frankel & Young, 1998). Once bound to RRE, Rev facilitates the export of singly spliced and un-spliced transcripts from the nucleus (Emery & Swanstrom, 2021).

After export to the cytoplasm, spliced viral mRNA is translated initially for quality control before it can be recruited to the 43S pre-initiation complex (43S PIC). The 43S PIC recognizes AUG as the start codon, and the formation of the 80S ribosome occurs (Guerrero, et al., 2015). Translation eukaryotic elongation factors, eEF1 and eEF2, help polypeptide elongation until the 80S ribosome reaches a stop codon, and new viral proteins are thus synthesized.

1.2.2.6 Assembly of Viral Components

Before new viral particles can be packaged into virions, some post-translational processing must occur. For instance, as Env proteins are being synthesized, newly synthesized host CD4 can form a complex with Env in the endoplasmic reticulum (ER), thus preventing the packaging of Env into virions (Gonzalez, 2015). Thus, HIV-1 viral protein U (Vpu) acts to downregulate this newly synthesized CD4. This downregulation has been shown to occur even in the absence of Env expression and has also been shown to regulate the expression of other host membrane proteins (Gonzalez, 2015). Env-CD4 complexes also occur at the host cell's membrane so, the HIV-1 protein, Nef, works to degrade this CD4 through lysosomal degradation (Gonzalez,

2015) (Frankel & Young, 1998). Like Vpu, Nef also targets other host cell membrane proteins (Buffalo, Iwamoto, Hurley, & Ren, 2019) thus contributing to immunosuppression associated with HIV-1 infection.

As mentioned above, newly synthesized mRNA can be exported outside the nucleus as full-length, unspliced RNA that serves as the viral genome. Two of these strands are associated with one another, along with the other viral components that have been newly translated (Fanales-Belasio, Raimondo, Suligoi, & Butto, 2010) (Figure 2).

When newly synthesized proteins are made from viral mRNA, the proteins Gag and Pol are produced as a larger precursor polyprotein (Gag-Pol) that must be cleaved to produce the mature functional proteins (Konnyu, et al., 2013). Therefore, PR, which is packaged inside the virion, catalyzes the hydrolysis of the peptide bonds that hold Gag and Pol together (Brik & Wong, 2003). PR is also responsible for cleaving Gag into the proteins MA, CA, NC, and p6 during the budding process (Sundquist & Krausslich, 2012).

1.2.2.7 Budding of Mature Virions

The viral packaging and budding process is mediated by the Gag polyprotein (Sundquist & Krausslich, 2012). Gag forms spherical microdomains and binds to the host cell's outer membrane. The proteolysis of Gag into its smaller protein domains causes viral components to come together at the host cell's membrane. At the same time, Env traffics to the host cell's membrane and interacts with Gag to be incorporated into the mature virion (Sundquist & Krausslich, 2012). After all viral components are incorporated, HIV-1 hijacks the host cell's machinery responsible for endosome

formation and transport. Thus, a newly formed virion is released from the infected cell and can go on and infect other cells (Figure 2).

1.3 Cell Targets

HIV-1 can infect cells as free virions or by cell-cell contact (Agosto, Herring, Mothes, & Henderson, 2018). Because the mode of viral transmission varies, the primary infected cell types vary as well depending on how the virus enters the body. However, certain cell types are primary targets for HIV-1 infection, and most of what we know today about the viral life cycle has been due to studying these cell types. Furthermore, HIV-1 has been known to affect multiple organ systems illustrating the fact that this virus is not limited to the canonical cell targets.

1.3.1   CD4 T Cells

Because HIV-1 entry into host cells is initiated by the binding of the gp120 subunit of Env to host cell CD4 receptors, one of the main targets for infection is CD4 T cells (Swanstrom & Coffin, 2012). Along with CD4, these T cells also express the CCR5 or CXCR4 co-receptor that is required for viral entry. In fact, one of the main mechanisms of HIV-1 pathogenesis is the depletion of these CD4 T cells, and CD4 T cell count is used as a marker for infection and antiretroviral therapy efficacy (Vijayan, Karthigeyan, Tripathi, & Hanna, 2017).

Because T cells aid in the immune response as key antigen-presenting cells (Tai, Wang, Korner, Zhang, & Wei, 2018), they are inhibitory to HIV-1 infection. Therefore, as more cells are infected by HIV-1, more naïve CD4 T cells are produced by the thymus that can then be infected themselves (Vijayan, Karthigeyan, Tripathi, &

Hanna, 2017). Memory CD4 T cells are also induced and activated as more cells become infected. Once a memory CD4 T cell is infected, it undergoes cell death (Vijayan, Karthigeyan, Tripathi, & Hanna, 2017) thus furthering the depletion of CD4 T cells. In this case, these CD4 T cells undergo either apoptosis or pyroptosis depending on whether they are permissive to infection (Vijayan, Karthigeyan, Tripathi, & Hanna, 2017).

As mentioned above, HIV-1 Vpr and Nef can downregulate CD4 expression in infected CD4 T cells. However, Vpr has also been shown to arrest the cell cycle at the G_2 phase and prevent the infected cell from entering mitosis (Emerman, 1996). This arrest in proliferation aids the virus in translation but has also been linked to apoptosis of these CD4 T cells (Andersen, Le Rouzic, & Planelles, 2008).

1.3.2   Macrophages

CD4 receptors are not exclusively expressed on CD4 T cells and have been found to be expressed on other immune cells such as monocytes and macrophages (Swanstrom & Coffin, 2012). Monocytes are precursors to macrophages, and both circulate in the blood stream as part of the immune system. Both have been shown to express low levels of CD4 and CCR5 and can thus become infected by HIV-1 (Swanstrom & Coffin, 2012). It is believed that HIV-1 that infects CD4 T cells can evolve to infect these low CD4-expressing cells at later stages of infection, especially in the brain (Swanstrom & Coffin, 2012).

Monocytes and macrophages circulate in the body but are also common in mucosal surfaces where HIV-1 transmission most often occurs (Kruize & Koostra,

2019). Therefore, monocytes and macrophages possess the ability for HIV-1 infection in early stages.

One of the hallmarks of HIV-1 pathology is viral latency where the virus is not actively producing new virions but is still present in infected tissues (Kumar, Abbas, & Herbein, 2014). Latency allows HIV-1 to persist and evade host immune responses as well as the effect of antiretroviral therapeutics. Though CD4 T cells play a role in HIV-1 latency (Agosto, Herring, Mothes, & Henderson, 2018), monocytes and macrophages have also been shown to be important viral reservoirs (Kumar, Abbas, & Herbein, 2014). For instance, HIV-1 has been detected in monocytes from patients on antiretroviral therapy (Kumar, Abbas, & Herbein, 2014). Macrophages in the central nervous system (CNS) and the gut have also been evaluated as possible viral reservoirs (Kumar, Abbas, & Herbein, 2014).

1.3.3   Other Cell Types

HIV-1 can infect other cell types in the body including other immune cells and other tissue-specific cells. For instance, natural killer cells have been shown to be infected with HIV-1 *in vitro,* as well as microglia in the brain (Swanstrom & Coffin, 2012). Dendritic cells, another antigen presenting cell type, have also been shown to be infected *in vitro* (Kandathil, Sugawara, & Balagopal, 2015). Along with immune cells, epithelial cells have also been shown to be infected with HIV-1 *in vitro,* however, they do not express CD4 or co-receptors for infection (Kandathil, Sugawara, & Balagopal, 2015).

These cell types, along with CD4 T cells and monocytes/macrophages, are located throughout the body in many different tissues, thus allowing HIV-1 infection to spread to and affect various organs and organ systems, causing comorbidities of primary HIV-1 infection.

CHAPTER 2

COMORBIDITIES OF HIV-1

2.1 Comorbidities

Patients diagnosed with HIV are immediately prescribed antiretroviral therapy (ART), medications that act on various stages of the HIV life cycle to inhibit replication and maturation of new virions (HIVinfo.NIH.gov, 2021). With continuous combination ART (cART), patients can reduce viral load and recover CD4 T cells (HIVinfo.NIH.gov, 2021). Therefore, cART can control HIV infection and people living with HIV are living longer lives. In fact, in a study conducted between the year 2000 and 2016 in the US, researchers found the difference in life expectancy between HIV-infected and uninfected adults to be reduced from 22 years from 2000-2003 to 9 years from 2014-2016 (Marcus, et al., 2020). However, it was also found that the difference in non-AIDS comorbidity-free years between the two groups in this same cohort was markedly larger at 16 years. Thus, though the implementation of cART does increase overall life expectancy, the aging population of people living with HIV is developing these age-related comorbidities prematurely (Collins & Armstrong, 2020).

Comorbidities experienced with cART-controlled HIV infection are diseases not associated with AIDS, or non-AIDS comorbidities (Lorenc, Ananthavarathan, Lorigan, Jowata, & Brook , 2014). The most common comorbidities affect parts of the body that are also affected during aging including the heart, lungs, liver, kidney, and brain. These comorbidities include diseases such as diabetes mellitus, cardiovascular disease, liver disease, cancer, and neurodegeneration as well as opportunistic infections

(Lerner, Eisinger, & Fauci, 2020) (Lorenc, Ananthavarathan, Lorigan, Jowata, & Brook, 2014). Furthermore, a study conducted in a French cohort over the ten-year period between 2004 and 2014 found that the prevalence of many comorbidities had increased (Bonnet, et al., 2020), most likely indicative of the longer life expectancy of these patients. However, the increased prevalence is also indicative of the premature aging that occurs in these patients.

2.1.1 Heart

One of the most common comorbidities seen in patients living with HIV is cardiovascular disease (Bonnet, et al., 2020) (Lerner, Eisinger, & Fauci, 2020). A study conducted in HIV-patients from the years 2009 to 2015 evaluated the incidence of cardiovascular events compared to uninfected controls and saw that the incidence rate was increased (Alonso, et al., 2019). Particularly, the incidence of heart failure and stroke were almost three times higher in HIV patients than in healthy uninfected patients.

The exact reason for the high HIV-associated cardiovascular disease incidence is unknown. It is postulated that the high incidence is because the common risk factors for cardiovascular disease, such as diabetes and hypertension, are also increased with HIV and aging (Triant, 2013). In addition, there has been some evidence that certain cARTs are more toxic to the body and contribute to cardiovascular risks however, newer, less toxic drugs have been developed since then that have shown have less toxicity (Triant, 2013).

The cellular mechanisms involved in HIV-associated cardiovascular comorbidities have been elucidated through many studies though our understanding is not complete. For instance, it is thought that HIV plays a role in development of atherosclerosis, inflammation, and endothelial dysfunction (Vachiat, McCutcheon, Tsabedze, Zachariah, & Manga, 2017). Atherosclerosis during HIV infection involves the accumulation of low-density lipoprotein (LDL) and subsequent nuclear factor-κB (NF-κB) activation (Fisher, Miller, & Lipshultz, 2006). NF-κB activation causes more proinflammatory cytokines to be produced and attracts infiltrating inflammatory cells to the area including foam cells, monocytes with accumulated oxidized LDL. Inflammation caused by activated macrophages trigger downstream events that promote the maturation and rupture of an atherosclerotic plaque formed by foam cells (Fisher, Miller, & Lipshultz, 2006). It has also been shown that HIV can directly infect arterial smooth muscle cells aiding in atherosclerotic pathology (Eugenin, et al., 2008).

2.1.2   Brain

HIV can affect the brain in many ways causing neurocognitive decline called HIV-associated neurocognitive disorder (HAND). However, in order to affect the brain, HIV must first cross the blood brain barrier (BBB) and enter the CNS. Researchers have found many ways in which the virus can infect and affect the various cell types of the CNS leading to neurotoxicity and the neurodegeneration seen in HAND (Figure 3).
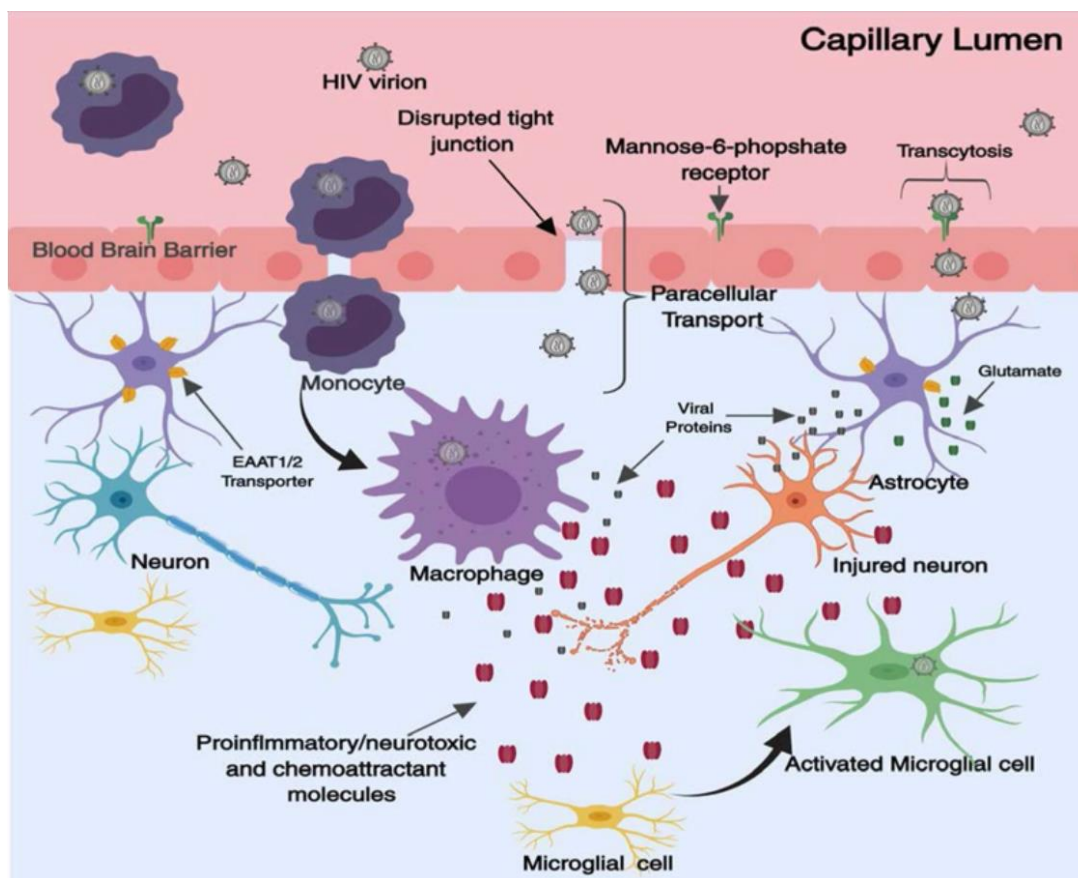
Figure 3. HIV Mechanisms of Neuropathogenesis.
Illustration of the mechanisms of HIV neuropathogenesis including entry into the CNS across the blood brain barrier, infection of resident macrophages and glial cells, and neurotoxic effects of resulting inflammation and viral proteins. (Omeragic, Kayode, Hoque, & Bendayan, 2020).

Entry into the CNS occurs very early on during infection where HIV can be found in the cerebral spinal fluid (CSF) of patients as early as eight days post infection (Valcour, et al., 2012). HIV-1 virions and infected cells circulate the body in the blood stream and can enter the CNS in various ways. For instance, infected monocytes and CD4 T cells can cross the BBB bringing the virus with them (Zayyad & Spudich, 2015) (Figure 3). Free HIV virions can also cross the BBB through gaps in tight junctions between endothelial cells (Zayyad & Spudich, 2015) (Figure 3). It has also been found

that the mannose-6-phosphate receptor, on endothelial cells, can bind to HIV-1 virions and transport them across the BBB via transcytosis (Dohgu, Ryerse, Robinson, & Banks, 2012).

Once inside the CNS, HIV-1 can infect non-neuronal cells, for instance, microglia and pericytes (Omeragic, Kayode, Hoque, & Bendayan, 2020), because neurons themselves do not express CD4 and cannot be infected (Mocchetti, Bachis, & Avdoshina, 2014). Microglia, as CNS immune cells, express antigen recognition receptors and secrete cytokines and chemokines thus initiating an immune response to HIV-1 (Figure 3) (Omeragic, Kayode, Hoque, & Bendayan, 2020). This proinflammatory response has been associated with neurocognitive impairment (Kamat, et al., 2012). The excess inflammation, for instance the oxidative stress, damages neuronal processes (Zayyad & Spudich, 2015). Furthermore, reactive oxygen species (ROS) have been shown to be elevated in the CSF of HIV patients with dementia (Turchan, et al., 2003) and ROS is known to cause neuronal cell death (Borrajo, Spuch, Penedo, Olivares, & Agis-Balboa, 2021).

Other aspects of the chronic neuroimmune response to HIV-1 infection in the CNS involve the release of cytokines such as interleukin 1 β (IL-1β), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) (Borrajo, Spuch, Penedo, Olivares, & Agis-Balboa, 2021). The release of IL-1β and IL-8 has been shown to be induced in the CNS after HIV-1 infection (Borrajo, Spuch, Penedo, Olivares, & Agis-Balboa, 2021). Moreover, TNF-α, IL-6, and other cytokines have been shown to be increased in the CSF of HAND patients leading to increased infiltration of more immune

cells. The accumulation of these immune cells promotes a proinflammatory environment that increases ROS production and can damage neurons.

2.1.2.1 Viral Protein Neurotoxicity

Neurons are not only damaged in HAND by the chronic oxidative stress caused by activated microglia and macrophages. In fact, neurons are also damaged by HIV proteins that are released from infected cells in the CNS (Mocchetti, Bachis, & Avdoshina, 2014). These proteins may be either actively or passively released from microglia or macrophages or may be shed from HIV virions during fusion with the host membrane. HIV proteins such as Tat, gp120, Nef, and Vpr have been detected in the CSF of HIV patients (Henderson, et al., 2019) and have been shown to interact with neurons promoting toxicity (Mocchetti, Bachis, & Avdoshina, 2014).

2.1.2.1.1   Gp120

Gp120 has been shown to induce IL-1β and TNF-α causing neuroinflammation and thus damaging neurons (Mocchetti, Bachis, & Avdoshina, 2014). It has also been shown that gp120 can interact with the glutamate receptor, N-methyl-D-aspartate (NMDA) receptor, on neurons (Jadhav & Nema, 2021). Binding to NMDA receptors causes and influx of calcium ions (Ca^{2+}) and altered Ca^{2+} homeostasis, which leads to neurotoxicity and cell death (Lannuzel, Lledo, Lamghitnia, Vincent, & Tardieu, 1995). Gp120 has also been shown to inhibit the glutamate uptake transporter, excitatory amino acid transporter-2 (EAAT2), on astrocytes and microglia, thus leading to more extracellular glutamate and NMDA receptor stimulation (Figure 3) (Omeragic, Kayode, Hoque, & Bendayan, 2020). Further, gp120 has been shown to upregulate the expression

of NMDA receptors on the surface of neurons by modifying intracellular trafficking of these receptors (Xu, et al., 2011). This altered trafficking is especially important since NMDA receptor trafficking is crucial for synaptic plasticity (Xu, et al., 2011). Further, altered NMDA receptor surface expression and trafficking has been implicated in other neurodegenerative diseases (Xu, et al., 2011).

Evidence of gp120 neurotoxicity comes from *in vitro* and *in vivo* studies. Transgenic mice that express gp120 in CNS cells (astrocytes) show evidence of damaged dendrites and synapses (Toggas, et al., 1994). In addition, some *in vitro* studies have shown that gp120 exhibits neurotoxicity independent of its interaction with NMDA receptors (Bachis & Mocchetti, 2003) (Bachis, Aden, Nosheny, Andrews, & Mocchetti, 2006). These same studies also revealed that gp120 can be internalized by neurons by binding to CXCR4 causing cell death suggesting gp120-mediated neurotoxicity involves many mechanisms.

2.1.2.1.2   Tat

Like gp120, Tat has been shown to bind to NMDA receptors and has been shown to upregulate TNF-α, IL-6, IL-8, and IL-1β (Nath, et al., 1996) (Jadhav & Nema, 2021). The resulting neurotoxicity is also mediated by an influx in Ca^{2+} and altered Ca^{2+} homeostasis that leads to cell death (Nath, et al., 1996). Further, the Tat-mediated increase in TNF-α has also been shown to contribute to neurotoxicity (Buscemi, Ramonet, & Geiger, 2007) and has been observed in HAND patients to promote cell death (Jadhav & Nema, 2021). Much like gp120, Tat has been shown to decrease the

expression of EAAT2 leading to increased glutamate excitotoxicity (Figure 3) (Omeragic, Kayode, Hoque, & Bendayan, 2020).

Tat can also induce the receptor, P2X7R, on astrocytes which can lead to increased release of proinflammatory chemokines and result in neuronal apoptosis (Tewari, Monika, Varghese, Menon, & Seth, 2015). However, one study showed Tat-mediated behavioral alterations *in vivo* in the absence of a robust proinflammatory response (Moran, Fitting, Booze, Webb, & Mactutus, 2015) indicative of multiple mechanisms in which Tat is involved in HAND pathogenesis. For instance, Tat has also been shown to increase ROS and treatment with antioxidants has been shown to prevent neuronal apoptosis (Agrawal, Louboutin, & Strayer, 2007).

In terms of neurocognitive effects, many studies have shown Tat alone is enough to produce neuropathology and behavioral changes (Dickens, et al., 2017) (Joshi, Stacy, Sumien, Ghorpade, & Borgmann, 2020). A study investigating chronic low-level Tat expression *in vivo* found evidence of synaptic damage and increased proinflammatory cytokine expression (Dickens, et al., 2017). Recently, studies in rodent models have shown that Tat expression in the CNS causes behavioral deficits including those in short-term and long-term memory and motor balance/coordination (Joshi, Stacy, Sumien, Ghorpade, & Borgmann, 2020) (Zhao, et al., 2020). Therefore, Tat must play a central role in HAND neuropathogenesis.

2.2 HIV-Associated Neurocognitive Disorder (HAND)

HAND is an evolving disorder that presents differently in each patient. With the ongoing development of new therapies for HIV infection and comorbidities, the

prevalence and symptoms of HAND are likely to change as they have in the past. However, it is important to know how the disease has evolved so patterns and trends can be analyzed to advance treatment.

2.2.1 Prevalence

The use of cART to treat HIV infections became widely available at the end of the 20th century (Heaton, et al., 2011). As mentioned above, cART can improve CD4 T cell number and lower viral load. However, the prevalence of HIV-associated comorbidities, including HAND, are still present in HIV patients (Heaton, et al., 2011). For instance, a study was conducted investigating the prevalence of HAND in patients in the pre-cART era (1988-1995) and the cART era (2000-2007) (Heaton, et al., 2011). This study found that the prevalence of neurocognitive impairment in HIV patients had slightly declined with cART in later stages of disease. However, the prevalence of neurocognitive impairment at early stages of HIV had increased in the cART era indicating a possible toxicity of these antiretroviral drugs.

This results from this study suggest that cART is not sufficient therapy to reduce the prevalence of HAND at a significant rate at any stage of HIV disease. In fact, a more recent meta-analysis of HAND in the cART era found that the estimated prevalence of HAND is ~44% (Wei, et al., 2020). Also, HAND can be categorized into three severities, asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) (Wei, et al., 2020). This meta-analysis concluded the prevalence of ANI (26.2%) to be higher than MND (8.5%) and HAD (2.1%).

2.2.2 Symptoms

Currently, the most common method for diagnosing all severities of HAND is the Frascati criteria (Matchanova, Woods, & Kordovski, 2020). These criteria categorize the neurocognitive impairment seen in HAND into ANI, MND, and HAD based on the level of functioning in daily life. Common areas of neurocognitive impairment with HAND are impairments in verbal skills, speed of information processing, attention/working memory, and motor skills (Figure 3) (Heaton, et al., 2011). Of note, executive function, recall, and learning/memory are areas of impairment that have not improved in the cART era (Figure 3) and have been shown to influenced by cART itself (Heaton, et al., 2011).
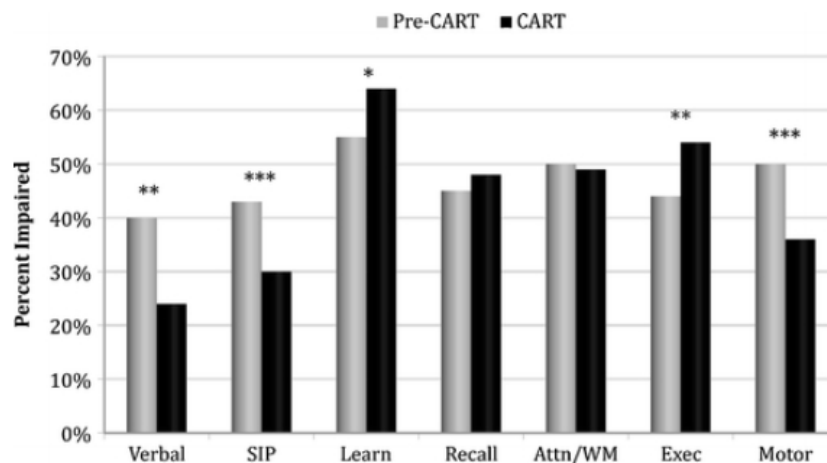
Figure 4. Areas of Neurocognitive Impairment in the Pre-cART and cART Eras. Percent impairment of different areas of neurocognitive ability in the pre-cART era and cART era represented as a bar graph. SIP stands for speed of information processing. Attn/WM stands for attention/working memory. *p<0.05; **p<0.01; ***p<0.001. (Heaton, et al., 2011).

2.2.3 Therapies

Current treatment for HAND begins with early diagnosis. Diagnostic testing for HAND is similar to that of other neurocognitive impairments where neuropsychometric performance testing is commonly used (Clifford & Ances, 2013). Neuropsychometric tests include the MiniMental State Exam (MMSE), the Montreal Cognitive Assessment (MoCA), and the International HIV Dementia Screen (IHDS) (Clifford & Ances, 2013). Typically, these tests evaluate a patient's ability to perform small tasks assessing areas of cognitive performance such as memory/recall, motor speed, and psychomotor speed as in the case of the IHDS (National HIV Curriculum , 2022). The MMSE and MoCA are used to test a variety of neurocognitive impairment diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and Fronto-temporal dementia (FTD). Because of their wide use in assessing other neurocognitive diseases, these tests are not specific for HAND and thus not sensitive enough for milder forms of HAND (Clifford & Ances, 2013). However, the neuropsychometric tests are still used to evaluate the progression of disease and efficacy of therapeutics.

As mentioned above, the use of cART to treat HIV infection in the brain has improved some but not all areas of decline in HAND. However, the inability of many of these cART drugs to cross the blood brain barrier and the neurotoxicity prevent cART from being the primary therapeutic to treat HAND. Therefore, many adjuvant therapies have been proposed targeting pathways seen to be important in other neurodegenerative diseases such as acetylcholinesterase inhibitors, NMDA receptor antagonists, and monoamine oxidase B (MAO-B) inhibitors (McGuire, Barrett, Vezina, Spitsin, &

Douglas, 2014). Clinical trials with these adjuvant therapies saw some but not significant improvement in HAND stressing the need for a better understanding of HAND pathogenesis and thus more targeted therapies.

CHAPTER 3

INTERORGANELLE COMMUNICATION

3.1 Introduction to Interorganelle Communication

The various functions carried out by a cell are mediated by organelles that are specialized in those functions. For instance, transcription of DNA into RNA is carried out in the nucleus. However, each organelle cannot act independently and is facilitated by the transport and exchange of biomolecules from other areas of the cell (Petkovic, O'Brien, & Jan, 2021).
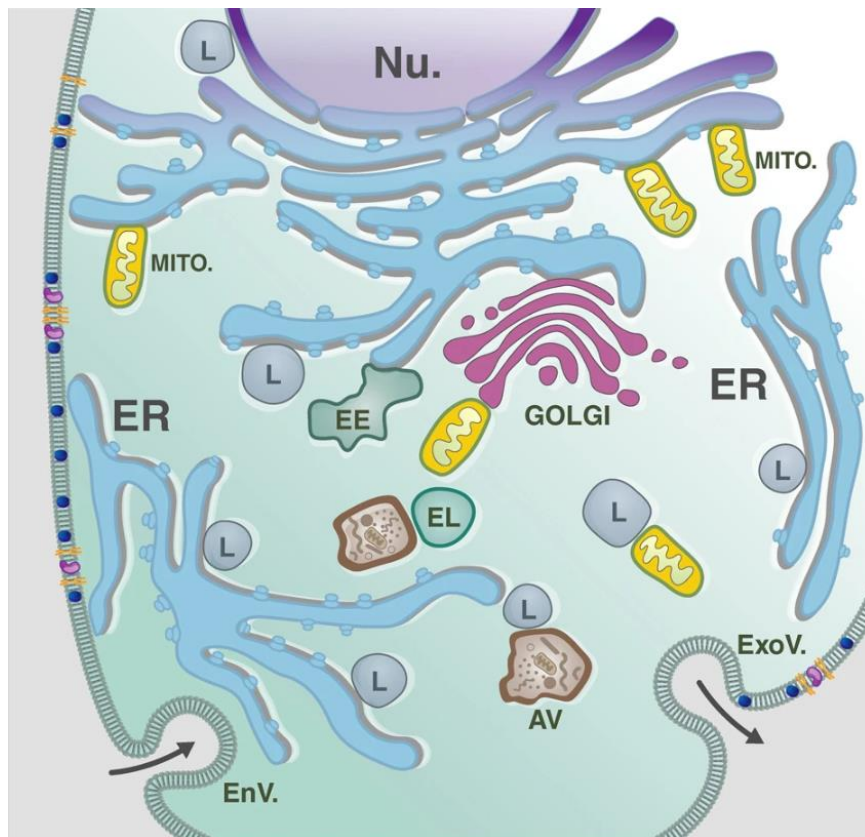
Figure 5. Illustration of Various Interorganelle Contacts.
Depiction of different interorganelle contacts in eukaryotic cells between mitochondria (MITO), ER, lysosomes (L), Golgi, early endosomes (EE), nucleus (Nu), autophagic vacuoles (AV), and endolysosomes (EL).
(Annunziata, Sano, & d'Azzo, 2018).

In order for this exchange to occur, organelles must interact and communicate with one another (Figure 5). This communication occurs when organelles are in close proximity to each other, and it is important in normal cellular function.

3.2 Mitochondria-associated ER Membranes (MAMs)

The interaction between the ER and mitochondria was first described in 1990 where the transfer of phospholipids between the ER and mitochondria was being investigated in rat livers (Vance, 1990). Here, it was noted that a previously undescribed membrane fraction possessed properties of both the ER and mitochondria and was thought to be a site for phospholipid synthesis. This "fraction X" would later be named mitochondria-associated ER membranes (MAMs). Since then, MAMs have been implicated in a wide variety of cellular functions and their dysregulation or disruption has been associated with age-related and disease pathology (Petkovic, O'Brien, & Jan, 2021).

3.2.1   Proteins Involved in Mitochondria-ER Communication

MAMs are the dynamic relationship between the ER and mitochondria. According to electron tomography studies, the contacts between the two organelles making up MAMs is between 10-20nm (Csordas, et al., 2006). This relationship is mediated by many different proteins that can either be enriched in the MAM fraction or function as tethering proteins to join the ER and mitochondria together in close proximity.

MAM tethering proteins are proteins found on outer mitochondrial membranes or ER membranes that physically interact with one another to facilitate the

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interorganelle communication (Figure 6). For instance, the proteins mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) have been shown to tether the ER and mitochondria together (Martins de Brito & Scorrano, 2008). Since Mfn1/2 are involved in mitochondrial biogenesis, the MAMs also play an important role in this process (Paillusson, et al., 2016). The ER Ca^{2+} channel, inositol trisphosphate receptor (IP_3R), has also been shown to tether the ER and mitochondria together when coupled to voltage-dependent anion channel (VDAC) on mitochondria facilitated by glucose-regulated protein 75 (Grp75) (Figure 6) (Shengnan & Ming-Hui, 2018). Other tethering proteins include vesicle-associated membrane protein (VAMP)- associated protein B (VAPB) and B-cell receptor-associated protein 31 (Bap31) on the ER bound to regulator of microtubule dynamics protein 3/protein tyrosine phosphatase interacting protein 51 (RMDN3/PTPIP51) and mitochondrial fission protein 1 (Fis1) on mitochondria, respectively (Figure 6) (Paillusson, et al., 2016).

Other proteins may be enriched in MAMs but do not directly participate in the physical tethering of the ER to mitochondria. These proteins include fatty acid-CoA ligase 4 (FACL4), mTOR complex 2, and others involved in many different signaling pathways (Gao, Yan, & Zhu, 2020). Still, recent proteomic studies on the MAM fraction revealed many other proteins enriched in MAMs and may also lead to the discovery of more tethering partners indicating the novelty of the field and the complexity of MAMs (Hung, et al., 2017) (Horner, Wilkins, Badil, Iskarpatyoti, & Gale Jr., 2015).
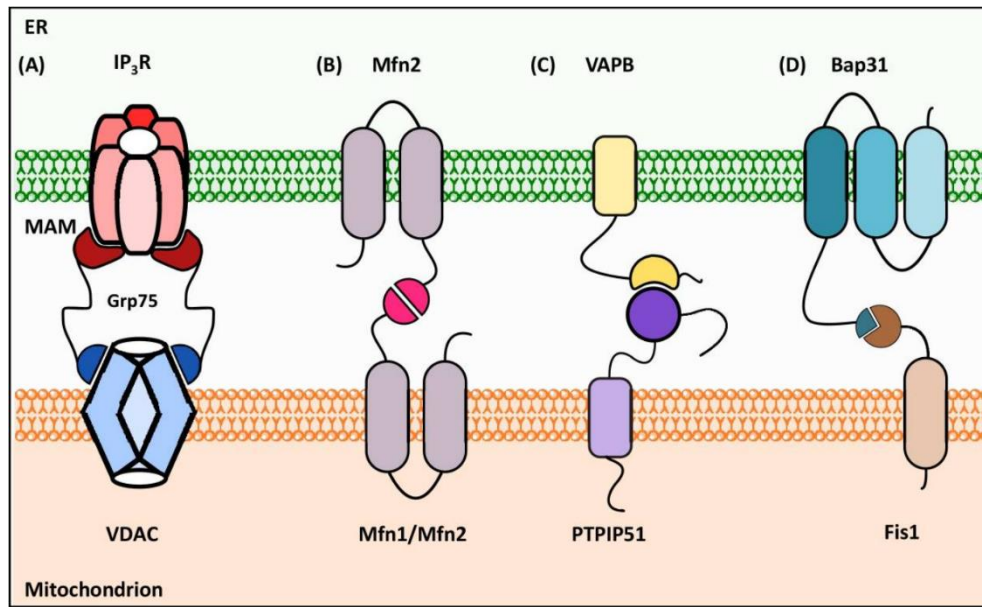
Figure 6. Schematic of Various MAM Tethering Proteins.
MAM tethering proteins on outer mitochondrial membranes (VDAC, Mfn1/Mfn2, PTPIP51 and Fis1) and their binding partners on the ER membrane (IP$_3$R, Mfn2, VAPB, and Bap31) including adapter proteins (Grp75) facilitating this binding. (Paillusson, et al., 2016).

3.2.2 Cellular Functions of MAMs

MAMs play roles in many different cellular functions including lipid synthesis and transfer, Ca^{2+} transfer and homeostasis, mitochondrial biogenesis, autophagy, and inflammasome formation as well as others (Gao, Yan, & Zhu, 2020) (Yang, et al., 2020) (Raturi & Simmen, 2013). In fact, the first paper describing MAMs showed enrichment of phosphatidylserine (PS) synthase 1 and 2 (PSS1/PSS2) and suggesting PS metabolism from phosphatidylcholine (PC) is a function of MAMs (Vance, 1990). Further, phospholipid metabolism has been shown to occur at MAMs thus facilitating the transfer of essential phospholipids between the ER and mitochondria (Annunziata, Sano, & d'Azzo, 2018). For instance, PS is transferred from MAMs to the inner

mitochondrial membrane to be converted to phosphatidylethanolamine (PE). PE is then converted to PC at MAMs to be transferred to the ER. This transfer and metabolism of phospholipids helps maintain organellar membranes (Annunziata, Sano, & d'Azzo, 2018). MAMs are also important in synthesis of cardiolipin (CL) from phosphatidic acid (PA) which can come from the ER (Osman, Voelker, & Langer, 2011). Sterols and sphingolipids (ceramides) are also important components of cellular membranes, including mitochondrial membranes, and are synthesized in the ER (Flis & Daum, 2013). Therefore, the interaction between the ER and mitochondria is also important for sterol metabolism.

One of the main functions of MAMs is the transfer of Ca^{2+} from ER stores to mitochondria (Shengnan & Ming-Hui, 2018). As mentioned above, this transfer is facilitated by the communication between the IP_3R on the ER and VDAC on the mitochondria at MAMs. Regulators of these Ca^{2+} channels, such as protein kinase B (PKB) and protein phosphatase 2A (PP2A), are also enriched in MAMs suggesting these contact sites are important in regulating Ca^{2+} transfer (Shengnan & Ming-Hui, 2018). Ca^{2+} signaling is important for many cellular processes so regulation of its release and uptake is crucial for maintain cellular health (Raturi & Simmen, 2013). For instance, the ER and mitochondria work together to regulate the amount of cytosolic Ca^{2+} by acting as intracellular stores. During high cytosolic Ca^{2+} concentrations, the ER releases Ca^{2+} to mitochondria to be able to take up the excess Ca^{2+} (Raturi & Simmen, 2013). These increases in mitochondrial Ca^{2+} concentrations also activate other mitochondrial enzymes essential for cellular metabolism and oxidative phosphorylation such as

pyruvate dehydrogenase. Interestingly, the release of Ca^{2+} from the ER has been shown to regulate mitochondrial motility inside the cell where mitochondria slow down when Ca^{2+} is released by IP_3Rs (Yi, Weaver, & Hajnoczky, 2004). These findings suggest that communication between the ER and mitochondria is not only important for Ca^{2+} signaling and homeostasis but also for the subcellular distribution of mitochondria. Since mitochondria are important for bioenergetics and cell death/survival signaling (Raturi & Simmen, 2013), their distribution inside a cell is important in regulating cellular responses to the extracellular environment. Therefore, MAMs play a crucial role in cell health.

Autophagic signaling has also been shown to be an important function of MAMs (Yang, et al., 2020). For instance, autophagosome formation has been shown to be localized at MAMs with several autophagosome markers enriched in these contact sites (Hamasaki, et al., 2013). Moreover, when MAMs are disrupted, the autophagosome formation is inhibited (Hamasaki, et al., 2013). The implication that MAMs regulate autophagy is mostly due to the role of phospholipids and Ca^{2+} in autophagosome formation. During autophagosome membrane expansion, PE and PS help bind autophagic proteins, such as microtubule-associated protein 1 light chain 3B (LC3), thus enabling the formation of autophagosomes (Yang, et al., 2020). Also, Ca^{2+} signaling between the ER and mitochondria is important for providing the energy needed to form autophagosomes. Furthermore, many autophagy-associated proteins are enriched in MAMs such as PTEN-induced putative kinase 1 (PINK1) and Parkin, two important players in mitophagy (Gelmetti, et al., 2017).

MAMs also play a role in inflammation with the formation of the inflammasome, a protein complex that aids in cellular inflammatory responses (Missiroli, et al., 2018). The inflammasome is formed and activated in response to toll-like receptor (TLR) activation, NLR family pyrin domain containing 3 (NLRP3) and IL-1β transcription, the presence of a stimulus such as P2X7 activation, lysosome rupture, or ROS (Missiroli, et al., 2018). Once formed, the inflammasome acts to aid in caspase-1 activation and interleukin 18 (IL-18) maturation (Figure 7).
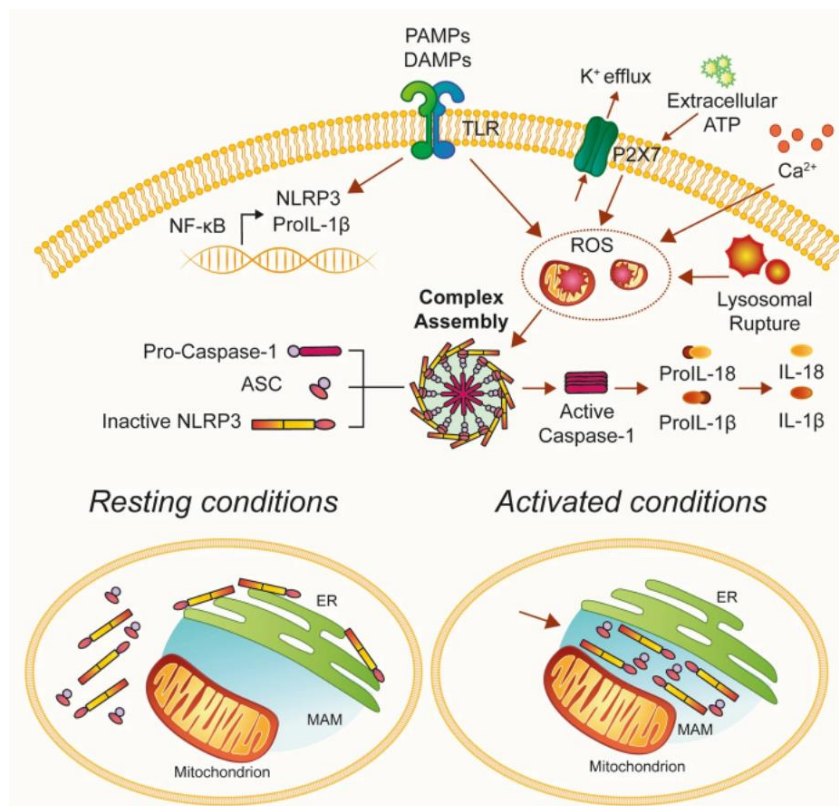
Figure 7. Inflammasome Formation at MAMs.
Schematic showing the induction of NLRP3 inflammasome formation by various stimuli and localization of complex formation in MAMs (Missiroli, et al., 2018).

The NLRP3 inflammasome is then localized to MAMs most likely in part by mitochondrial ROS (Shengnan & Ming-Hui, 2018). Further, since the ER and

mitochondria are important in ROS production and release (van Vliet, Verfaillie, & Agostinis, New functions of mitochondria associated membranes in cellular signaling, 2014), MAMs may use ROS to recruit ROS-sensing proteins involved in inflammasome formation as well as other cellular processes.

3.2.3   Neurodegenerative Diseases Associated with MAM Dysfunction

Because of the many important roles that MAMs play in normal cellular functions, disruption of MAMs has been implicated in disease pathology (Annunziata, Sano, & d'Azzo, 2018) (Paillusson, et al., 2016) (Yu, Sun, Gong, & Feng, 2021). In the past few years, MAMs have especially been linked to neuro-aging and neurodegenerative diseases (Paillusson, et al., 2016) (Liu & Yang, 2022) as many MAM-associated functions are altered in these conditions.

3.2.3.1 Alzheimer's Disease (AD)

Studies have shown that MAMs may be involved in Alzheimer's disease (AD) pathology in part because many pathogenic molecules and proteins are enriched in MAMs. For instance, presenilin 1 (PS1) and presenilin 2 (PS2) have been shown to be highly enriched in MAMs (Area-Gomez, et al., 2009). PS1/2 are part of the γ-secretase complex that cleaves the membrane-bound 99 amino acid (aa) long C-terminal part of amyloid precursor protein (APP) into 40-42aa amyloid β (Aβ) (Liu & Yang, 2022). This C-terminal end of APP has been shown to be enriched in MAMs in AD models (Area-Gomez, et al., 2009). Aβ self-aggregates and accumulates in the form of plaques which are toxic to cells and are well known players in AD pathology. Further, Aβ has been shown to increase the interactions between IP$_3$R and VDAC at MAMs *in vitro*

(Hedskog, Pinho, Filadi, & Ankarcrona, 2013). These interactions, as well as the interactions between other MAM tethering proteins, have also been shown to be altered in AD patient brain samples (Liu & Yang, 2022). These studies demonstrate the importance of MAMs in Aβ pathology (Figure 8).
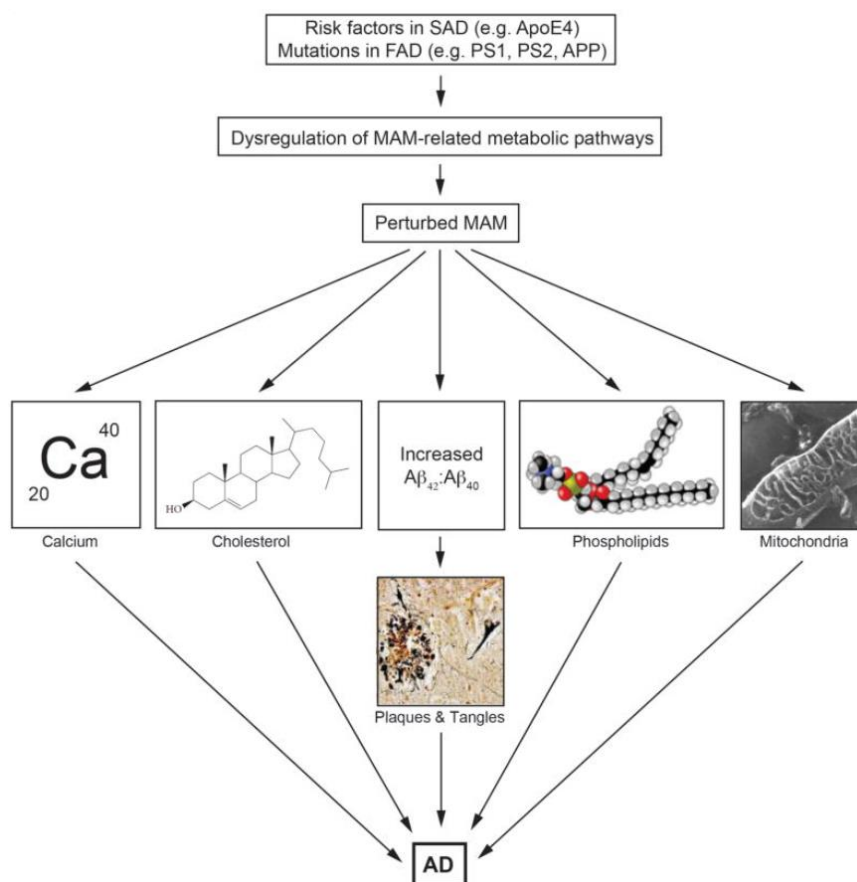
Figure 8. Proposed Involvement of MAMs in AD.
Schematic showing how MAM dysregulation my lead to dysregulated calcium, cholesterol, Aβ, phospholipid, and mitochondria contributing to AD pathology. (Area-Gomez & Schon, 2016).

AD pathology involves other factors that have been shown to be regulated by MAMs. For instance, Ca^{2+} excitotoxicity has been shown to be involved in AD pathology where Ca^{2+} affects neuronal cytoskeleton and cell death processes

(Bezprozvanny & Mattson, 2008). Elevated Ca^{2+} levels have also been seen in AD patient brain tissue (Bezprozvanny & Mattson, 2008) and there has been evidence that Ca^{2+} may contribute to Aβ production (Querfurth & Selkoe, 1994). In addition, Ca^{2+} release from the ER has been shown to be altered in AD models (Mattson, 2011). Therefore, MAMs' importance for Ca^{2+} signaling and homeostasis further implicates them in AD pathogenesis (Figure 8).

Other MAM-associated functions that are also dysregulated in AD include phospholipid and cholesterol synthesis/transfer (Figure 8) (Area-Gomez & Schon, Mitochondria-asscociated ER membranes and Alzheimer's disease, 2016). *In vitro* and *in vivo* studies revealed that phospholipid homeostasis is affected (Area-Gomez, et al., 2012) including acetyl-CoA acetyltransferase (ACAT1) (Area-Gomez, et al., 2009). ACAT1 is an enzyme localized at MAMs and has been shown to be essential for Aβ production (Puglielli, Ellis, Ingano, & Kovacs, 2004). Moreover, mitochondrial health and bioenergetics are known MAMs functions and have been shown to be perturbed in AD (Figure 8) (Area-Gomez, et al., 2009). Overall, MAM-associated cellular processes are also processes that are disrupted in AD pathogenesis thus demonstrating MAMs as key players in AD.

3.2.3.2 Parkinson's Disease (PD)

One of the main pathological markers for Parkinson's disease (PD) is accumulation of the lipid-binding protein, α-synuclein, in Lewy bodies. Aggregation of α-synuclein disrupts normal cellular functions including Ca^{2+} homeostasis, lipid metabolism, autophagy, and mitochondrial transport (Paillusson, et al., 2017). It has

been shown that α-synuclein can control Ca^{2+} homeostasis by enhancing ER and mitochondria contacts (Cali, Ottolini, Negro, & Brini, 2012). Further, α-synuclein is enriched in MAMs and can bind to the MAM tethering protein, VAPB (Guardia-Laguarta, et al., 2014) (Paillusson, et al., 2017). As mentioned above, VAPB, located on the ER, binds to PTPIP51 on mitochondria at MAMs. This interaction facilitates C^{2+} transfer in addition to that of IP_3R and VDAC. It has been shown that α-synuclein can disrupt this interaction in neurons which subsequently leads to disrupted Ca^{2+} transfer from the ER to mitochondria (Paillusson, et al., 2017). In addition, α-synuclein has been shown to alter IP_3R and VDAC interactions at MAMs thus further altering Ca^{2+} transfer (Erustes, et al., 2021).

Also mentioned above, the mitophagy-associated proteins PINK1 and parkin are enriched in MAMs. Common in familial PD are mutations in the genes encoding these proteins, *PARK2* and *PARK6,* respectively (Liu & Yang, 2022). Parkin ubiquitinates many MAM-associated proteins that are important for mitochondrial function such as VDAC, Mfn1/2, and dynamin-related protein 1 (Drp1) (Cali, Ottolini, Negro, & Brini, Enhanced parkin levels favor ER-mitochondria crosstalk and guarentee Ca2+ transfer to sustain cell bioenergetics, 2013). It has also been shown that parkin is important for maintaining physical and functional ER-mitochondria interactions including Ca^{2+} transfer (Cali, Ottolini, Negro, & Brini, Enhanced parkin levels favor ER-mitochondria crosstalk and guarentee Ca2+ transfer to sustain cell bioenergetics, 2013). Therefore, mutated parkin in familial PD cases disrupts normal MAM function thus implicating MAMs in PD pathogenesis.

3.2.3.3 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease that affects motor neurons in the brain, brainstem, and spinal cord (Parakh & Atkin, 2021). In some familial forms of ALS, the MAM-associated protein, VAPB, is mutated causing it to misfold and aggregate (Parakh & Atkin, 2021). The TAR DNA binding protein 43 (TDP-43) is also mutated in familial ALS and has been shown to disrupt VAPB-PTPIP51 interactions at MAMs (Paillusson, et al., 2016). TDP-43 has been shown to activate glycogen synthase kinase-3β (GSK-3β) and subsequently regulate VAPB-PTPIP51 interactions where overexpression or expression of mutated TDP-43 decreases these interactions (Figure 9) (Stoica, et al., 2014).
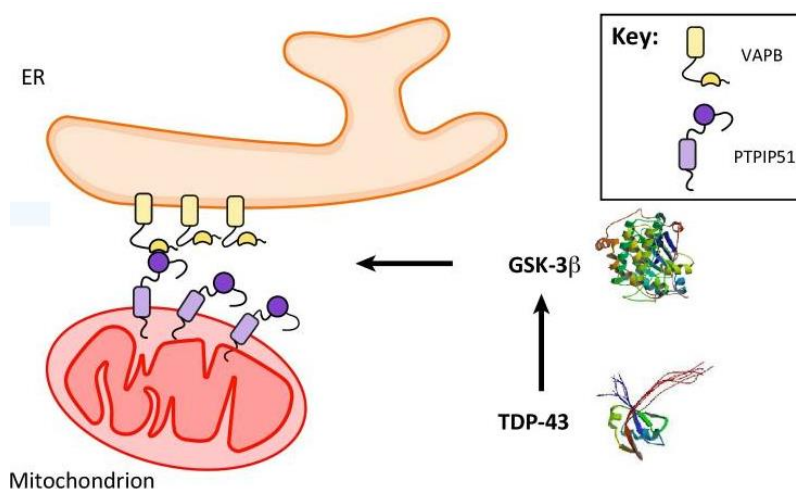
Figure 9. Altered VAPB-PTPIP51 Interactions in ALS.
Illustration showing TDP-43 activating GSK-3β to disrupt the interaction between VAPB and PTPIP51 at MAMs. (Modified from Paillusson, et al.,

In addition to mutations in VAPB and TDP-43, some other MAM-associated proteins are mutated in some forms of ALS. For instance, the sigma-1 receptor (Sig-1R)

has been shown to be mutated in juvenile forms of ALS (Sakai, Watanabe, Komine, Sobue, & Yamanaka, 2021). In animal models of ALS, disrupted Sig-1R leads to ALS pathology and phenotype (Luty, et al., 2012). Furthermore, the ALS-associated Sig-1R mutation has been shown to reduce ER-mitochondria contacts and lead to Ca^{2+} dysregulation (Bernard-Marissal, Medard, Azzedine, & Chrast, 2015). Therefore, MAMs play a role in ALS pathology though the extent of that role is still not elucidated.

3.3 ER-Golgi Communication

The Golgi apparatus is an organelle that is responsible for intracellular trafficking and the secretory pathway (David, Castro, & Schuldiner, 2021). It is made up of three substructures, the cis-cisterna, medial-cisterna, and trans-cisterna. One of the main functions of the Golgi is mediating post-translational modifications of newly formed proteins coming from the ER (David, Castro, & Schuldiner, 2021). Post-translational modifications are important for protein folding and subsequent trafficking to other parts of the cell. Therefore, communication between the ER and Golgi (the trans-Golgi) is vital for cellular function.

3.3.1   Proteins Involved in ER-Golgi Communication

ER-Golgi communication involves many proteins including VAPA and VAPB on the ER. As mentioned above, VAPB can bind to PTPIP51 on mitochondria in MAMs. However, VAPB, and VAPA, can also bind proteins on the trans-Golgi thus tethering these two organelles together (Masone, Morra, & Venditti, 2019). For instance, VAPA/B can bind to oxysterol binding protein 1 (OSBP1), and OSBP-related proteins 4L (ORP4L), 9 (ORP9), and 10 (ORP10) (Figure 10) (Masone, Morra, & Venditti,

2019). VAPA/B have also been shown to bind ceramide transfer protein (CERT) (Figure

10). Other proteins associated with ER-Golgi interactions include pleckstrin homology

domain containing A8 (PLEKHA8/FAPP2) and phosphatidylinositol transfer protein

membrane associated 1 (PTPNM1/Nir2) that play roles in lipid exchange between the

two organelles (Figure 10) (David, Castro, & Schuldiner, 2021). Together, these

tethering proteins help facilitate multiple cellular functions associated ER-Golgi

communication.
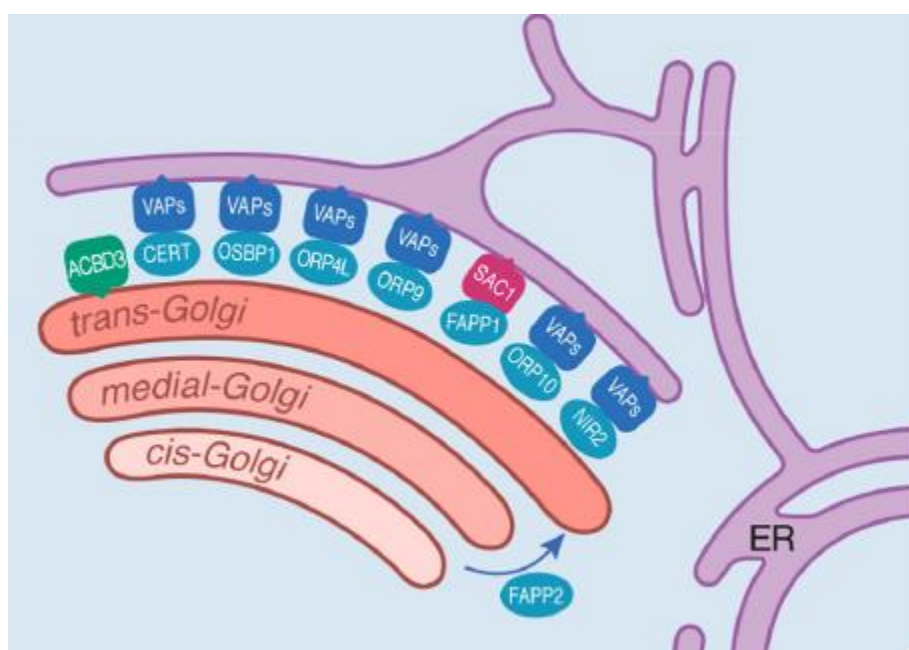
Figure 10. Proteins Involved in ER-Golgi Tethering.
Illustration of ER-Golgi contacts and the proteins involved in tethering the two
organelles together. (Modified from David, Castro, & Schuldiner, 2021).

3.3.2   Cellular Functions of ER-Golgi Communication

ER-Golgi contacts are important for lipid transfer between the organelles.

Since lipids make up various membranes in a cell, this lipid transfer is vital for cellular

functions like membrane trafficking. For instance, CERT, the ceramide transfer protein

enriched in ER-Golgi contact sites, has been shown to be important in membrane trafficking during neurogenesis (Xie, Hur, Zhao, Abrams, & Bankaitis, 2018). CERT is also important for transferring ceramides to be used in sphingomyelin synthesis, an important sphingolipid in myelin sheaths (Venditti, Masone, & De Matteis, 2020).

An important component of the trans-Golgi and other membrane vesicle is phosphatidylinositol 4-phosphate (PI4P). PI4P aids in sterol transfer between the ER and the Golgi by recruiting and binding OSBP in the contact site OSBP (Phillips & Voeltz, 2016). OSBP then binds with VAPA/B on the ER and facilitates the transfer of lipids. Further, PI4P is synthesized in the Golgi but must be transferred to the ER to continue the cycle of sterol transfer (Figure 11) (Venditti, Masone, & De Matteis, 2020) (Phillips & Voeltz, 2016). PI4P is also important in regulating anterograde membrane trafficking, especially in the case of secretion (Venditti, Masone, & De Matteis, 2020). It has been shown that ER-Golgi contact sites can regulate PI4P, therefore, this communication is important for membrane trafficking (Venditti, Masone, & De Matteis, 2020).
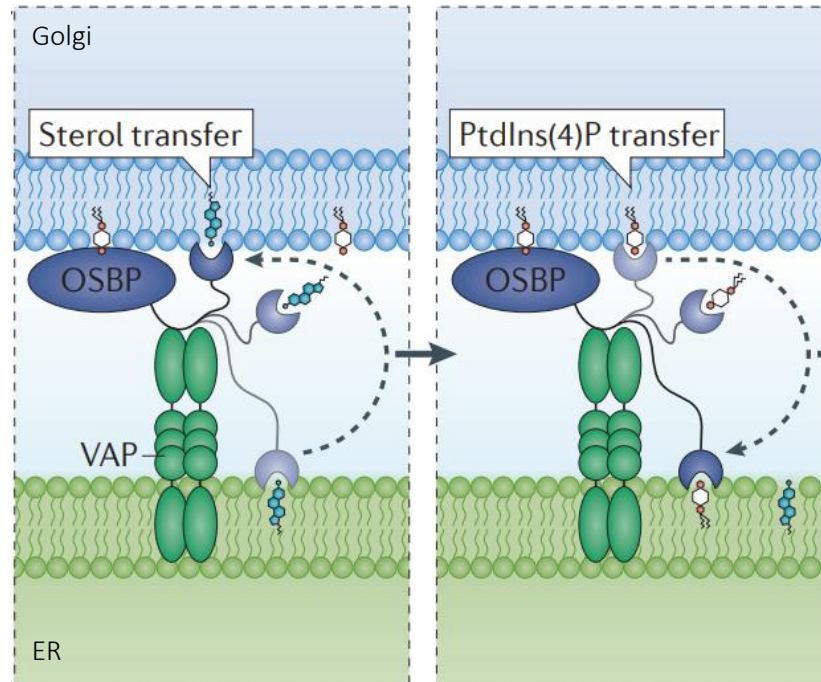
Figure 11. Sterol and PI4P Transfer at ER-Golgi Contacts.
Illustration of the cycle of sterol and PI4P transfer between the ER and
Golgi facilitated by OSBP and VAPA/B proteins. (Modified from
Phillips & Voeltz, 2016).

3.3.3 Diseases Associated with Disrupted ER-Golgi Communication

Dysfunction of the Golgi alone has been implicated and described in many
diseases including Hepatitis, osteoporosis, neurodegenerative diseases, and heart failure
(Liu, et al., 2021). However, the role of ER-Golgi communication has not been studied
as in depth. A mutation in the ER-Golgi protein, CERT has been linked to some
developmental disorders (The Deciphering Developmental Disorders Study, 2015).
CERT mutations have also been found in patients with severe intellectual disability
(Murakami, et al., 2020). Therefore, ER-Golgi contacts may also play a role in these
diseases.

Since the ER proteins VAPA/B are important in tethering to the Golgi through interactions with many proteins, any disruption in VAPA/B function may also alter ER-Golgi contacts. As mentioned above, VAPB has been shown to be mutated in some familial forms of ALS. This mutation alters its binding to mitochondrial proteins and most likely alters its binding to Golgi proteins as well. For instance, it has been shown that VAPB binds to Yip1-interacting factor homologue A (YIF1A) on the Golgi and this interaction is important in maintaining dendrite morphology in neurons (Kuijpers, et al., 2013). However, the mutated form of VAPB associated with ALS binds to YIF1A in protein aggregates preventing localization to ER-Golgi contacts (Kuijpers, et al., 2013). Thus, ER-Golgi communication and function is linked to ALS pathology.

Moreover, aggregates of α-synuclein have also been shown to inhibit ER-Golgi trafficking in a cellular model of PD (Cooper, et al., 2006). Manipulating ER-Golgi trafficking through overexpression of Rab1 has been shown to override α-synuclein toxicity in PD models further implicating ER-Golgi contacts in PD pathogenesis (Wang, Stanford, & Kundu, 2020).

3.4 Evidence for Altered Interorganelle Communication in HAND

Though altered interorganelle communication has been implicated in neurodegenerative diseases such as AD, PD, and ALS, little has been shown in the context of HAND. However, since HAND shares some similarities in pathology with these other neurodegenerative diseases, it may be possible that interorganelle communication is involved HAND as well.

3.4.1 Altered MAMs in HAND

Recently, MAMs have been implicated in HIV-1 pathogenesis. A study from 2012 showed HIV-1 Vpr could integrate into both the mitochondrial and ER membranes especially at MAMs (Huang, Chiang, Lin, Chiou, & Chow, 2012). Here, Vpr can disrupt the contacts between the ER and mitochondria as well as decrease the expression of the MAM tethering protein, Mfn2. However, this study was conducted in non-neural cells (kidney cells and CD4 T cells). MAMs however, have also been implicated in HAND.

Many cellular processes that are affected in HAND are also those that are regulated by MAMs. For instance, Ca^{2+} signaling and homeostasis is disrupted during HAND pathogenesis and is also regulated by MAMs (Figure 12) (Proulx, Park, & Borgmann, 2021). This Ca^{2+} dysregulation also contributes to upregulation of the unfolded protein response (UPR) and ER stress seen in HAND. Further, mitochondrial dysfunction and inflammasome formation is seen with HAND in neurons and astrocytes, where decreased ATP levels and increased ROS has been seen *in vitro* (Figure 12) (Proulx, Park, & Borgmann, 2021). It has also recently been shown that gp120 can manipulate mitochondrial bioenergetics and cellular metabolism (Allen, et al., 2022).

Many MAM-associated proteins have also been shown to be altered in the context of HAND. For instance, mitophagy-associated proteins such as PINK1 and parkin have been shown to be increased in response to Tat and gp120 (Teodorof-Diedrich & Spector, 2018) (Thangaraj, et al., 2018). Also, Tat and Vpr have been shown to decrease VDAC expression and manipulation of these MAM-associated proteins has been shown to reverse neurotoxic effects (Proulx, Park, & Borgmann, 2021).

Overall, there have been many overlaps between the disrupted cellular processes seen in HAND and those regulated by MAMs. Because MAMs are known to regulate processes such as Ca^{2+} signaling and homeostasis, mitochondrial bioenergetics, and UPR/ER stress, MAMs may prove to be potential therapeutic targets for treatment of HAND.
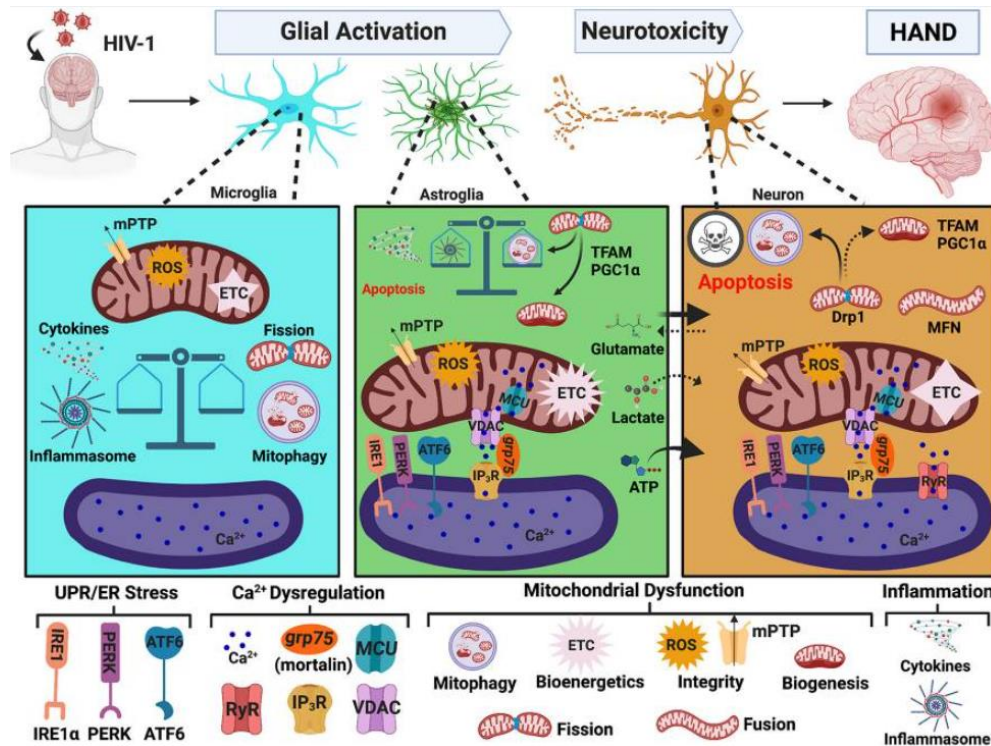
Figure 12. Involvement of MAMs in HAND.
Schematic showing various ways MAMs may be involved in glial activation and neurotoxicity leading to HAND. Electron transport chain (ETC), unfolded protein response (UPR), inositol-requiring protein 1α (IRE1), activating transcription factor 6 (ATF6), mitochondrial Ca^{2+} uniporter (MCU), ryanodine receptors (RyR) mitochondrial permeability transitional pore (mPTP), proliferator-activated receptor γ coactivator 1α (PGC-1α) transcription factor A (TFAM). (Proulx, Park, & Borgmann, 2021).

3.4.2 Altered ER-Golgi Communication in HAND

There is little direct evidence that ER-Golgi contacts are disrupted in HAND. As mentioned above, the interaction between VAPB and YIF1A has been shown to be important in membrane trafficking and delivery to dendrites in neurons as well as maintaining dendrite morphology. In HAND models, dendritic spine density and morphology is greatly reduced (Irollo, Luchetta, Ho, Nash, & Meucci, 2021). For instance, in HIV-1 Tat-treated neurons, dendrite number and length is decreased (Liu, et al., 2018). Though studies have attributed the loss of dendrites to other damaged cellular processes, they have not ruled out dysfunction in ER-Golgi communication. Vesicle trafficking has also been shown to be altered in HAND (Fields, et al., 2013). In addition, vesicle trafficking is also important for HIV-1 infection and some HIV-1 proteins aid in hijacking the host cells' trafficking machinery. Therefore, as intracellular trafficking is an important role of ER-Golgi contacts, the communication between these two organelles may play a role in HAND pathogenesis.

CHAPTER 4

HYPOTHESIS AND SPECIFIC AIMS

4.1 Hypothesis and Specific Aims

HIV-1 leads to non-AIDS related comorbidities including those of the heart, liver, and brain. Among these comorbidities are HAND. To date, there are no efficient treatments for HAND and even in the era of cART where mild forms of HAND are seemingly stabilized with the suppression of viral load, there is a chance for the severity of the neurocognitive function to increase especially in the older population of people living with HIV-1. Therefore, it is important to study the mechanisms leading to the development of HAND in order to develop better treatments that target this aging population of people living with HIV-1.

HIV-1 enters the CNS and goes on to infect non-neuronal cells such as monocytes and microglia. Neurons, though not infected, are affected by the influx of inflammatory cytokines or by the toxicity of viral proteins shed by infected cells. Some viral proteins including Tat, gp120, and Vpr have been found in the CSF and brain tissue of HIV patients and have been shown to disrupt cellular processes in the CNS. Tat alone is used in many studies of HAND and Tat transgenic mice have been widely used to study the underlying mechanisms that lead to neurodegeneration. In these studies, Tat has been shown to reduce the number of neuronal dendritic spines, alter organelle ultrastructure, and cause mitochondrial defects. **Thus, the goal of this project is to link the disruption of these cellular functions that lead to HAND with a common regulator that may be altered in the presence of TAT.**

The interaction between mitochondria and the ER is important for regulating many cellular functions including calcium exchange, lipid exchange, intracellular trafficking, and mitochondrial biogenesis. MAMs serve as communication sites for these two organelles and have been shown to play a role in other neurodegenerative diseases. For instance, many Alzheimer's disease-associated proteins are enriched in MAMs such as amyloid beta (Aβ), amyloid precursor protein (APP), and presenilin 1/2 (PS1/2). Dysfunction of MAMs have also been studied in the context of fronto-temporal dementia and related amyotrophic lateral sclerosis (FTD/ALS). FTD/ALS is characterized by cellular dysfunction of processes known to be regulated by MAMs. These include altered bioenergetics, unfolded protein response (UPR), Ca2+ homeostasis disruption, and defects in axonal transport.

The interaction between the ER and the Golgi apparatus is also important for normal cell functions including membrane protein sorting and trafficking and lysosome formation. Evidence from the literature suggests these same cellular functions are affected early during aging, AD, PD, ALS/FTD, and other neurodegenerative conditions including HAND. This suggests that not only are MAMs important but that overall interorganelle communication may play a role in the pathogenesis of these diseases. Therefore, I hypothesize that HIV-1 Tat disrupts interorganelle communication and that this disruption leads to cellular changes that have been associated with the pathogenesis of HAND. To test this hypothesis, the following specific aims are proposed:

Aim 1. To characterize the disruption of MAMs caused by HIV-1 Tat protein. Expression levels of known MAM tethering proteins and MAM-associated

proteins will be analyzed in the presence and absence of Tat protein. The interaction between MAM tethering proteins will be visualized and quantified in the presence and absence of Tat. The direct downstream MAM regulation of calcium dynamics and mitochondrial energy usage will be assessed in the presence and absence of Tat.

Aim 2. To characterize the disruption of ER-Golgi contacts caused by HIV-1 Tat protein. Expression levels of known ER-Golgi interacting proteins will be analyzed in the presence and absence of Tat protein. The interaction between ER and Golgi proteins will be analyzed in the presence and absence of Tat.

CHAPTER 5

**HIV-1 TAT AFFECTS MITOCHONDRIAL-ASSOCIATED ER
MEMBRANES (MAM) IN A NEURONS IN HIV-1-ASSOCIATED
NEUROCOGNITIVE DISORDERS (HAND)**

5.1 Introduction

HIV-1 has been shown to accelerate aging in many different organ systems in the body causing various comorbidities (Meir-Shafrir & Pollack, 2012) (Cohen & Torres, 2017). These include comorbidities of the heart, liver, kidney, and brain including HAND (Gallant, Hsue, Shreay, & Meyer, 2017) (Clifford & Ances, 2013). A recent meta-analysis identified the prevalence of HAND to be about 44% (Wei, et al., 2020). HAND has been shown to persist even with the antiretroviral therapies (Heaton, et al., 2011) and the exact mechanism of pathogenesis is still unknown. Thus, it is important to continue to study the mechanisms of HAND to develop better therapies.

Though neurons in the CNS are affected by HIV-1 and contribute most to the cognitive defects seen in HAND, they cannot be infected by HIV-1 (Irollo, Luchetta, Ho, Nash, & Meucci, 2021). Instead, astrocytes, microglia, endothelial cells, and infiltrating macrophages are infected by the virus leading to blood brain barrier defects and persistent neuroinflammation (Scutari, Alteri, Perno, Svicher, & Aquaro, 2017). These infected cells can also produce individual viral proteins, such as gp120, Tat, Nef, and Vpr, that are released through various mechanisms into the CNS (Nath, 2002). For instance, Tat can be actively produced and released by infected CNS cells (Nath, 2002). The presence of Tat, as well as other HIV-1 proteins, in the extracellular environment

has been shown to cause neuronal dysfunction including calcium dysregulation and increased ROS (Nath, 2002) (Hu, 2016).

MAMs are the dynamic functional relationship between the ER and mitochondria (Csordas, et al., 2006). MAMs have been the focus of multiple studies in recent years aimed at elucidating the many functions of this relationship. Mitochondria and ER contacts are important for calcium and lipid transfer between the two organelles (Peretti, Kim, Tufi, & Lev, 2020) and thus are important in cellular homeostasis. Because of the role MAMs play in homeostasis, their dysfunction has recently been implicated in disease pathology. For instance, studies have looked at MAMs in cancer pathology (Sassano, van Vliet, & Agostinis, 2017) and metabolic diseases (Theurey & Rieusset, 2017). MAMs have also been shown to regulate important cellular functions in neurons, specifically postsynaptic energetics (Leung, Ohadi, Pekkurnaz, & Rangamani, 2021) and neuronal homeostasis (Xu, et al., 2020) (Fowler, Garcia-Pardo, Simpson, & O'Sullivan, 2019). In addition, MAMs have also been implicated in neurodegeneration (Paillusson, et al., 2016) (Krols, et al., 2016) and aging and senescence (Picca, et al., 2020) (Janikiewicz, et al., 2018) (Ziegler, et al., 2021).

Recently MAMs have been implicated in HAND in various ways (Proulx, Park, & Borgmann, 2021). HIV-1 has been shown to induce ER stress and the UPR in both astrocytes and neurons (Akay, et al., 2012). Additionally, the HIV-1 protein Tat alone has been shown to produce similar effects to that of the whole virus (Norman, et al., 2008). MAMs have been implicated in regulating these ER stress and UPR signals (van Vliet & Agostinis, 2016) suggesting they play a role in HAND pathology. HIV-1

has also been shown to alter calcium signaling and homeostasis (Proulx, Park, & Borgmann, 2021) including HIV-1 Tat specifically (Hu, 2016) (Santerre, et al., 2019). Calcium signaling and mitochondrial function have been shown to be regulated by MAMs (van Vliet, Verfaillie, & Agostinis, 2014) (Ziegler, et al., 2021), which further supports the idea that MAMs contribute to HAND pathology.

Therefore, I hypothesize MAMs are affected by HIV-1 contributing to neuronal dysfunction seen in HAND. To test this hypothesis, I am using recombinant HIV-1 Tat protein added directly to the media of cultured cells. Here I use the SH-SY5Y cell line, an established cell line previously used to study HAND (Wang, et al., 2017) (Santerre, et al., 2019). I also utilize a non-cancer neuronal cell line, Lund human mesencephalic (LUHMES) cells. These cells have been used to study other neurodegenerative diseases (Leah, Vazquez-Villasenor, Ferraiuolo, Wharton, & Mortiboys, 2021) and viruses (Edwards & Bloom, 2019) but this will be the one of first studies to use LUHMES for investigating HAND. This study is the beginning of a larger investigation into the relationship between MAMs and HAND and will bring us one step closer to elucidating potential therapeutic targets for HAND.

5.2 Materials and Methods

5.2.1   Cell Culture

SH-SY5Y neuroblastoma cells were purchased from ATCC (CRL-2266) and were maintained as previously described (Santerre, et al., 2019). Cells were differentiated with 10μM retinoic acid for at least four days prior to treatment and subsequent experiments.

LUHMES cells were purchased from ATCC (CRL-2927) and Abm (T0284) and were maintained and differentiated as previously described (Allen, et al., 2022) (Zhang, Yin, & Zhang, 2014).

5.2.2 Tat Treatment

Recombinant Tat protein was obtained from the NIH HIV Reagent Program (HIV-1 IIIB Tat Protein, ARP-2222). The protein was reconstituted according to datasheet in PBS containing 1mg/mL BSA and 0.1 mM DTT. All experimental cells were treated with 100ng/mL Tat or PBS with BSA and DTT (labeled control) for 24 hours as indicated.

5.2.3 Proteomics

SH-SY5Y cells were treated with Tat or PBS for 24 hours and then collected with 0.25% trypsin, centrifuged at 4°C at 200 x g for five minutes to form cell pellets. Mass spectrometry analysis was then performed at the Temple University Proteomics Facility as previously described (Santerre, et al., 2021). Heat maps were made using Microsoft Excel.

5.2.4 Western Blot

Cells were collected and lysed with radio-immunoprecipitation assay (RIPA) buffer (25mM Tris-HCl pH7.6. 150mM NaCl, 1% Triton X-100, 0,1% SDS). Western blot analysis was performed as described (Santerre, et al., 2019) using primary antibodies as indicated. Secondary antibodies were used to detect protein bands: Anti-mouse IgG-HRP 1:10,000 (Advansta, R-05071-500) and Anti-rabbit IgG-HRP 1:10,000

(Advansta, R-05072-500). Densitometry of protein bands was determined using ImageJ software.

5.2.5 Antibodies and Dilutions for Western Blot

Anti-VDAC 1:1000 (Cell Signaling, 4661), Anti-PTPIP51 1:1000 (Proteintech, 20641-1-AP), Anti-VAPB 1:1000 (Invitrogen, MA5-24348), Anti-Grp75 1:5000 (Proteintech, 14887-1-AP), Anti-ACSL4 1:5000 (Proteintech, 66617-1-Ig), Anti-Bap31 1:1000 (Enzo Life Sciences, ALX-804-601-C100), Anti-Fis1 1:1000 (GeneTex, GTX111010), Anti-Tomm40 1:1000 (Novus Biologicals, NBP2-94075), Anti-YIF1A 1:500 (Abnova, PAB20773), Anti-IP3R-I/II/III 1:500 (Santa Cruz Biotechnology, sc-377518).

5.2.6 Proximity Ligation Assays

LUHMES were differentiated for one day and then split onto glass coverslips coated with poly-L-ornithine and fibronectin as previously described (Allen, et al., 2022) and allowed to differentiate for at least three more days. Cells were then treated with Tat or PBS as described above. After 24 hours, cells were fixed in 4% formaldehyde in PBS for 20 minutes, then washed in PBS twice for five minutes. Next, the cells were permeabilized in PBS with 0.2% Triton X-100 for five minutes and washed in PBST twice for five minutes. Afterwards, the cells were blocked in Duolink 1x blocking solution for one hour at 37°C. Cells were probed for protein interactions with a Duolink In Situ PLA kit (Sigma-Aldrich DUO92001, DUO92005, DUO92008) according to manufacturer's instructions. Primary antibodies were diluted to 1:350 in Duolink Antibody Diluent. Anti-pTyr 1:350 (Cell Signaling, 9411) was used to detect

phosphorylation of PTPIP51. Images were taken on a Leica EL6000 DMI3000 confocal microscope and analyzed using ImageJ.

5.2.7 Immunohistochemistry

HIV-1 transgenic rat brain tissue was obtained from Rosemarie Booze at the University of South Carolina. This animal model was first described by Reid et al. (Reid, et al., 2001) and constitutively expresses an HIV-1 provirus lacking *gag* and *pol* to render these animals noninfectious. The animals were housed and treated as previously described (Li, McLaurin, Illenberger, Mactutus, & Booze, 2021). Frozen brains were sent to the Fox Chase Cancer Center Histopathology Facility, where they were fixed and sectioned. The tissue was stained with anti-PTPIP51 and anti-VAPB antibodies and counterstained with hematoxylin. Images were taken using Aperio ImageScope software and analyzed in ImageJ.

5.2.8 MAM Fractionation

SH-SY5Y cells were differentiated for at least four days then treated with Tat or PBS for 24 hours as described above. Cells were then collected and lysed in a sucrose homogenization buffer and subjected to fractionation as previously described (Williamson, Wong, Bozidis, Zhang, & Colberg-Poley, 2015). MAM and cytosolic fractions were collected and subjected to western blot analysis as described above.

5.2.9 Seahorse Mito Stress Test

A Seahorse Mito Stress test measuring changes in oxygen consumption rate was conducted using a Seahorse XFe96 Analyzer (Agilent Technologies). LUHMES were differentiated for five days and then split onto a XFe96-well microplate coated

with poly-L-ornithine and fibronectin and allowed to grow overnight. The cells were then treated with Tat or PBS for 24 hours. Afterwards, the Mito Stress test was performed as previously described (Gu, Ma, Liu, & Wan, 2021) and per the Agilent Technologies protocol.

5.2.10 Reactive Oxygen Species Measurement

RedoxSensor Red CC-1 (R-14060) and MitoTracker Green FM (M-7514) were purchased from Fisher Scientific. LUHMES were differentiated for one day and then split onto glass coverslips and allowed to grow for four days. Afterwards, cells were treated with Tat or PBS for 24 hours as described above. Cells were incubated with 1μM Redox Sensor Red CC-1 and 1μM MitoTracker Green FM at 37°C for 10 minutes. The cells were then washed with PBS, the medium was replaced, and allowed to sit for 20 minutes in a cell culture incubator. Cells were then imaged using a Leica EL6000 DMI3000 confocal microscope and images were analyzed using ImageJ.

5.2.11 Metabolomics for Glutamate and ATP/ADP Expression

LUHMES were differentiated as described above. On the last day of differentiation, glucose-free medium with D-Glucose-13C6 (Aldrich, 389374-250MG) was added to the cells for 24 hours. The cells were then treated with 100ng/mL Tat or PBS for eight hours. Polar metabolites were extracted in ice-cold 80% methanol as described previously (Allen, et al., 2022). Mass spectrometry was conducted at the Proteomics and Metabolomics Center at the Wistar Institute in Philadelphia as previously described (Di Marcantonio, et al., 2021).

5.2.12 Glutamate Assay

LUHMES were differentiated and treated with 100ng/mL PBS or Tat as described above. Cell medium (supernatant) was collected after 24 hours and subjected to a colorimetric Glutamate Assay Kit (Cell BioLabs, MET-5080) according to manufacturer's instructions. Absorbance at 450nm was read on a GloMax Multi Detection System plate reader (Promega) and analyzed in Prism 9 (GraphPad).

5.2.13 Calcium Labeling and Imaging

LUHMES were grown on glass coverslips coated in PLO and fibronectin and were then differentiated and treated with Tat or PBS as described above. On the day of the experiment, 2µM Rhod-2 AM (Invitrogen, R1245MP) and 0.5M ci-IP$_3$/PM (Tocris, 6210) were added to the cells for one hour. Afterward, the medium was replaced by a calcium buffer (107mM NaCl, 7.2mM KCl, 1.2mM MgCl$_2$·6H$_2$O, 11.5mM glucose, 20mM HEPES-NaOH, and 1mM CaCl$_2$, pH7.2) containing 2µM Fura-2 AM (Invitrogen, F1221) and 0.5M ci-IP$_3$/PM for 30 minutes. This solution was replaced by calcium buffer without Fura-2 AM or ci-IP$_3$/PM for another 30 minutes. The cells were then imaged on a Leica microscope in a calcium-free buffer (107mM NaCl, 7.2mM KCl, 1.2mM MgCl$_2$·6H$_2$O, 11.5mM glucose, 20mM HEPES-NaOH, pH7.2) for two minutes for a baseline reading of intracellular calcium levels. Next, 1mM CaCl$_2$ was added to the cells and imaging was continued for another two minutes. Caged-IP$_3$ (ci-IP$_3$) was uncaged by exposure to UV light (λ395) for 20 seconds and imaging was continued for two minutes. Another dose of UV light was done for 20 seconds to ensure all IP$_3$ was in fact, uncaged. Imaging was continued until calcium levels in the cytoplasm and

mitochondria began to plateau (~500 seconds). Cells were imaged on a DMI 6000B fluorescence microscope (Leica Microsystems) as previously described (Samakai, et al., 2016). Fluorescence intensity was measured and graphed over time using Prism 9 (GraphPad).

5.2.14 Kinase Inhibitors

Dasatinib (Cayman Chemical Company, 11498) is an inhibitor of non-receptor tyrosine kinases such as Abl and members of the Src family. Gefitinib (Cayman Chemical Company, 13166) is an inhibitor of EGFR and EGFR-associated kinases. Rp-cAMPs (Enzo Life Sciences, BML-CN135-0001) is an inhibitor of cAMP-dependent protein kinases such as PKA. All three kinase inhibitors have been shown to modulate PTPIP51 phosphorylation and the ability for PTPIP51 to interact with other proteins (Brobeil, Koch, Eiber, Tag, & Wimmer, 2014). LUHMES were differentiated on glass coverslips coated in PLO and fibronectin as described above. Cells were treated with Tat or PBS for 24 hours as described above. Dasatinib (400nM), Gefitinib (80μM), and Rp-cAMPs (40μM) were added to the cells at the same time and allowed to incubate for four hours. The cells were then washed in PBS and fixed in 4% formaldehyde in PBS for 20 minutes. PLAs were conducted as described above using anti-pTyr 1:350 (Cell Signaling, 9411) and anti-PTPIP51 (Proteintech, 20641-1-AP) primary antibodies. Images were taken on a Leica EL6000 DMI3000 confocal microscope and analyzed using ImageJ.

5.2.15 Statistics

Statistical analysis was conducted in Prism 9 (GraphPad) where Mann-Whitney tests were calculated assuming a non-parametric distribution. Results were considered statistically significant if $p < 0.05$.

5.3 Results

5.3.1 Tat Alters MAM-associated Cellular Functions in LUHMES

MAMs are associated with many cellular functions such as mitochondrial dynamics, autophagy, calcium signaling, and the ER stress response (Proulx, Park, & Borgmann, 2021), (Paillusson, et al., 2016). I wanted to assess some of these MAM-associated functions in the presence of Tat in the LUHMES cell line since this is a relatively new cell line and has not previously been used to show cellular effects of Tat.

5.3.1.1 Tat Alters Mitochondrial Bioenergetics and Cellular Metabolism

First, I wanted to see the alteration in mitochondrial bioenergetics caused by Tat. To do this, I measured changes in the oxygen consumption rate (OCR) of LUHMES in the presence of Tat by using a Seahorse Mito Stress Test (Figure 13A). After the addition of FCCP, an uncoupling agent, maximal respiration was decreased in the presence of Tat suggesting that Tat was affecting the ability of these cells to generate energy through the electron transport chain (Allen, et al., 2022) (Figure 13B). Spare respiratory capacity, the difference between maximal respiration and basal respiration, was also reduced. (Figure 13B). Because both maximal respiration and spare respiratory capacity are indicative of the cells' ability to function in a high energy-demanding state, these data suggest that Tat is altering regulators of cellular energy, for instance, MAMs.
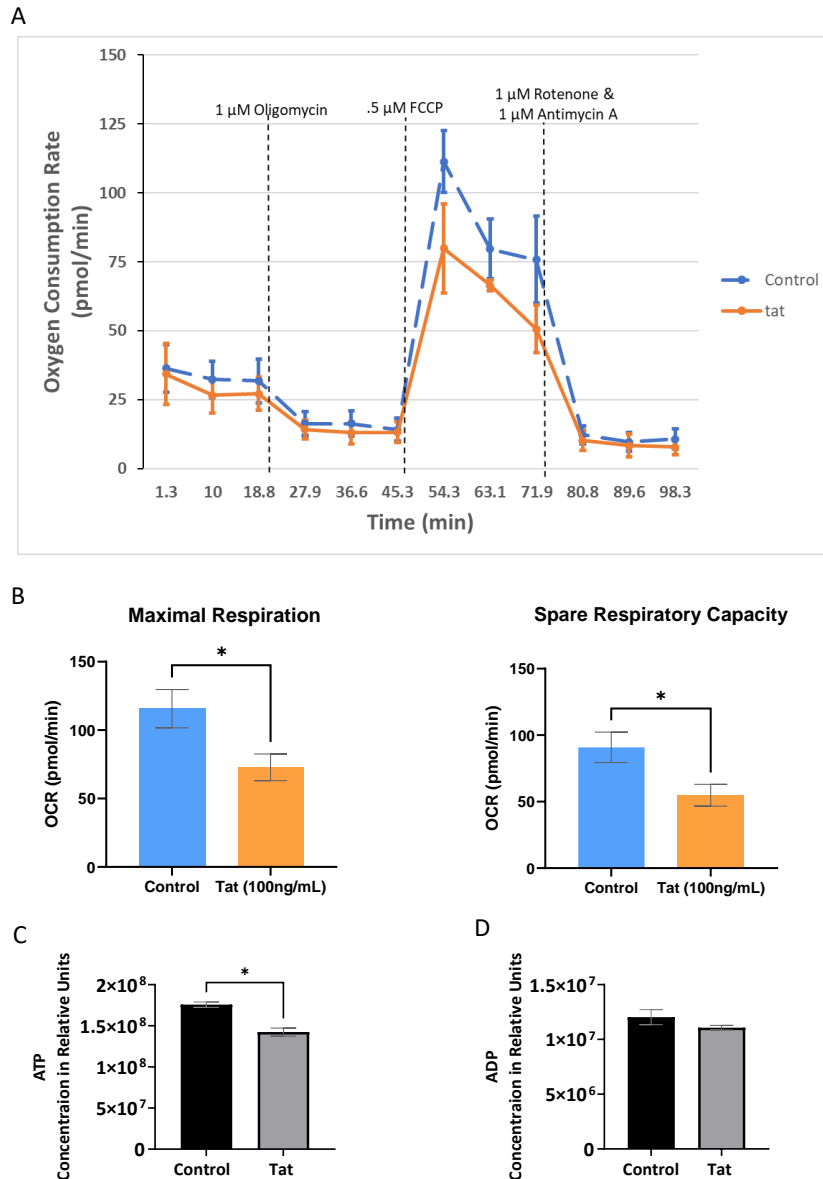
Figure 13. Altered Mitochondrial Bioenergetics in the Presence of Tat.
A) Graph illustrating a Seahorse Mito Stress Test in LUHMES treated
with PBS or Tat. B) Representative bar graphs showing OCR associated
with maximal respiration and spare respiratory capacity. Bar graphs
showing intracellular C) ATP and D) ADP from metabolomics analysis of
control LUHMES and cells treated with Tat in relative concentration units.
* indicates $p < 0.05$. Error bars are shown as S.E.M.

Mitochondria are important producers of ATP and mitochondrial energy
dynamics are known to be regulated by MAMs (Yu, Sun, Gong, & Feng, 2021).

Therefore, I measured the concentration of ATP and ADP in LUHMES treated with Tat or PBS using mass spectrometry for metabolites (Figure 13C and D). Here, ATP levels are significantly decreased in the presence of Tat, thus supporting the reduction in OCR seen in the Seahorse Mito Stress Test experiment. This data suggests that MAMs, as regulators of mitochondrial energy, are affected by Tat.

5.3.1.2 Tat Alters Reactive Oxygen Species

Since MAMs have also been shown to regulate mitochondrial ROS (Janikiewicz, et al., 2018), I also wanted to measure the ROS levels in cells treated with Tat using a redox-sensitive dye, RedoxSensor Red (Figure 14A). RedoxSensor Red can be oxidized and localized to mitochondria or lysosomes where it fluoresces. In the presence of Tat, the total ROS levels, indicated by total RedoxSensor Red fluorescence intensity, were significantly elevated compared to control cells (Figure 14B). Mitochondria can also be labeled with a counterstain (MitoTracker Green), and the colocalization of the RedoxSensor Red dye and MitoTracker Green can be imaged to visualize redox potential in the mitochondria specifically. In the presence of Tat, colocalization of RedoxSensor Red and MitoTracker Green was increased suggesting that mitochondrial ROS was also increased in these cells (Figure 14C). To see if the increase of mitochondrial ROS in Tat-treated cells was due to an increase in mitochondrial number, I measured the overall fluorescence intensity of MitoTracker Green. In the presence of Tat, there was a decrease in MitoTracker Green indicating that Tat increased total ROS and mitochondrial ROS without increasing the mitochondrial

number (Figure 14D). These results suggest that Tat may be altering MAMs, which in

turn, can cause altered ROS production in these cells.

Figure 14. Tat Increases ROS in LUHMES.
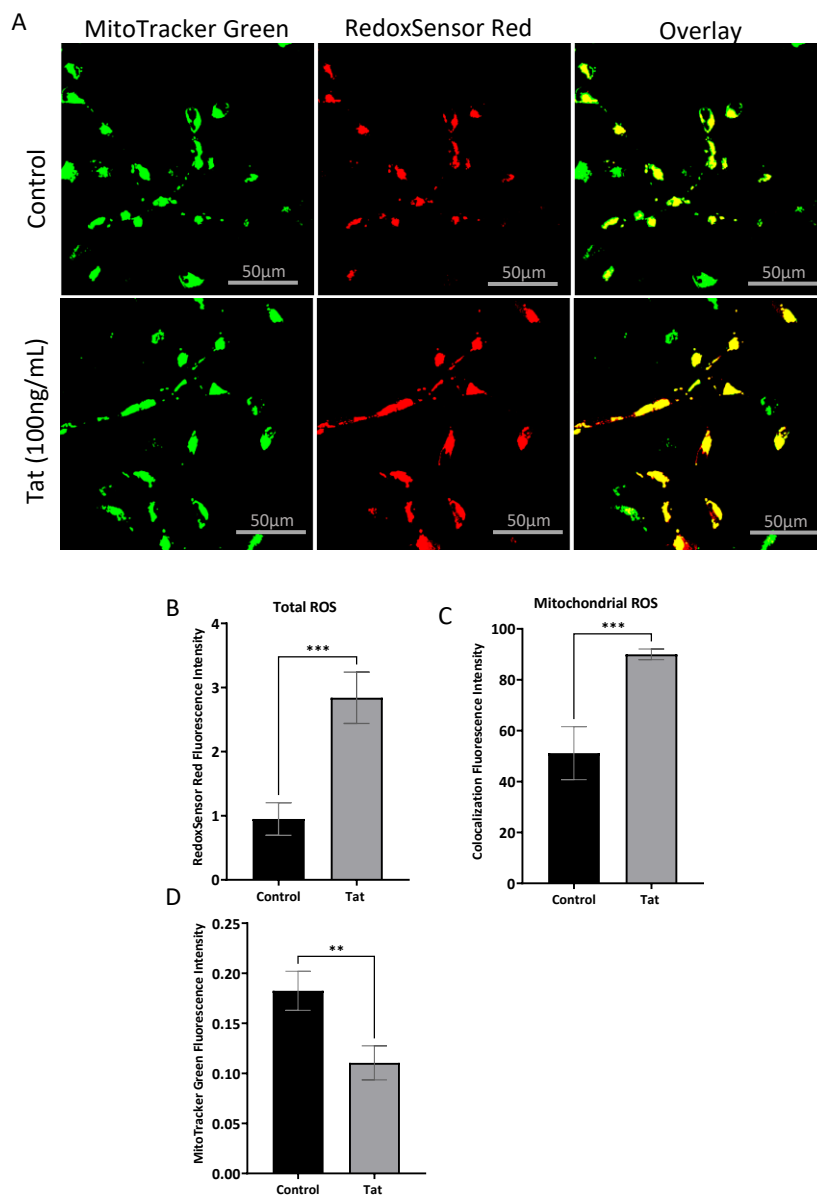A) Representative images of RedoxSensor Red and MitoTracker Green
staining for ROS in control LUHMES and those treated with Tat.
Quantification of B) total ROS, C) mitochondrial ROS, and D)
mitochondrial number (represented as fluorescence intensity of MitoTracker
Green stain) represented as bar graphs. ** indicates p<0.01. *** indicates
p,0.001. Error bars are shown as S.E.M.

5.3.1.3 Tat Alters Calcium Dynamics in Response to IP_3

Mitochondrial bioenergetics and ROS production are highly regulated by calcium signaling (Brookes, Yoon, Robotham, Anders, & Sheu, 2008). MAMs have been shown to be important in calcium homeostasis (Hirabayashi, et al., 2017) and calcium signaling (Yang, et al., 2020). Specifically, ER-mitochondria calcium transfer has been shown to be important in ATP production and ROS homeostasis. Disrupted calcium transfer has been implicated in many disease pathologies (Hirabayashi, et al., 2017). To investigate the effect of Tat on calcium transfer between the ER and mitochondria, I visualized calcium levels in the cytoplasm and mitochondria using live LUHMES treated with Tat in response to IP_3. IP_3 binds to IP_3Rs leading to calcium release from ER stores (Keebler & Taylor, 2017). Since IP_3 is very reactive with IP_3Rs, caged-IP_3 can be loaded into cells where it can be uncaged by exposure to UV light at the desired time to visualize immediate calcium localization (Decrock, et al., 2015). Here, LUHMES were loaded with calcium indicator dyes that localize to the cytoplasm (Fura2) and mitochondria (Rhod2). Fluorescence was then measured in response to the addition of $CaCl_2$ and IP_3 uncaging (Figure 15). Here, baseline mitochondrial calcium levels were higher in Tat-treated cells than in control cells, consistent with previous reports that Tat causes an influx of extracellular calcium into the cytoplasm (Santerre, et al., 2019) where it can be taken up by mitochondrial stores. In response to $CaCl_2$, cytoplasmic calcium remained relatively unchanged, whereas mitochondrial calcium levels slightly increased suggesting that mitochondria were taking up this calcium in both control and Tat-treated cells. However, after IP_3 was uncaged, mitochondrial

calcium levels increased in control cells but did not change in Tat-treated cells suggesting that even though calcium was being released from the ER, mitochondria were prevented from taking up this new calcium. The second uncaging event did not alter calcium levels in the mitochondria and cytoplasm suggesting all the IP_3 was uncaged the first time. Together, these data indicate that calcium dynamics, which MAMs regulate, are altered in the presence of Tat.
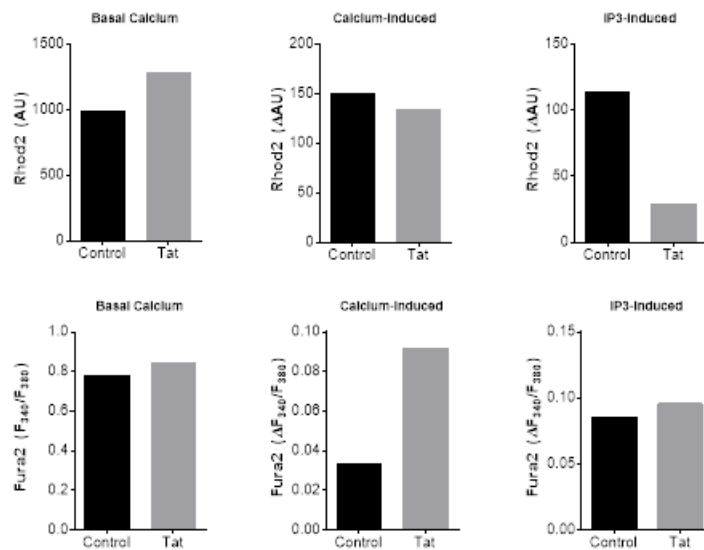
Figure 15. Altered Ca^{2+} Dynamics in the Presence of Tat.
Mitochondrial (Rhod2) and cytosolic (Fura2) calcium levels in live control LUHMES and those treated with Tat at basal levels, in response to additional extracellular calcium, and uncaged IP_3. Results are from a single experiment and represented as arbitrary units (AU) and delta arbitrary units (ΔAU) in bar graphs.

5.3.1.4 Tat Increases Glutamate Toxicity

Tat has been shown to increase glutamate exocytosis and glutamate excitotoxicity (Musante, et al., 2010), (Haughey, Nath, Mattson, Slevin, & Geiger, 2001). Glutamate excitotoxicity has been linked to neurodegenerative diseases

(Lewerenz & Maher, 2015) and the glutamatergic system has been proposed as a target

for HAND (Potter, Figuera-Losada, Rojas, & Slusher, 2013). Therefore, I measured the

expression of glutamate in LUHMES in the presence of Tat. Analysis of the data from

the mass spectrometry of polar metabolites in cells treated with PBS or Tat, showed that

the glutamate concentration was significantly higher in Tat-treated cells (Figure 16A)

suggesting that Tat increased the intracellular concentration of glutamate. I also

measured the extracellular concentration of glutamate to look at extracellular toxicity

by performing a glutamate concentration assay on the cell supernatant (Figure 16B).

Here, there was an increase in extracellular glutamate suggesting that Tat is affecting

the glutamatergic system in these LUHMES. Taken together, these results indicate that

the functional communication between the ER and mitochondria is altered in the

presence of Tat thus suggesting that Tat is affecting MAMs.
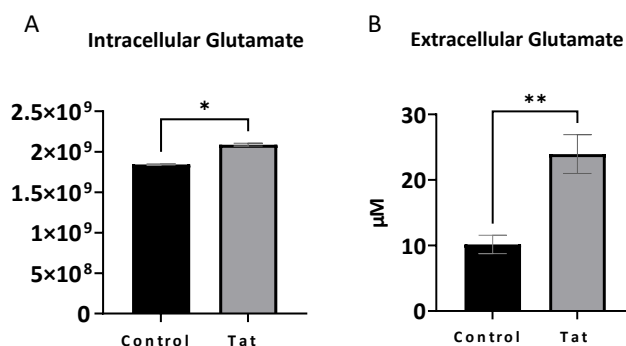
Figure 16. Glutamate Toxicity in LUHMES Treated with Tat.
A) Glutamate concentration in LUHMES in the absence and presence of
Tat as obtained from metabolomics. B) Extracellular glutamate
concentration in LUHMES in the absence and presence of Tat using a
glutamate assay and cell supernatant. * indicates $p < 0.05$. ** indicates
$p < 0.01$. Error bars are shown as S.E.M.
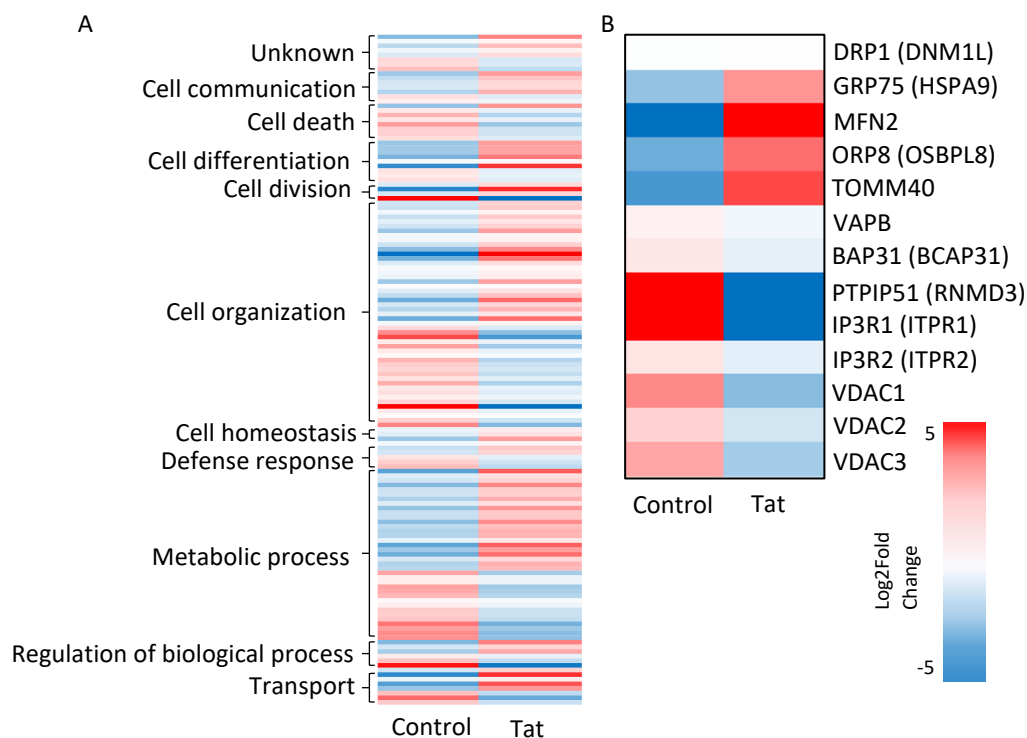
Figure 17. Heatmaps Representing Proteomics Analysis.
Heatmaps representing differentially expressed proteins from proteomics analysis organized by A) cellular functional pathway and B) association with MAMs between control SH-SY5Y cells and Tat-treated cells. Colors represent Log2fold change.

5.3.2 Tat Alters Expression of MAM-Associated Proteins

Since MAM-associated cellular functions are altered with Tat here, and in previous reports, I wanted to characterize the expression pattern of MAM-associated proteins in the presence of Tat. To begin, I conducted a proteomic analysis of global protein expression in differentiated SH-SY5Y cells treated with 100ng/mL Tat or PBS for 24 hours. Pathway analysis of the most differentially expressed proteins revealed that many cellular processes that were altered with Tat treatment were also those regulated by MAMs (Figure 17A). Among these processes are cell organization, cell homeostasis, and transport, all of which are regulated in some way by MAMs (Yang, et

al., 2020), (Gao, Yan, & Zhu, 2020), (Annunziata, Sano, & d'Azzo, 2018). When analyzing the data further, I saw that individual MAM-associated proteins were also differentially expressed in Tat-treated cells compared to control cells (Figure 17B) suggesting that protein expression, in MAMs, was altered. Together, these results suggest that Tat is altering MAMs in a way that may affect cellular processes associated with MAMs.

Figure 18. Interaction Between Bap31 and Tomm40 in the Presence of Tat.
A) Representative images of PLA for the interaction between MAM tethering proteins Bap31 and Tomm40 in LUHMES cells in the absence and presence of Tat. Red dots indicate individual interactions. Dapi staining indicates individual nuclei. B) Quantification of assays represented by bar graphs. C and D) Quantification of western blot analyses for whole cell expression of Bap31 and Tomm40. N.S. – not significant. Error bars are shown as S.E.M.

Figure 19. Interaction Between Bap31 and Fis1 in the Presence of Tat.
A) Representative images of PLA for the interaction between MAM tethering proteins Bap31 and Fis1 in LUHMES cells in the absence and presence of Tat. Red dots indicate individual interactions. Dapi staining indicates individual nuclei. B) Quantification of assays represented by bar graphs. C and D) Quantification of western blot analyses for whole cell expression of Bap31 and Tomm40. N.S. – not significant. ** indicates $p<0.01$. Error bars are shown as S.E.M.

5.3.3 Tat Alters Interactions Between MAM-Tethering Proteins *in vitro*

Many proteins play roles in MAM maintenance and communication between the ER and mitochondria. These include proteins that are important for ER and/or mitochondrial function and proteins involved in transiently tethering the two organelles together (Yang, et al., 2020). MAM tethers are proteins that help form the physical connection between the ER and the mitochondria and facilitate the crosstalk between the two (Paillusson, et al., 2016). Therefore, I wanted to see whether Tat altered the interaction between MAM tethering proteins. Here, I treated differentiated LUHMES cells with 100ng/mL Tat or PBS for 24 hours then performed Duolink proximity ligation assays (PLA). Using PLAs, I visualized the interaction between the tethering proteins BAP31, IP3R, and VAPB on the ER and translocase of outer mitochondrial membrane

40 (Tomm40), Fis1, PTPIP51, and VDAC on mitochondria (Figures 18A, 19A, 20A, and 20E). These protein pairs have been shown to tether the ER and mitochondria together and regulate important MAM-associated functions (Paillusson, et al., 2016). Quantification of each PLA shows that Tat significantly increased the interaction between Bap31 and Fis1 and decreased the interaction between IP3R and VDAC as well as VAPB and PTPIP51 (Figures 19B, 20B, and 20F). The interaction between Bap31 and Tomm40 was not significantly altered with Tat treatment however, there was a slight increase (Figure 18B). These data suggest that Tat is altering the interactions between key MAM tethering proteins. To assess whether these altered interactions were due to altered protein expression, I performed western blots using whole cell lysates (Figure 18C-D, 19C, 20C-D, and 20D-H). Tat did not significantly change the whole cell expression of these tethering proteins, except VDAC, suggesting that Tat is altering MAM interactions by some other mechanism other than protein expression.
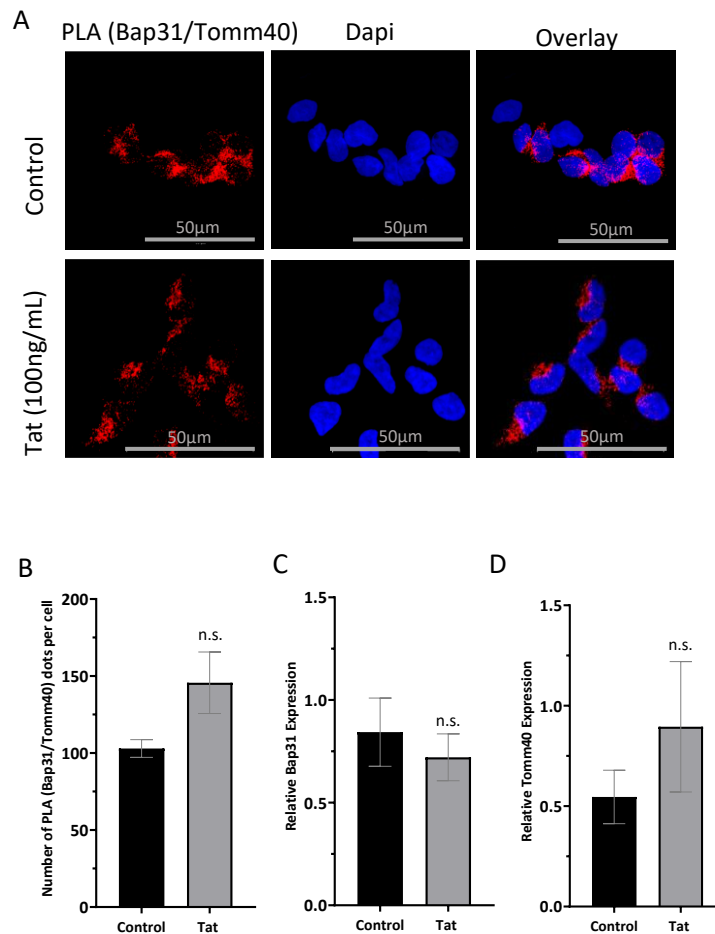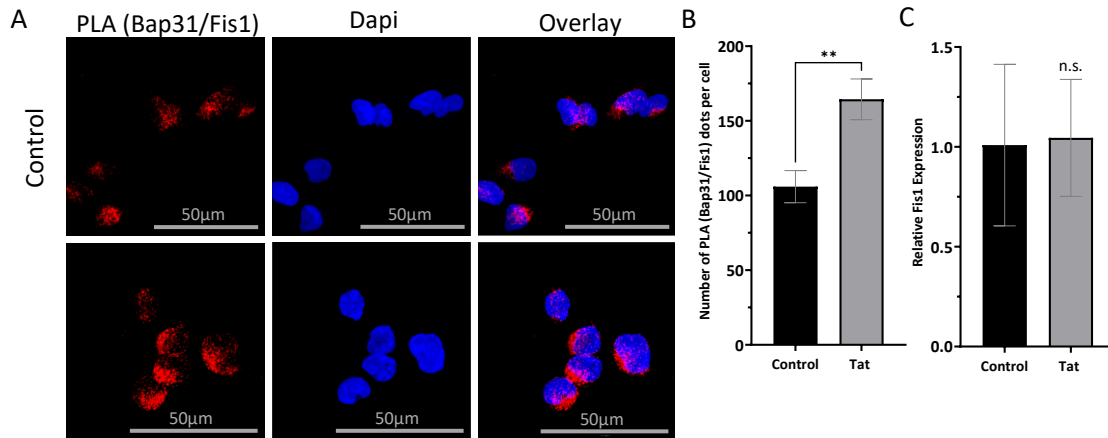
Figure 20. Interaction Between MAM Tethering Proteins in the Presence of Tat.
A) Representative images of PLA for the interaction between MAM tethering proteins IP$_3$R and VDAC and E) PTPIP51 and VAPB in LUHMES cells in the absence and presence of Tat. Red dots indicate individual interactions. Dapi staining indicates individual nuclei. B and F) Quantification of assays represented by bar graphs. C, D, G and H) Quantification of western blot analyses for whole cell expression of these proteins. N.S. – not significant. * indicates $p<0.05$. ** indicates $p<0.01$. *** indicates $p<0.001$. Error bars are shown as S.E.M.
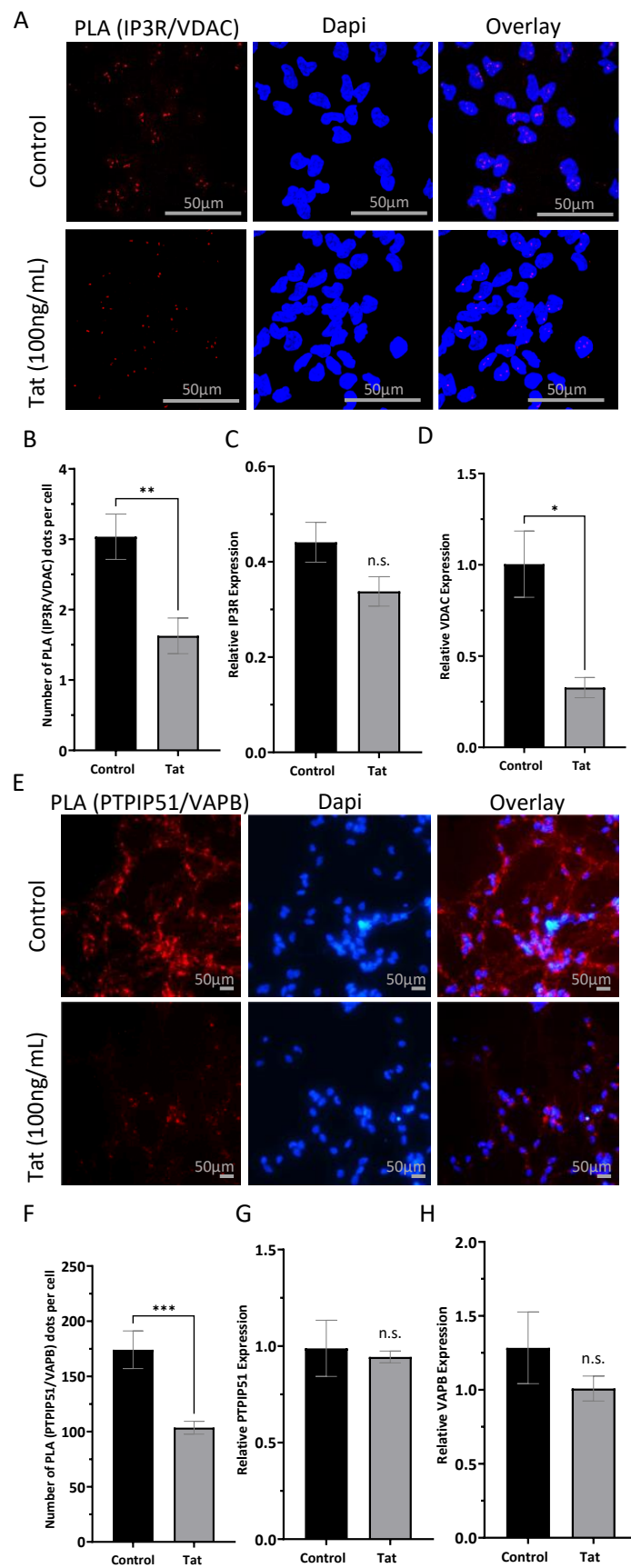
Figure 20. (Legend on the previous page)

5.3.4 Tat Alters Interactions Between PTPIP51 and VAPB in HIV Tg Rats

One of the MAM tethering proteins that I showed was altered with Tat is PTPIP51, which has recently been implicated in memory formation and its interaction with its MAM tethering partner, VAPB, has been shown to regulate synaptic activity (Brobeil, et al., 2015) (Gomez-Suaga, et al., 2019). Therefore, I wanted to look at PTPIP51 and VAPB interactions specifically.
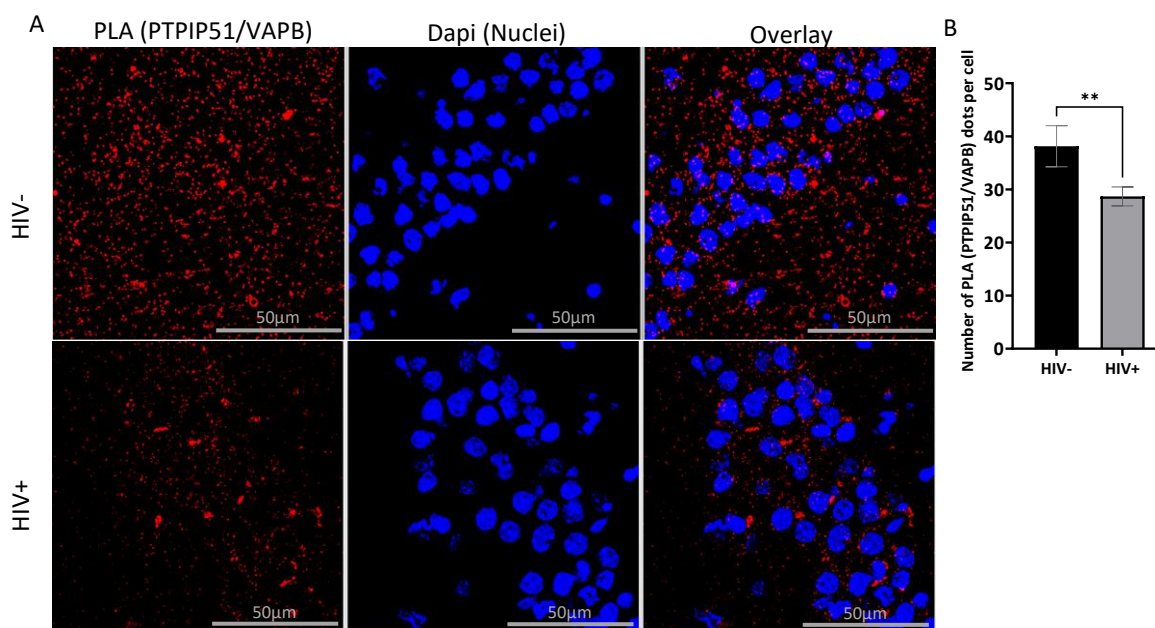
Figure 21. Altered PTIPI51 and VAPB Interactions in HIV Tg Rats.
A) Representative images of PLA for PTPIP51 and VAPB in hippocampal sections of Tg rat brains. Red dots indicate individual interactions. Dapi staining indicates individual nuclei. B) Quantification of PLA analysis represented as a bar graph. ** indicates $p < 0.01$. Error bars are shown as S.E.M.

To see if PTPIP51 and VAPB interactions are altered in an *in vivo* model, I obtained frozen brain tissue from HIV-1 transgenic (Tg) rats. PLAs were performed to visualize PTPIP51 and VAPB interactions (Figure 21A). Here there was a significant decrease in the interaction between PTPIP51 and VAPB in HIV Tg rat hippocampi (Figure 21B) similar to the decrease I showed before *in vitro*. To see if global expression

of these proteins is also altered and if protein expression could explain the decrease in interactions, immunohistochemistry was performed on the hippocampi of these same animals. Images were taken of the CA1, CA2/3 and DG regions of the hippocampi (Figure 22A). Quantification of these regions revealed that the expression of PTPIP51 was significantly increased in both the CA1 and CA2/3 regions (Figures 22B-E). The expression of VAPB was significantly decreased in DG region as well (Figure 22F and G). These results suggest that the expression of PTPIP51 and VAPB is region-specific, and that HIV-1 is altering the interaction between the two.

Figure 22. Altered PTPIP51 and VAPB Expression in HIV Tg Rats.
Representative images of IHC for the expression of PTPIP51 and VAPB in the B) CA1 region, E) CA2/3 region, and F) DG region of the hippocampus as indicated in A. Quantification of the IHC analysis in the C) CA1 region, E) CA2/3 region, and G) the DG region of the hippocampus represented as bar graphs. N.S. – not significant. * indicates $p<0.05$. *** indicates p,0.001. Error bars are shown as S.E.M.

5.3.5   Tat Alters PTPIP51 and VAPB Protein Expression in the MAM Fraction

As mentioned above, the whole cell expression of the MAM-associated proteins PTPIP51 and VAPB was not significantly changed with Tat treatment (Figures 20G and H). Since these proteins have other functions in the cell that are not associated with the MAM (Dietel, et al., 2019) (Dietel, et al., 2019), (Gkogkas, et al., 2008), I wanted to see if the expression of these proteins was altered in the MAM fraction, specifically, in the presence of Tat. To do this, I isolated the MAM and cytosolic fractions of SH-SY5Y cells treated with 100ng/mL Tat or PBS using a Percoll/sucrose gradient. After fractionation, I blotted for PTPIP51 and VAPB expression (Figure 23). PTPIP51 and VAPB expression was decreased in the MAM fraction in the presence of Tat. These results suggest that Tat may be altering the localization of PTPIP51 and VAPB, thus altering their interaction at MAMs.
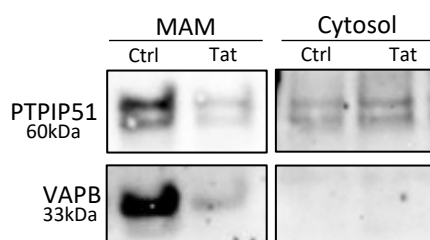
Figure 23. Altered Expression in the MAM Fraction in the Presence of Tat. Western blot analysis of the expression of PTPIP51 and VAPB in different fractions of control SH-SY5Y cells and those treated with Tat.

5.3.6   PTPIP51 Phosphorylation is Increased with Tat

Protein interactions with PTPIP51 have been shown to be regulated by the phosphorylation of PTPIP51 (Brobeil, Bobrich, Tag, & Wimmer , 2012) (Brobeil, Koch, Eiber, Tag, & Wimmer, 2014). For instance, phosphorylated PTPIP51 (pTPTPI51) can

bind with 14-3-3 and Raf-1 in the cytosol of the cell to signal through the mitogen activated protein kinase (MAPK) pathway (Brobeil, Chehab, Dietel, Gattenlohner, & Wimmer, 2017). Subcellular localization of PTPIP51 is also regulated by phosphorylation where tyrosine 176 (Tyr176) phosphorylation has been shown to cause PTPIP51 to localize to the mitotic spindle (Brobeil, Graf, Eiber, & Wimmer, 2012). In these cases, phosphorylation of PTPIP51, particularly on the Tyr176 residue, allows for protein interactions outside the mitochondria. Therefore, when PTPIP51 is phosphorylated, it does not localize to the mitochondria and thus does not interact with VAPB at MAMs.
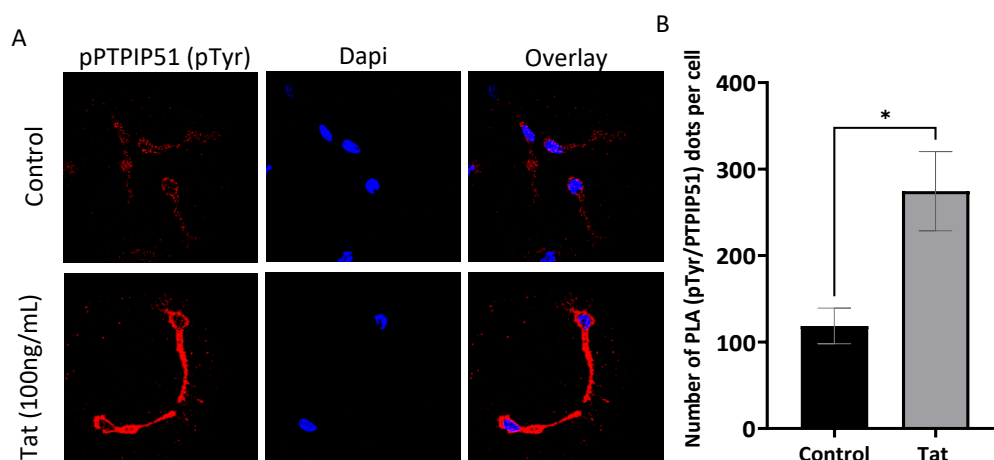
Figure 24. Altered PTPIP51 Phosphorylation in the Presence of Tat.
A) Representative images of PLA for tyrosine phosphorylated PTPIP51 (pPTPIP51) in LUHMES cells in the absence and presence of Tat. Red dots indicate phospho-tyrosine residues. Dapi staining indicates individual nuclei. B) Quantification of the PLA for pPTPIP51 represented as a bar graph. * indicates $p<0.05$. Error bars are shown as S.E.M.

To see if Tat may be altering the phosphorylation status of PTPIP51 and if this could explain why there is a decrease in PTPIP51 expression in MAMs, I performed PLAs in LUHMES treated with Tat or PBS for 24 hours using anti-PTPIP51 and anti-

pTyr antibodies (Figure 24A). Here, PTPIP51 phosphorylation on tyrosine residues was significantly increased in the presence of Tat (Figure 24B). This result suggests that Tat is altering tyrosine phosphorylation of PTPIP51, thus leading to decreased localization to MAMs and, subsequently, decreased interaction with VAPB.

5.3.7 Inhibiting PTPIP51 Phosphorylation Rescues PTPIP51 and VAPB
Interaction

Phosphorylation is important for regulating the localization of PTPIP51. When PTPIP51 is phosphorylated, it does not localize to the mitochondria and thus cannot interact with binding partners in MAMs. Lyn and Src family kinases have been shown to phosphorylate PTPIP51 (Brobeil, et al., 2011). Dasatinib is an inhibitor of tyrosine kinases such as Src family kinases, and Lyn (Ryu, Ka-Young, et al., 2019). Gefitinib, an EGFR inhibitor, has also been shown to alter PTPIP51 phosphorylation (Brobeil, Koch, Eiber, Tag, & Wimmer, 2014). Rp-cAMPS is an inhibitor of cAMP-dependent kinases (Dostmann, 1995) and has also been shown to alter PTPIP51 phosphorylation (Brobeil, Koch, Eiber, Tag, & Wimmer, 2014). To see if phosphorylation of PTPIP51 affects the interaction between PTPIP51 and VAPB at MAMs, I treated LUHMES with these three kinase inhibitors in the absence and presence of Tat. I performed PLAs in these LUHMES for PTPIP51 and VAPB interactions (Figure 25A). Here, the interactions between PTPIP51 and VAPB remained unchanged in the presence of Tat (Figure 25B). These results suggest that phosphorylation of PTPIP51 is being altered with Tat, which can lead to decreased localization to MAMs, and thus, decreased interaction with VAPB.
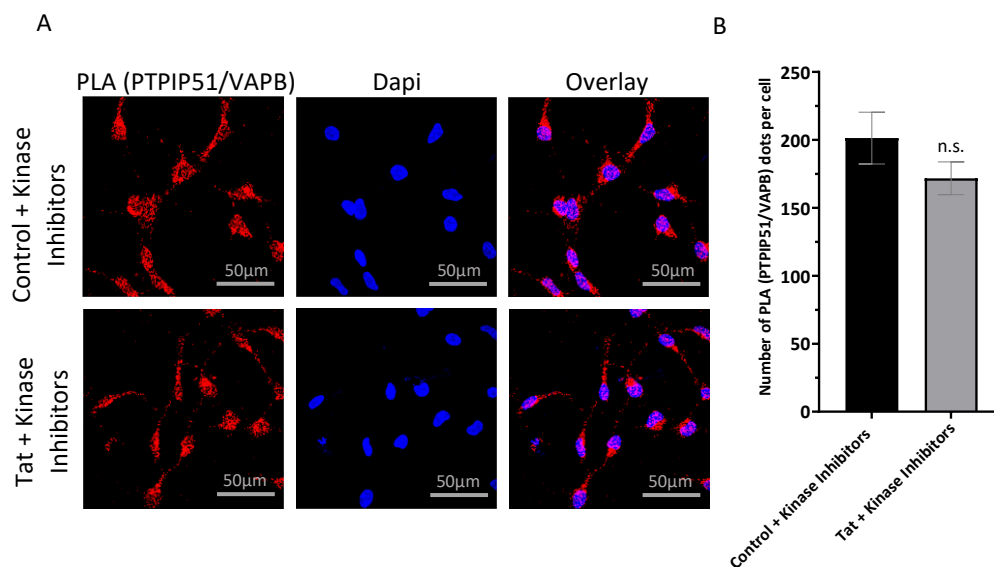
Figure 25. Inhibiting PTPIP51 Phosphorylation Prevents Loss of PTPIP51 and VAPB Interactions in the Presence of Tat.
A) Representative images of PLA for PTPIP51 and VAPB in LUHMES cells treated with kinase inhibitors (gefitinib, dasatinib, and RpcAMPs) for 4 hours in the absence and presence of Tat. Red dots indicate individual interactions. Dapi staining indicates individual nuclei. B) Quantification of the number of PTPIP51 and VAPB interactions represented as a bar graph. N.S. – not significant. Error bars are shown as S.E.M.

5.4 Discussion and Future Directions

In this study, I showed that MAM-associated cellular functions were altered in the presence of HIV-1 Tat. These same functions have also been shown to be altered in HAND. For instance, I showed that maximal respiration and spare capacity in LUHMES cells treated with Tat were decreased. Altered brain energy metabolism has been shown to play a role in HAND including altered metabolism in glial cells and neurons (Cotto, Natarajaseenivasan, & Langford, 2019). HIV-1 gp120 has also been shown to lead to metabolic reprogramming in neurons, thus contributing to HAND (Allen, et al., 2022). Also, I showed that total ROS and mitochondrial ROS were increased in the presence of Tat (Figure 26). ROS has been implicated in HAND pathology (Buckley, et al., 2021) and is known to be regulated, in part, by MAMs (Missiroli, et al., 2018).
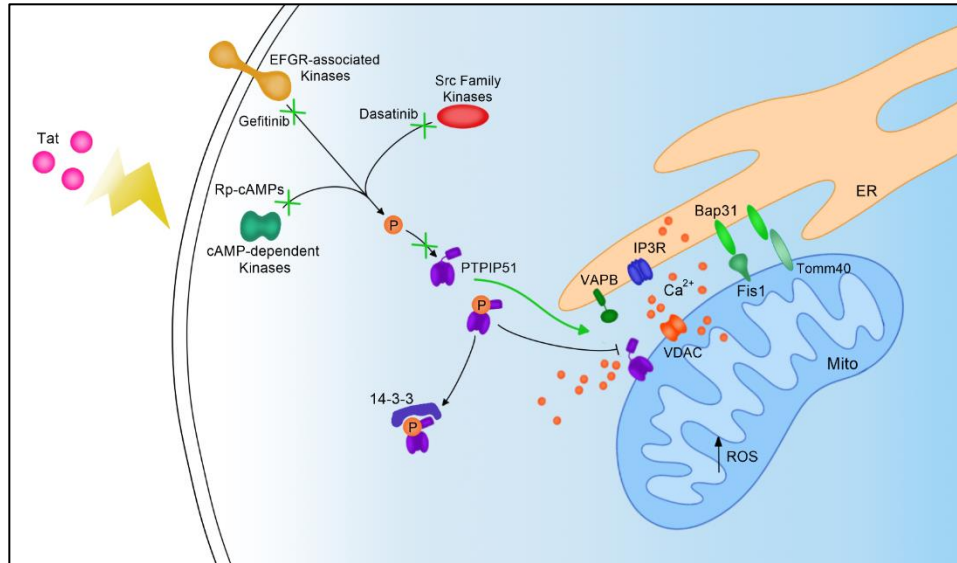
Figure 26. Schematic Illustrating the Effect of Tat on MAMs in Neurons.
Tat affects the interaction between MAM tethering proteins IP3R and VDAC, Bap31 and Fis1, Bap31 and Tomm40, and PTPIP51 and VDAC. The affected MAM tethering leads to dysregulated calcium transfer between the ER and mitochondria and increased ROS. Tat also affects PTPIP51 phosphorylation and thus its localization to MAMs. Kinase inhibitors gefitinib, dasatinib, and Rp-cAMPs together can block PTPIP51 tyrosine phosphorylation even in the presence of Tat and can lead to PTPIP51 and VAPB interactions.

Most of these MAM-associated cellular functions can be attributed to the regulation of calcium signaling between the ER and mitochondria (van Vliet, Verfaillie, & Agostinis, 2014) and calcium dysregulation has been implicated in HAND (Irollo, Luchetta, Ho, Nash, & Meucci, 2021). Here, I showed that calcium signaling between the organelles is disrupted with Tat, most likely due to the decrease in PTPIP51 and VAPB interactions, IP_3R and VDAC interactions, and the decrease in VDAC expression. In this experiment, I used IP_3 to provoke the release of calcium from ER stores to visualize calcium entering mitochondria or the cytosol. Though I did not see the same mitochondrial response in Tat-treated cells as in the control cells after the initial uncaging of IP_3, this response was not as drastic as expected. Most likely, the

elevated baseline levels of mitochondrial calcium in the Tat-treated cells were skewing the results as Tat has been shown to cause an influx of calcium (Hu, 2016) (Santerre, et al., 2019). Tat has also been shown to stimulate the production of IP_3 in cortical neurons (Hu, 2016) thus, the addition of IP_3 to the Tat-treated cells would not elicit much of an effect on calcium release if IP_3 levels were already elevated.

Another MAM-associated function not investigated in this study is autophagy (Janikiewicz, et al., 2018) (Yang, et al., 2020), which has also been implicated in HAND (Dever, Rodriguez, Lapierre, Costin, & El-Hage, 2015) (Santerre, et al., 2021). Calcium has been shown to be important in autophagic signaling and autophagosomes have been shown to form at MAMs (Hamasaki, et al., 2013). Thus, the important connection between MAMs and autophagy in the context of HAND needs to be investigated further.

Though the MAM-associated functions investigated in this study have been established in the literature as being altered by HIV-1 and Tat specifically, they have not been shown in the LUHMES cell line in the context of HIV-1 or HAND. Thus, it was important to establish LUHMES could be affected by Tat added directly to the medium. LUHMES were used to investigate MAM-associated cellular functions and to visualize MAM tethering interactions. Most importantly, since LUHMES are not a cancer-based cell line, they can be used to measure oxygen consumption during a Seahorse Mito Stress Test without proliferative and cancer-associated factors influencing the results. Also, even though LUHMES are derived from mesencephalic tissue from an 8-week-old (Scholz, et al., 2011), they have been used to study age-related neurodegeneration (Leah, Vazquez-Villasenor, Ferraiuolo, Wharton, &

Mortiboys, 2021) (Zhang, Yin, & Zhang, 2014) and more recently HAND (Allen, et al., 2022).

PTPIP51, and its binding to the MAM tethering protein VAPB, has been implicated in memory and synaptic regulation (Brobeil, et al., 2015) (Gomez-Suaga, et al., 2019) (Leung, Ohadi, Pekkurnaz, & Rangamani, 2021). The interaction between these two proteins has been shown to be localized to synapses and loss of this interaction can decrease synaptic activity (Gomez-Suaga, et al., 2019). At the same time, PTPIP51 and VAPB interaction has also been shown to increase with synaptic activity suggesting a close relationship between these MAM tethering proteins and neuronal signaling (Gomez-Suaga, et al., 2019). Tat has been known to lead to synaptic dysfunction *in vivo* (Fitting, et al., 2012) and *in vitro* (Hargus & Thayer, 2013), though the exact mechanism is still unknown. Here, I showed that the interaction between PTPIP51 and VAPB in the presence of HIV-1 Tat was decreased in neurons implicating PTPIP51 and VAPB tethering in Tat-mediated synaptic dysfunction. It is important to note that I did not see a consistent decrease in all MAM tethering protein interactions and in fact, I saw an increase in Bap31 and Fis1 interactions. Thus, Tat appears to affect MAM tethering proteins differently and suggests that the dysregulation of MAMs is important in pathogenesis rather than an overall upregulation or downregulation.

I attributed the decrease in PTPIP51 and VAPB interactions to an alteration in the localization of PTPIP51 after measuring the protein expression in the MAM fraction compared to whole cell expression. Phosphorylation regulates PTPIP51-protein binding and thus subcellular localization (Brobeil, Graf, Eiber, & Wimmer, 2012) (Brobeil,

Chehab, Dietel, Gattenlohner, & Wimmer , 2017). Here, I showed that PTPIP51 tyrosine phosphorylation is increased in the presence of Tat. PTPIP51 can, however, also be phosphorylated on serine and threonine residues so I used three different kinase inhibitors that target various classes of kinases (gefitinib, dasatinib, and RpcAMPs) and showed restored interaction with VAPB even in the presence of Tat. PTPIP51 interactions and phosphorylation has been mostly studied in cancer pathology (Brobeil, et al., 2011) (Dietel, et al., 2019) where MAMs have also been implicated (Sassano, van Vliet, & Agostinis, 2017). In addition to cance, other studies have been conducted in keratinocytes (Brobeil, Bobrich, Tag, & Wimmer , 2012) (Brobeil, Koch, Eiber, Tag, & Wimmer, 2014). Therefore, more work needs to be conducted in neurons investigating the possible PTPIP51-protein interactions and the role PTPIP51 phosphorylation plays in localization and function. Mutating the known phosphorylated amino acids on PTPIP51 and then treating cells with Tat will help better understand which phosphorylated residue is most involved in subcellular localization.

Furthermore, PTPIP51 has also been shown to interact with oxysterol-binding protein-related proteins 5 and 8 (ORP5/8) at MAMs to facilitate phospholipid transfer between the ER and mitochondria (Galmes, et al., 2016) (Santos, Girik, & Nunes-Hasler, 2020). Future studies looking at the interaction between PTPIP51 and ORP5/8 in the presence of Tat would further elucidate the role of MAMs in HAND.

So far, the direct mechanism of Tat leading to the dysfunction of MAMs is unknown. However, a study investigating the HIV-1 protein Vpr and mitochondrial dysfunction that showed that Vpr localizes and, in fact, integrates into the outer

membrane of mitochondria and the ER (Huang, Chiang, Lin, Chiou, & Chow, 2012). This study also showed that Vpr disrupts MAMs mediated by the expression of Mfn2, a MAM-associated protein. Therefore, future studies should be conducted investigating the localization and protein interactions with Tat in neurons to elucidate a possible direct mechanism of MAM dysregulation.

Overall, MAMs remain a novel area of interest in studying aging and neurodegeneration, specifically HAND. This present study illuminates multiple potential targets for further study in HAND pathogenesis including VDAC expression, Bap31 and Fis1 interactions, PTPIP51 and VAPB interactions, and PTPIP51 phosphorylation/localization. Though MAMs have only recently been implicated in HAND, this study suggests that Tat leads to PTPIP51 tyrosine phosphorylation preventing PTPIP51 from localizing to MAMs where it can interact with VAPB and facilitate the essential communication between the ER and mitochondria.

CHAPTER 6

HIV-1 TAT AND ER-GOLGI COMMUICATION

6.1 Introduction

The Golgi apparatus is an organelle made of stacks of cisternae called the cis-, medial-, and trans-Golgi (Li, Ahat, & Wang, 2019). The cis-Golgi is located closest to the nucleus and the trans-Golgi is located further from the nucleus. These cisternae work together to perform many cellular functions. For instance, the Golgi is important in protein modification, glycoprotein/glycolipid synthesis, and the secretory pathway (Li, Ahat, & Wang, 2019). However, is has been shown that the Golgi does not act alone and can form contacts with the ER to mediate other functions such as vesicular trafficking, Ca^{2+} homeostasis, and endosome/lysosome formation and trafficking (Figure 27) (Venditti, Masone, & De Matteis, 2020). For instance, proteins newly synthesized in the ER may need to be delivered to the plasma membrane or packaged in vesicles (Gurel, Hatch, & Higgs, 2014). This process requires communication with the Golgi.

Since ER-Golgi contacts are important in mediating many cellular functions, the disruption of this communication has been implicated in some diseases (Wang, Stanford, & Kundu, 2020). ER-Golgi contacts have been shown to be especially important in neuron health and disruption has been implicated in neurological disorders (Wang, Stanford, & Kundu, 2020). For instance, some genes that encode proteins that regulate ER-Golgi trafficking are mutated in some neurological diseases such as *SEC31A*. A mutation in this gene has been linked to a recessive neurological syndrome

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and developmental delay (Halperin, et al., 2019). Moreover, other mutations in ER-Golgi trafficking genes have been shown to be present in patients with Charcot-Marie-Tooth disease, hereditary motor and sensory neuropathy, and other neurological diseases (Wang, Stanford, & Kundu, 2020).
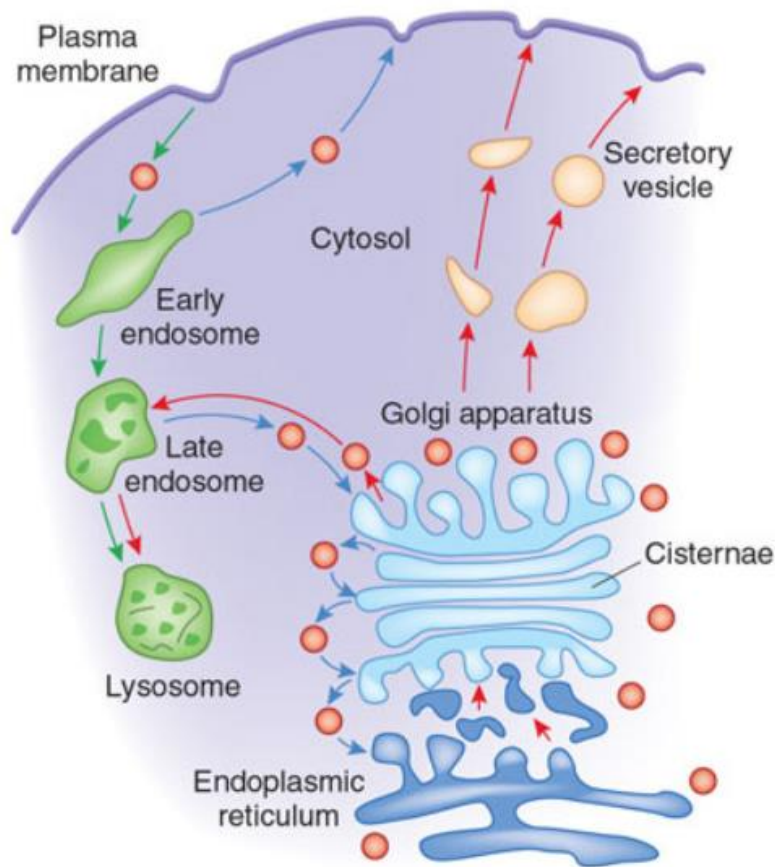
Figure 27. ER-Golgi Communication in Membrane and Protein Trafficking.
Illustration showing communication between the ER and Golgi and the function of these contacts in secretory vesicle trafficking, endosome formation and trafficking, and protein trafficking. (Modified from Xu & Esko, 2009).

It has been found that alterations in ER-Golgi-associated vesicular trafficking also occurs in HAND (Fields, et al., 2013). HIV-1 proteins Vpu and Nef have also been

shown to alter membrane trafficking (Tokarev & Guatelli, 2011). However, alteration of ER-Golgi communication has so far not been directly linked to HAND pathogenesis.

6.2 Materials and Methods

6.2.1 Cell Culture

LUHMES cells were maintained and differentiated as described above.

6.2.2 Tat Treatment

LUHMES were treated with 100ng/mL Tat or PBS for 24 hours as described above.

6.2.3 Immunofluorescence

LUHMES were treated with Tat or PBS for 24 hours as described above. For a positive control, some cells were treated with 2ng/mL tunicamycin for 30 minutes (Millipore Sigma, CAS 11089-65-9). The cells were then fixed in 4% formaldehyde in PBS for 20 minutes and washed twice with PBS for five minutes. Then, the cells were permeabilized in PBS with 0.2% Triton X-100 for five minutes and washed again with PBST. The cells were blocked in 3% bovine serum albumin (BSA) in PBST for one hour at room temperature. Cells were then incubated overnight with anti-GOLGA2/GM130 antibody 1:350 (Proteintech, 11308-1-AP). Afterward, the cells were incubated with a fluorescent secondary antibody, Alexa Fluor 488 donkey anti-rabbit (Life Technologies, A21206). Images were taken on a Leica EL6000 DMI3000 confocal microscope and analyzed using ImageJ.

6.2.4 Western Blot

Whole cell lysates were collected as described above. Western blots were conducted as described above using the anti-GOLGA2/GM130 antibody 1:2000. Expression was measured using ImageJ as described above.

6.2.5 Proximity Ligation Assays

PLAs were performed as described above and according to manufacturer's instructions. Anti-YIF1A antibody (Abnova, PAB20773) was diluted 1:350 with Duolink Antibody Diluent. PLA images were taken and analyzed as described above.

6.2.6 Statistics

Statistical analysis was conducted in Prism 9 (GraphPad) as described above where Mann-Whitney tests were calculated assuming a non-parametric distribution. Results were considered statistically significant if $p<0.05$.

6.3 Results

6.3.1 Golgi Morphology is Unchanged with HIV-1 Tat

The Golgi is a dynamic organelle that changes morphology in different intracellular conditions. Under normal healthy conditions, the Golgi cisternae is stacked surrounding the nucleus in association with microtubules (Figure 28A) (Makhoul, Gosavi, & Gleeson, 2019). Under certain conditions, these stacks can disperse into smaller stacks not associated with the nucleus (Figure 28B1), or smaller fragments that no longer exhibit any organization to the stacking (Figure 28B2 and B4) (Makhoul, Gosavi, & Gleeson, 2019). These morphologies have been associated with disease and disease pathologies including altered autophagy (Gosavi, Houghton, McMillan,

Hanssen, & Gleeson, 2018), membrane transport, and Aβ aggregation (Joshi, Chi, Huang, & Wang, 2014). The trans-Golgi can also disperse on its own (Figure 28B3) in the case of inflammasome formation (Chen & Chen, 2018).
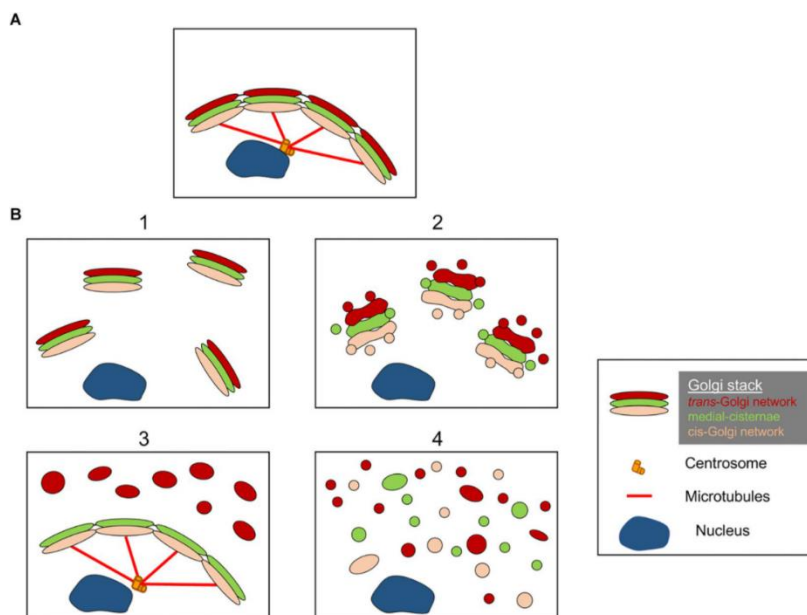
Figure 28. Different Golgi Cisternae Morphologies.
Illustration showing the varying cisternae morphologies that the Golgi can exhibit in different intracellular conditions in health and disease. A) Trans-, medial-, and cis-Golgi stacked neatly around the nucleus in association with microtubules. B) Golgi stacks dispersed and/or fragmented in various conditions. (Makhoul, Gosavi, & Gleeson, 2019).

Therefore, I wanted to see whether Golgi fragmentation or dispersal was induced with the addition of Tat. To visualize Golgi fragmentation, I treated differentiated LUHMES with 100ng/mL Tat or PBS for 24 hours and performed an immunofluorescent stain using anti-GOLGA2/GM130 antibody, a cis-Golgi matrix protein (Chang, et al., 2012) (Figure 29A). Tunicamycin, an antibiotic that has been shown to induce ER and cellular stress (Wu, et al., 2018), was used as a positive control for Golgi fragmentation. Here, I saw that Tat did not induce Golgi fragmentation or

dispersal (Figure 29A). However, morphology does not always correlate to dysfunction

and though the cisternae are intact, Golgi-associated functions may still be altered.
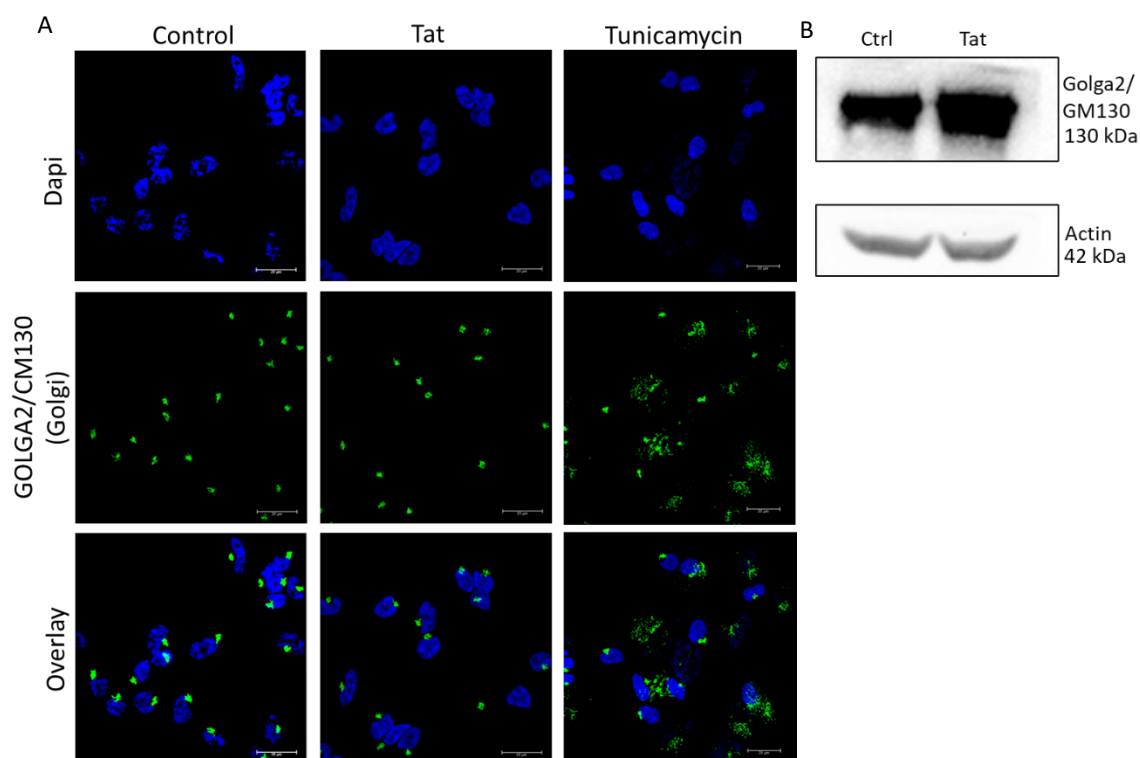
Figure 29. Tat Does Not Affect Golgi Morphology.
A) Immunofluorescent staining of LUHMES cells treated with 100ng/mL Tat or PBS for 24 hours then stained for GOLGA2/GM130 (green) and counterstained with Dapi to visualize nuclei (blue). Tunicamycin was used as a positive control for Golgi fragmentation. B) Western blot images using whole cell lysate to visualize expression of GOLGA2/GM130 in the presence of Tat.

In order to see if GM130 protein expression of may be affecting the cisternae,

I conducted a western blot on whole cell lysate of cells treated with Tat or PBS. Here, I

saw that GM130 expression was increased in the presence of Tat (Figure 29B). This

increase may be a compensatory mechanism to help the cell maintain Golgi morphology

in the event of Tat-induced cellular stress.

6.3.2 VAPB and YIF1A Interaction is Unchanged with HIV-1 Tat

The ER protein VAPB and the Golgi protein YIF1A have been shown interact and this interaction is important in membrane delivery into dendrites in neurons (Kuijpers, et al., 2013). It has also been shown that loss of this interaction negatively affects membrane trafficking and dendrite morphology (Kuijpers, et al., 2013). Therefore, I wanted to see if Tat affected YIF1A and VAPB interactions in LUHMES cells. To visualize and measure the interactions between these two proteins, I performed PLAs on cells treated with Tat or PBS for 24 hours (Figure 30A). Here, the interactions between YIF1A and VAPB are increased in the presence of Tat (Figure 30B) suggesting that Tat is upregulating this interaction at ER and Golgi contacts. Since ER-Golgi contacts are important for vesicle trafficking in neurons, including those carrying neurotransmitters, this increase in interactions may be contributing the excitotoxicity associated with Tat and HAND.

Figure 30. Altered YIF1A and VAPB Interactions in the Presence of Tat.
A) Representative images of PLA for YIF1A and VAPB in LUHMES cells in the absence and presence of Tat. Red dots indicate individual interactions. 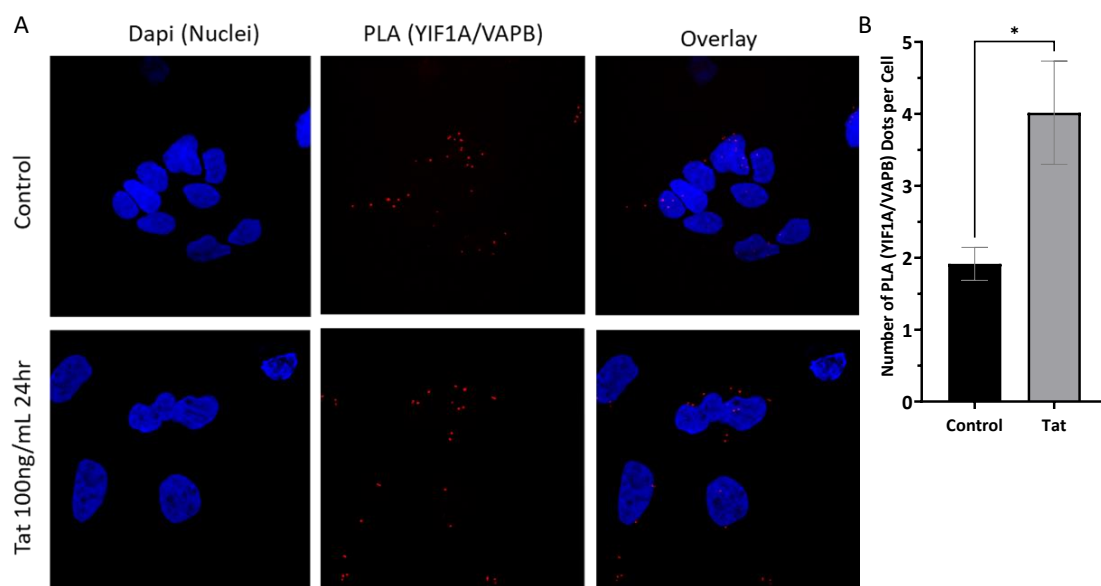Dapi staining indicates individual nuclei. B) Quantification of the number of YIF1A and VAPB interactions represented as a bar graph. * indicates $p<0.05$. Error bars are shown as S.E.M.

6.4 Discussion and Future Directions

In this study, I show Golgi morphology is unchanged in the presence of HIV-1 Tat. However, I also show that the interaction between YIF1A on the Golgi and VAPB on the ER is altered in the presence of Tat. This interaction has been shown to be important in neuronal health and when deregulated, has been shown to be deleterious to neurons (Kuijpers, et al., 2013). Since neurons can span long distances in the human brain and body, membrane and vesicular trafficking is especially important to deliver cargo from the cell body to the distal dendrites (Wang, Stanford, & Kundu, 2020). Therefore, ER-Golgi contacts, that have been shown to mediate trafficking, are especially important for maintaining neuronal health. In this study, the presence of Tat increased the interactions between YIF1A and VAPB suggesting ER-Golgi communication is also increase. This increase in ER-Golgi contacts could be indicative of heightened vesicle trafficking and secretion including neurotransmitter vesicles. Increased neurotransmitter release could explain the excitotoxicity associated with Tat and HAND. A future electrophysiology study investigating the role of ER-Golgi contacts, and YIF1A and VAPB interactions specifically, is needed to further implicate this interorganelle communication in HAND-associated excitotoxicity.

More work needs to be done to explore the role of ER-Golgi communication in HAND as well as other diseases. For instance, some work has been done identifying the ER-Golgi contact protein, ER-Golgi intermediate compartment 53 (ERGIC-53) as a marker for the communication between these two organelles (Hauri, Kappeler, Andersson, & Appenzeller, 2000). It may be important to tag ERGIC-53 and visualize

the expression and subcellular localization of this protein in live cells treated with Tat to better understand the ER-Golgi connection in a disease model. In addition, exploring vesicle trafficking and release of neurotransmitters in the presence of Tat may also help elucidate the role of ER-Golgi communication in HAND.

CHAPTER 7

CONCLUSIONS

7.1 Conclusions

In conclusion, this study explored the role of interorganelle communication in the pathogenesis of HAND. The communication between the ER and mitochondria at MAMs was shown to be altered in the presence of Tat. Specifically, the interaction between PTPIP51 on mitochondria and VAPB on the ER was shown to be decreased possibly due to increased PTPIP51 phosphorylation. In addition, the communication between the ER and the Golgi apparatus was also shown to be altered with Tat. The interaction between YIF1A on the Golgi and VAPB on the ER as shown to be increased without any alteration in Golgi morphology. These sites of interorganelle communication are dynamic relationships that change overtime and in response to many stimuli. They mediate many cellular functions and work together to maintain cellular homeostasis. Therefore, exploring the dysfunction and dysregulation of these interorganelle contacts may help elucidate the exact mechanisms HIV-1 exploits in the pathogenesis of HAND.

7.1 Limitations

One of the main limitations in this study was the use of cell culture to model HIV infection in the brain. Here, cell lines were used that could be differentiated into mature dopaminergic neuron-like cells and were treated for 24 hours with recombinant Tat protein. However, HIV infection and HAND are chronic conditions where time is a major factor in pathogenesis. Multiple cell

types are involved in HAND pathogenesis. Also, HAND is a behavioral disease where patients neurocognitive behavior dictates the efficacy of treatments and progression of disease. Therefore, it is difficult to model HAND in cell culture alone, albeit molecular mechanisms are often easier to elucidate in cell culture.

Another limitation in this study is lack of a widely available antibody for the phosphorylated forms of PTPIP51. These antibodies are difficult to make and therefore expensive and uncommon. Therefore, though using PLAs to measure phosphorylation of PTPIP51 was feasible, specific antibodies for specific phosphorylated residues would help identify the exact amino acids responsible for subcellular localization.

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