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By

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ABSTRACT

Human immunodeficiency virus (HIV) infection of the central nervous system promotes neuronal injury and apoptosis culminating in HIV-associated neurocognitive disorders (HAND). Viral proteins, like transactivator of transcription, Tat, are leading candidates to explain HIV-mediated neurotoxicity. In the recent years, mitochondrial dysfunction has become identified as a causal factor in neurodegenerative diseases, including HAND. While disturbances to ATP production, Ca^{2+} buffering, and reactive oxygen species (ROS) production have been identified in investigations of Tat toxicity, the precise cause of mitochondrial dysfunction remains unclear. Impairments to mitochondrial dynamics may be behind the dysfunction observed following Tat exposure in vitro.

First, I examined the time course of effects of Tat. Rat cortical neurons were exposed to Tat for various time points. Within an hour, Tat induced ROS production followed by other indices of mitochondrial destabilization; all preceding the induction of DNA double-strand breaks (DSBs). I next investigated the neuroprotective activity of pituitary adenylate cyclase-activating polypeptide 27 (PACAP27) against these cardinal features of Tat toxicity. PACAP27 inhibited all Tat-mediated toxic effects including DNA DSBs. Importantly, PACAP27 prevented Tat-induced neuronal loss. My data support a mechanism of Tat neurotoxicity through mitochondrial destabilization, which increases the release of ROS and causes DNA DSBs leading to cell death.
To further evaluate the mechanism of Tat-mediated mitochondrial toxicity, I examined alterations to mitochondrial dynamics following Tat exposure. Within 30 min, Tat caused a significant reduction in mitochondrial membrane potential, a process regulated by fusion and fission. By 2 hr, Tat caused a significant change in mitochondrial size, supporting the hypothesis of altered fusion and fission. To further assess whether Tat changes these processes, fusion and fission proteins Mfn2 and Drp1, respectively, were measured. Tat caused increased levels of Drp1 and increased association with the mitochondrial membrane while Mfn2 remained unchanged. Furthermore, Drp1 and calcineurin inhibitors prevented Tat-mediated mitochondrial fragmentation. These findings implicate mitochondrial fission as the leading factor in Tat-mediated alterations to mitochondrial morphology. This disruption in mitochondrial homeostasis likely contributes to neuronal cell death following Tat exposure. Overall, these findings indicate Tat-induced mitochondrial impairment as a key event preceding cell death and a neurotoxic mechanism contributing to HAND.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>ANT</td>
<td>adenine nucleotide translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CMT2A</td>
<td>Charcot-Marie-Tooth type 2A</td>
</tr>
<tr>
<td>CRI</td>
<td>Co-receptor inhibitor</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CTD</td>
<td>C-Terminal Domain</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dynamin-related protein-1</td>
</tr>
<tr>
<td>DSIF</td>
<td>DRB Sensitivity inducing factor</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone</td>
</tr>
<tr>
<td>FI</td>
<td>Fusion inhibitor</td>
</tr>
<tr>
<td>Fis1</td>
<td>Mitochondrial fission protein 1</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
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</table>
HAD - HIV-associated dementia
HAND - HIV associated neurocognitive disorders
HD - Huntington’s disease
HIV - Human immunodeficiency virus
INI - Integrase inhibitor
mCU - mitochondrial Calcium Uniporter
Mdivi-1 - Mitochondrial division inhibitor 1
Mff - Mitochondrial fission factor
Mfn1 - Mitofusin-1
Mfn2 - Mitofusin-2
MiD49 - Mitochondrial dynamics proteins 49 kDa
MiD51 - Mitochondrial dynamics proteins 51 kDa
MND - Mild neurocognitive disorder
mRNA - Messenger ribonucleic acid
mtDNA - Mitochondrial DNA
NELF - Negative elongation factor
NGF - Nerve growth factor
NNRTI - non-nucleoside RT inhibitor
NRTI - nucleoside RT inhibitor
NtRTI - nucleotide inhibitor
OPA1 - Optic Atrophy 1
PACAP27 - Pituitary adenylate cyclase activating polypeptide 27
PACAP38 - Pituitary adenylate cyclase activating polypeptide 38
PARP - poly ADP ribose polymerase
PD - Parkinson’s Disease
PI - Protease inhibitor
PINK1 - Mitochondrial phosphatase and tensin homolog (PTEN)-induced kinase 1
PTP - permeability transition pore
RNA - Ribonucleic acid
RNAP - RNA Polymerase
RT - Reverse transcriptase
SNPH - Syntaphilin
Spt5 - Transcriptional elongation factor 5
STORM - Stochastic Optical Reconstruction Microscopy
A. Overview: HIV-1 and Mitochondria

Postmortem brains of HIV-positive individuals with cognitive alterations exhibit signs of impaired mitochondrial metabolism (Bennett, Doyle, & Salvemini, 2015; Opii, Sultana, Abdul, & Ansari, 2007). In addition, when compared to HIV-positive with no cognitive alterations, HIV associated neurocognitive disorder (HAND) brains contain mitochondria with abnormal morphology (Valeria Avdoshina et al., 2016; J. A. Fields et al., 2016), including the lack of integrity of inner membrane cristae (Figure I.1). This scenario is similar to that observed in other neurological diseases such as Alzheimer’s disease in which mitochondrial ultrastructure is altered in such a way that cristae are disrupted (Reddy & Reddy, 2011). Mitochondria play a role in neuronal survival through a variety of metabolic mechanisms (Mattson, Gleichmann, & Cheng, 2008), Ca$^{2+}$ homeostasis and the reduction of reactive oxygen species (ROS), among them. In addition, mitochondria control the production of high-energy intermediates, such as ATP. Neurons are highly energetically dependent and require ATP at distant regions such as axonal and dendritic synapses, linking their function and survival tightly with ATP production (Berthet et al., 2014; Dickey & Strack, 2011; Merrill et al., 2011). Thus, it is not surprising that mitochondrial dysfunction and disruption of energy production have been proposed to cause neurodegenerative diseases (Burté, Carelli, Chinnery, & Yu-wai-man, 2014). The molecular mechanism of HIV-mediated mitochondrial impairment is still unclear.

It is widely accepted that mitochondria in neurons are susceptible to a number of neurotoxins. Included in this are viruses and associated proteins, not the least of which is HIV. Moreover, impairments to mitochondrial homeostasis have been implicated in a causal role of a number of neurodegenerative diseases, including Parkinson’s, Alzheimer’s, and Huntington’s
diseases (Eckmann, Eckert, Leuner, Muller, & Eckert, 2013; Itoh, Nakamura, Iijima, & Sesaki, 2013; Reynolds, Malaiyandi, Coash, & Rintoul, 2004; Sheng & Cai, 2012). The prevalence of neuronal diseases associated with mitochondrial impairment underscores the important functional relationship between neurons and mitochondria. Efficient mitochondrial function is essential for the health of highly energetic and polarized neurons. Inefficient mitochondrial function leading to the overproduction of cellular waste products and underproduction of ATP not only impairs neuronal function, but can contribute to neuronal cell death (Pahnke, Fröhlich, Krohn, Schumacher, & Paarmann, 2013). Regulation of mitochondrial health is tightly controlled by the dynamic processes of fusion and fission, which in turn directly affect the size of the organelles and their ability to be trafficked throughout sub-compartments of the neuron (H. Chen & Chan, 2009). Aberrations to any of these processes can contribute to organelle inefficiencies, impair cellular functions, and lead to cell death.
Figure I.1 Neuronal mitochondria in HAND display disrupted cristae. Cortical sections from HIV+ subjects with no cognitive alterations and HAND subjects were analyzed by transmission electron microscopy (TEM) as previously described (Avdoshina et al. 2016a) to visualize mitochondria. (bar = 1 μM). Magnification ×25,000

B. Mitochondria

a. Structure

Mitochondria are thought to have originated from a symbiotic relationship that resulted when a nucleated cell engulfed a prokaryote (Nunnari & Suomalainen, 2012). Over time, the engulfed cell became reliant upon the protective environment of the host cell, and conversely, the host cell became reliant upon the engulfed prokaryote for energy production. Descendants of the engulfed prokaryote became mitochondria and their ability to use oxygen to create energy became critical to eukaryote evolution.
The mitochondria contains two membranes: an outer membrane that has pores large enough for the passage of ions and small proteins, and an inner membrane that contains embedded proteins that make up the electron transport chain (ETC) necessary for the aerobic respiration (Osellame et al., 2012). Between the two membranes is the intermembrane space, to which ions are pumped by components of the ETC in order to create an ionic potential enabling the flow of ions into the matrix and the phosphorylation of ADP to ATP for the generation of energy.

The outer membrane contains many copies of a transport protein called porin, which forms large aqueous channels through the lipid bilayer, allowing ions and small proteins up to 5000 daltons in size to pass. Thus, the intermembrane space is chemically equivalent to the cytosol with respect to the small molecules it contains (Lane & Martin, 2010). The matrix space, the internal compartment located within the inner membrane, is an environment distinct from the cytosol. The inner membrane is highly specialized and contains a variety of transport proteins that make it selectively permeable to those small molecules that are metabolized or required by the many mitochondrial enzymes concentrated in the matrix space (Osellame et al., 2012).

b. Energy Production

The citric acid cycle, or Krebs cycle, takes place within the matrix, beginning following the metabolism of pyruvate and fatty acids to produce acetyl CoA. Acetyl CoA reacts with the four-carbon compound oxaloacetate to produce the six carbon citric acid, for which the cycle is named. Ultimately, after seven sequential enzyme-mediated reactions, two carbon atoms of CO₂, three electrons, temporarily bound the NADH and FADH₂, are produced. One ATP is also formed, by way of GTP by direct transfer of a phosphate from the intermediate GDP. Perhaps the most important products from these reactions are the electrons, as they are essential for
oxidative phosphorylation, the primary source of energy in eukaryotic cells (Hatefi, 1985). These electrons, carried by NADH and FADH$_2$ are then combined with molecular oxygen by means of the electron transport chain (ETC). The ETC consists of protein complexes I-IV and is located in the inner mitochondrial membrane (Nunnari & Suomalainen, 2012). As the high-energy electrons from the hydrogens in NADH and FADH$_2$ are transported down the respiratory chain, the energy released as they pass from one carrier molecule to the next is used to pump protons across the inner membrane from the mitochondrial matrix into the intermembrane space. This establishes an electrochemical proton gradient across the inner membrane, and the backflow of protons down this gradient drives the membrane bound ATP synthase, which catalyzes the conversion of ADP+ P$_i$ to ATP.

c. Calcium Buffering

In addition to their significant role in energy production, mitochondria are also crucial for Ca$^{2+}$ buffering within mammalian cells. Ca$^{2+}$ buffering is especially important in neurons which are highly susceptible to excitotoxicity following over activity and large influxes of Ca$^{2+}$ through glutamate receptor channels (Bertholet et al., 2016). The electrochemical proton gradient established by the ETC is also used to import Ca$^{2+}$ from the cytosol. The import of Ca$^{2+}$ into the mitochondria is thought to be important for the maintenance of certain matrix enzymes (Glancy & Balaban, 2012) but is also important for removing Ca$^{2+}$ from the cytosol when cytosolic Ca$^{2+}$ levels become dangerously high (Werth & Thayer, 1994). Thus, Ca$^{2+}$ buffering is an essential function of the organelle in maintaining cellular homeostasis.

Mitochondria are trafficked to areas of high Ca$^{2+}$, such as synapses, where they can both supply energy in the form of ATP as well as buffer excess Ca$^{2+}$ following synaptic activity. The predominant mechanism for Ca$^{2+}$ uptake by mitochondria is through the mitochondrial
calcium uniporter (mCU), which has a very low Ca\textsuperscript{2+} affinity (K\textsubscript{m} above 10\textmu M). The basal levels of Ca\textsuperscript{2+} within most cells is nearly 10 times lower than this (1-2\textmu M), even after cell stimulation, thus mitochondria are not physiological Ca\textsuperscript{2+} stores. However, mitochondrial Ca\textsuperscript{2+} levels are able to undergo rapid changes during cell activation (Rizzuto, Stefani, Raffaello, & Mammucari, 2012). These rapid changes are due to the precise localization of mitochondria within high-Ca\textsuperscript{2+} microdomains (Fonteriz et al., 2016), such as synapses. The low affinity of the uniporter requires the precise localization of mitochondria within subcellular domains of high Ca\textsuperscript{2+} for the maintenance of cellular function and health. Likewise, mitochondria are very well-adapted to take up Ca\textsuperscript{2+} in local high-Ca\textsuperscript{2+} microdomains such as those following activation of the plasma membrane or endoplasmic reticulum (ER) Ca\textsuperscript{2+} channels. In fact, mitochondria have been shown to be in close contact with the ER and with plasma membrane channels (Rizzuto et al., 1998).

**d. Biogenesis and Dynamics**

**i. Trafficking**

Neurons are highly polarized and energetic cells, requiring mitochondria to be trafficked appropriately to meet demands throughout the cell (Wong-Riley, 1989). Axons have a relatively uniform microtubule polarity, with the positive ends located in distal regions. This maintains that mitochondria moving away from the soma (anterograde) are trafficked by kinesin motor protein and mitochondria moving toward the soma (retrograde) are trafficked by dynein motor protein (Pilling, Horiuchi, Lively, & Saxton, 2006). In each case, a complex of several proteins is required for the adhesion of mitochondria to the motor protein (Figure I.2). Adaptor proteins
milton, syntabulin, and miro are necessary for the binding of mitochondria to kinesin, whereas dynactin is essential for transport by dynein.

Mitochondrial trafficking varies by cell type. In most neuronal subtypes, only a small fraction of mitochondria are motile at any one moment (Obashi & Okabe, 2013). Mobile mitochondria have been reported to account for 5–20 or 35–45% of the total mitochondrial pool in cultured hippocampal neurons (Ligon & Steward, 2000; Overly, Rieff, & Hollenbeck, 1996). Mitochondrial movement is dynamic, with individual organelles exhibiting various velocities and motility patterns in axons and dendrites (Ligon & Steward, 2000; Overly et al., 1996). Mitochondrial motility is much lower in mature cortical neurons than in developing neurons, with the majority of stationary mitochondria localized to sites of synaptic terminals (Lewis, Turi, Kwon, Losonczy, & Polleux, 2016). Mitochondrial movement can also vary across subcompartments of a single cell. In an investigation of cortical neurons, more mitochondrial movement was observed in axons compared with dendrites (Chang & Reynolds, 2006).

Mobile mitochondria exhibit saltatory movement, pausing briefly along their trajectory and also changing direction midcourse. Neuronal mitochondria pause most often at sites that lack other mitochondria, resulting in a well-spaced axonal mitochondrial distribution (Miller & Sheetz, 2004). The halting of mitochondrial movement is regulated by increasing cytosolic Ca2+ in many cell types (Chang & Reynolds, 2006; Yi, Weaver, & Hajnoczky, 2004), a phenomenon that is independent of mitochondrial membrane potential (Yi et al., 2004). Ca2+ regulation of mitochondrial motility is regulated by the EF-hands of Miro, a kinesin adaptor protein (Fransson, Ruusala, & Aspenström, 2006; Xinnan Wang & Thomas L, 2009). Ca2+ signaling does not cause the dissociation of mitochondria from the motor, but rather interferes with the binding of kinesin to microtubules (Xinnan Wang & Thomas L, 2009) causing mitochondria to stop. When immobilized, mitochondria can be docked in place in an activity-dependent manner by syntaphilin (SNPH), which acts as a “static anchor” (Kang et al., 2008). Deleting snph in mice
drastically increases mitochondrial movement and decreases mitochondrial immobilization. SNPH targets to axonal mitochondria and mediates their docking by anchoring them to the microtubule-based cytoskeleton.

**Figure I.2 Mitochondrial trafficking mechanisms within the axon.** Microtubules serve as tracks along which mitochondria move either towards the presynaptic terminal (anterograde transport) or towards the cell body (retrograde transport). ATP-dependent motor proteins that move mitochondria along microtubules include kinesins (anterograde) and dynein (retrograde). Mitochondria associate with the motor proteins through specific adaptor proteins. Adaptor proteins (AP) for kinesins include Milton, syntabulin and a Rho GTPase called Miro. Dynactin is an adaptor protein for dynein. Within the axonal growth cone and presynaptic terminal, mitochondria may be anchored and moved along actin filaments by a myosin-mediated
mechanism. Permission to reproduce this figure from Mattson, Gleichman, & Cheng (2008), Neuron, granted by Elsevier.

ii. Role in Synaptic Transmission

Mitochondria maintain synaptic transmission through the production of ATP and buffering of Ca\textsuperscript{2+}. The distribution of mitochondria in axons and dendrites correlates closely with the predicted energy usage of these compartments (Attwell & Laughlin, 2001). The trafficking to, and density of, mitochondria at subcellular locations with high energy demands and Ca\textsuperscript{2+} buffering requirements is important for correct neuronal function (MacAskill, Atkin, & Kittler, 2010). In fact, mitochondria tend to be more fully distributed throughout processes that have high synaptic density (D. T. W. Chang, Reynolds, Honick, & Reynolds, 2006) and are known to accumulate in the regions of active growth cones in developing neurons (Morris & Hollenbeck, 1993). Neuronal activity increases the delivery of mitochondria to the synapse (Tong, 2007) and regulates their motility, fusion/fission balance, and dendritic distribution of mitochondria (Z. Li, Okamoto, Hayashi, & Sheng, 2004). Moreover, defective synaptic transmission is associated with the loss of mitochondria from axon terminals (Stowers, Megeath, Meinertzhagen, Schwarz, & Avenue, 2002).

iii. Fusion and Fission

The biosynthesis of mitochondria requires the contribution of both nuclear and mitochondrial DNA (Jianhui Zhu, Wang, & Chu, 2013). Mitochondria are never created de novo, but rather are inherited and arise from the growth and division of existing mitochondria. Mitochondria can replicate their DNA as well as divide and fuse locally within the axon,
suggesting that the biogenesis of mitochondria is not limited to cell body (Amiri & Hollenbeck, 2008). Throughout their life cycle, a mitochondrion exists both as a solitary organelle, as well as part of a large interconnected network. The dynamic changes in mitochondrial morphology are regulated by the processes of fusion, wherein two or more organelles become conjoined, and fission, wherein a single mitochondrion divides into two daughter mitochondria (Figure I.3). Mitochondrial morphology and distribution may be optimized differentially to best serve the synaptic distributions in axons and dendrites. While numerous investigations have been conducted into mitochondrial dynamics, the purpose of a hyperfused network of mitochondria remains unclear, though speculation as to its role are plenty (Hoitzing, Johnston, & Jones, 2015). The length, shape, size, and number of mitochondria are all controlled by fusion and fission. These dynamic processes allow for the exchange of organelle contents such as solutes, metabolites, proteins, and mitochondrial DNA (mtDNA) (fusion) or the isolation of damaged proteins, mtDNA, and reactive oxygen species (ROS) (fission). Fusion and fission events are correlated to the bioenergetic state change of individual mitochondria (Gerencser & Nicholls, 2008). The delicate balance of these two processes is essential for the maintenance of a healthy mitochondrial network, and thus a healthy neuron (Jianhui Zhu et al., 2013). In fact, when either side of the scale is tipped in favor of the other through use of inhibitors, dominant negatives, or constitutive activity, organellar and cellular dysfunction ensue, commonly resulting in cell death (Palikaras & Tavernarakis, 2014).
Figure I.3 Mitochondrial fusion and fission cycle and its key players. (A) Mitochondrial interconnectivity is maintained by fusion which is regulated by proteins such as Mfn1, Mfn2, Opa1, SLP2, and PLD. Mitochondrial fragmentation follows fission, governed by several factors including Fis1, MiD49/51, Mff, miR-30, miR-499, and Drp1. Mitochondrial fission is suppressed via inhibition of Drp1 by the synthetic small molecule Mdivi-1. Permission to reproduce this figure modified from Rosdah et al. 2016, Pharmacology Research & Perspectives, granted by John Wiley and Sons.
iv. Fusion Regulation by Mitofusions and Optic Atrophy

Fusion has been suggested to be a restorative process for the organelle (H. Chen, Chomyn, & Chan, 2005), though the precise signal that initiates fusion remains to be elucidated. Fusion allows the exchange of membrane and matrix contents and therefore may help to restore local depletions and maintain mitochondrial function. (H. Chen et al., 2003). Fusion events can be separated into two categories: transient (outer membrane only) and complete (inner and outer membrane) fusion, each assumed to serve a separate and necessary biological function (Xingguo Liu & Hajnóczky, 2009).

In mammals, mitochondrial dynamic proteins mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) have been shown to be critical for mitochondrial membrane fusion (Y. Lee, Jeong, Karbowskii, Smith, & Youle, 2004; Santel & Fuller, 2001). Mfns are responsible for both tethering apposing mitochondria and also the fusion of the organelar membranes (H. Chen & Chan, 2005). These proteins belong to the dynamin superfamily of large GTPases. GTPases within this superfamily are distinguished from small Ras-like and other regulatory GTPases but their oligomerization-dependent GTPase activation, low GTP-binding affinities, and the ability of many to interact with lipid membranes (Praefcke & Mcmahon, 2004). Indeed, Mfn1 and Mfn2, which are 81% homologous, reside on the outer membrane of mitochondria (Rojo, Legros, Chateau, & Lombès, 2002) and homodimerize or heterodimerize to induce fusion of the mitochondrial membrane (H. Chen et al., 2003). It is their GTPase domain of that is required for the fusion to take place (H. Chen et al., 2003). Their similar structures give them relatively redundant functions.

Knockouts of either Mfn1 or Mfn2 in cultured mouse embryonic fibroblasts (MEFs) results in a clear alteration to mitochondrial morphology. In each instance, the mitochondria of Mfn mutants are significantly smaller than those in wild-type cells (H. Chen et al., 2003). Loss of Mfns not only impairs mitochondrial morphology, but also disrupts mitochondrial mobility,
resulting in organelles moving in “Brownian-like” patterns rather than along radial tracts as seen in healthy cells (H. Chen et al., 2003). Additionally, both endogenous and uncoupled respiration rates are reduced in Mfn-null cells (H. Chen et al., 2005).

Fusion of the inner mitochondrial membrane is regulated by optic atrophy 1 (OPA1). No biochemical interactions have been shown between the Mfn s and OPA1 in mammalian cells. However, they work in sequence for complete fusion of both outer and inner mitochondrial membranes. OPA1 is an intermembrane space protein, closely associated with the inner membrane (Olichon et al., 2003), which is the membrane on which it acts during fusion. OPA1 is necessary for the fusion of the inner mitochondrial membrane and creates a tubular mitochondrial network in a Mfn-dependent manner (Cipolat, Brito, Zilio, & Scorrano, 2004). Like Mfn mutants, deletion of OPA1 results in alterations to mitochondrial morphology and organellar dysfunction (H. Chen et al., 2005).

v. Fission Regulation by Dynamin Related Protein 1 and its Adaptors

Key regulators of fission by the individual mitochondrion are Ca\(^{2+}\), membrane potential, and ATP levels (Guillery, 2005; Z. Song, Chen, Fiket, Alexander, & Chan, 2007; Z. Song, Ghochani, Mccaffery, Frey, & Chan, 2009). In addition regulating mitochondrial size, fission may also have protective effects in Ca\(^{2+}\)-dependent apoptosis (Szabadkai et al., 2004). A depolarized mitochondrion may divide, subjugating this depolarization to only one of the daughter mitochondria. This depolarized organelle can then either re-fuse with the network or undergo selective degradation through mitophagy. Fission may help to isolate damaged segments of mitochondria and thus promote their autophagy (Twig et al., 2008). It is important to note that while opposing processes, the inhibition of fusion itself is not sufficient to elicit a fission event.
In mammals, mitochondrial fission is regulated by dynamin-related protein 1 (Drp1) (Frank et al., 2001), which is a member of a family of GTPases known to have a mechanical activity that pinches lipid membranes (Praefcke & Mcmahon, 2004). The recruitment of Drp1 to the mitochondrial surface can occur through interactions with several mitochondria outer membrane proteins including mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins 49 and 51 kDa (MiD49 and MiD51, respectively) (Losón, Song, Chen, & Chan, 2012), all of which will be discussed in more detail later in this section. Current understanding suggests three key steps to mitochondrial fragmentation: (a) organization of Fis1 proteins around a fission site (Jofuku, Ishihara, & Mihara, 2005); (b) recruitment of Drp1 from the cytosol to the sites (Yoon, Krueger, Oswald, & Mcniven, 2003); and (c) inhibition of fusion in response to local processing of OPA1 in individual mitochondria.

Drp1 exists primarily in a cytosolic pool, however a fraction will localize to the mitochondrial membrane (Smirnova, Grippari, Shurland, & van der Bliek, 2001). Regulation of Drp1 recruitment to the mitochondrial membrane is Ca²⁺-dependent (Yoon et al., 2003). The activity of Drp1 is heavily regulated by post-translational modifications including phosphorylation, ubiquitination, SUMOylation, and S-nitrosylation (D.-H. Cho et al., 2009; Karbowski, Neutzner, & Youle, 2007; Wasiak, Zunino, & McBride, 2007). Phosphorylation is the most well-studied mechanism, and currently thought to be the most prevalent, with known sites for phosphorylation being the serine residues 616, which promotes Drp1 activation, as well as 637 and 656, which inhibit translocation of Drp1 to the mitochondria (T.-H. Cho et al., 2005). Drp1 dephosphorylation by calcineurin at serine 637 has been shown to cause Drp1 to localize to mitochondria (Scorrano, 2005).

Like its fission regulating-counterparts, Drp1 is essential for the health of the cell. Downregulation of Drp1 in HeLa cells causes slower cell growth, loss of mtDNA, uncoupling of the ETC, decreased cellular respiration, and increased ROS levels (Parone et al., 2008).
Expression of Drp1-K38A (dominant negative mutant, mutated in GTPase domain) causes a drastic decrease in dendritic mitochondria (Z. Li et al., 2004). Conversely, overexpression of wild-type Drp1 results in a significant increase in dendritic mitochondria. Thus, Drp1 is critical for the distribution of mitochondria to dendrites. In fact, Drp1 is required for activity-dependent regulation of mitochondrial movement and enhances synaptogenic response to potentiating stimuli in hippocampal neurons (Z. Li et al., 2004).

Mitochondrial fission is often thought to lead to apoptosis, however, fission occurs in an apoptosis-independent manner in most instances and fission events do not imply apoptosis (Meuer et al., 2007). There are interactions between fission regulator Drp1 and proteins involved in the regulation of apoptosis. Drp1 has been implicated in Bax-dependent apoptosis (Perfettini, Roumier, & Kroemer, 2005). In healthy cells, Bax is found throughout the cytosol. Upon the initiation of apoptosis, Bax translocates to the mitochondrial membrane where it interacts with the mitochondrial voltage-dependent anion channel (VDAC), inducing its opening. Bax is also able to form oligomeric pores in the mitochondrial outer membrane, allowing the release of cytochrome c and other pro-apoptotic factors from the mitochondria, leading to caspase activation. Drp1 co-localizes with Bax in punctate structures (Karbowski et al., 2002). Bax translocation to mitochondria can still occur in cells devoid of Drp1, implying that Drp1 operates downstream of Bax mitochondrial localization (Y. Lee et al., 2004). Dominant negative Drp1 and Drp1-RNAi prevent mitochondrial fragmentation during apoptosis and also reduce cell death (Frank et al., 2001).

Drp1 associates with the mitochondrial membrane through interactions with several receptor proteins. In mammals, these receptor proteins include Fission 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics protein of 49 kDa and 51 kDa (MiD49 and MiD51, respectively). Fis1 is anchored to the mitochondrial outer membrane and binds to Drp1. Fis1 distributes evenly across the mitochondrial surface and does not wholly colocalize with
Drp1 puncta (D. I. James, Parone, Mattenberger, & Martinou, 2003; Suzuki, Jeong, Karbowski, Youle, & Tjandra, 2003). Deletion or overexpression of Fis1 does not appear to affect Drp1-mitochondria association (Y. Lee et al., 2004; Suzuki et al., 2003) which raises the question of whether Fis1 is the true receptor for Drp1. However, overexpression of Fis1 results in Drp1-mediated fragmentation while inhibition of Fis1 leads to mitochondrial elongation (Losón et al., 2012). Similar to depletion of Drp1, Fis1-RNAi also inhibits caspase-dependent cell death (Lee et al., 2004). Mff is another receptor protein for Drp1 (Gandre-babbe & Bliek, 2008). Artificial tagging of Mff to the plasma membrane redirected Drp1 to this location, indicating that Mff recruits Drp1 (Otera & Mihara, 2011). Mff knockdowns have been shown to reduce Drp1 association with mitochondria and to induce mitochondrial elongation. The overexpression of Mff enhances Drp1 translocation to the mitochondria, leading to mitochondrial fragmentation (Otera & Mihara, 2011). MiD49 and MiD51 have also been shown to bind and recruit Drp1 to mitochondria (Palmer et al., 2011; Zhao, Liu, Jin, Lendahl, & Niste, 2011). Cells overexpressing MiD49 or MiD51 had enriched Drp1 at the mitochondrial surface compared to those overexpressing Milt1 (Palmer et al., 2011). MiD49/51 proteins may be involved in the transient association of actin to mitochondria (some have reported that these associations are necessary for recruiting Drp1 to the mitochondria surface to facilitate fission in mammalian cells) (Palmer et al., 2011). Each of these proteins may act as a Drp1 receptor. Cell lines null for Fis1 and/or Mff indicated that Mff and Fis1 contribute to mitochondrial localization of Drp1 independent of one another, with Mff being more dominant (Losón et al., 2012). MiD49 and MiD51-mediated Drp1 recruitment are also independent of Mff and Fis1 (Losón et al., 2012; Palmer et al., 2011). Further studies are needed to better elucidate the roles of each of these proteins and how they coordinate the recruitment of Drp1 to the mitochondria to control morphology.
vi. Mitophagy

When insults to a mitochondrion are too great to be repaired through the dynamic processes of fusion and fission, the organelle can be isolated by the autophagosome to be degraded through the specific process of mitophagy. While precise parameters that indicate the necessity of mitophagy are not entirely clear, depolarization of the mitochondrial membrane below a certain potential may indicate impaired mitochondrial function and is a prerequisite for mitophagy (Elmore, Qian, Grissom, & Lemasters, 2001; Twig et al., 2008). Depolarized mitochondria may result from impairments to an organelle or be due to an asymmetrical division of a mitochondrion (Barsoum et al., 2006; Mannella, 2006). In the majority of fission events one of the two daughter mitochondria will leave the fission event with some level of depolarization (Twig & Shirihai, 2011). Thus, fission could then be a major route for generating mitochondria that are targeted for mitophagy. Although mitochondrial fragmentation is permissive for mitophagy, it is not a sufficient signal for mitophagy (Narendra, Tanaka, Suen, & Youle, 2008; Twig et al., 2008).

The predominant pathway leading to mitophagy involves the cytosolic E3 ubiquitin ligase Parkin and the mitochondrial phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) (Figure I.4). In response to mitochondrial damage, PINK1 becomes stabilized on the outer mitochondrial membrane and recruits Parkin (Lazarou, Jin, Kane, & Youle, 2012). Parkin then ubiquitylates several outer mitochondrial membrane proteins, resulting in fragmentation and isolation of impaired mitochondria from healthy mitochondria (N. C. Chan et al., 2011; Gegg, Cooper, Chau, Rojo, & Schapira, 2010; Toyama et al., 2016). These impaired mitochondria are then recognized and degraded by autophagic machinery. The precise control of mitophagy is essential for the maintenance of a healthy mitochondrial population. Perturbations to the mitophagic process, either an increase or decrease, can result in disruption
to cellular homeostasis and is in fact implicated in several pathologic conditions (Malpass, 2013).

**Figure I.4** Parkin-mediated mitophagy. In a healthy mitochondrion with an active membrane potential (↑mtΔΨ), PINK1 is imported to the inner mitochondrial membrane (IMM) and constitutively turned over by proteolysis. However, following a compromise of mitochondrial activity and loss of membrane potential (↓mtΔΨ), PINK1 stabilizes on the outer mitochondrial membrane (OMM) allowing recruitment of the E3-ubiquitin ligase parkin. Parkin activation results in the ubiquitination of OMM proteins that act as an ‘eat-me’ signal recognized by a nascent autophagosome. LC3 can bind ubiquitin directly or may interact indirectly via adapter protein p62. *Permission to reproduce this figure from MacVicar (2013), Biochemical Society Essays in Biochemistry, granted by Portland Press, Ltd.*
C. Implications in Disease

Increasing evidence suggests that structural and functional abnormalities in mitochondria are implicated in aging and age-related neurodegenerative diseases (Table I.1). Even subtle disruptions to mitochondrial dynamics can, over time, give rise to severe defects in neurons. Many mtDNA mutations also cause neurological problems (Keogh & Chinnery, 2015). In addition, there are several neuropathologies arising from specific mutations in genes coding for mitochondrial machinery proteins. Abnormalities in mitochondrial dynamics, including the inhibition of mitochondrial fission or fusion and the abnormal activation of mitochondrial fission, have been suggested to occur as early events during the pathogenesis of many diseases (H. Chen & Chan, 2009).

Table I.1 Mitochondrial fission and fusion proteins in neurological diseases. Many of the proteins regulating mitochondrial fission and fusion are disrupted in neurological diseases. This table summarizes the proteins as well as their changes. Permission to reproduce this table from Flippo & Strack 2017, Journal of Cell Science, granted by The Company of Biologists, Ltd.
a. Mutations in Mitochondrial Dynamics Machinery Leading to Mitochondrial Dysfunction and Neuropathology

Charcot-Marie-Tooth (CMT) Disease is a cluster of hereditary peripheral motor neuropathies, some of which have concomitant sensory loss (Reilly, Murphy, & Laur, 2011). CMT Type 2A (CMT2A), the most common form of the disease, arises from a mutation in mfn2 gene which regulates the fusion of outer mitochondrial membranes. As many as 15 different mutations have been identified, most of which cluster in the GTPase domain or just upstream of it (Kijima et al., 2005; Zuchner, Vorgerd, Sindern, & Schrö, 2004) hampering the normal fusion function of the protein. CMT2A is primarily a disorder of motor neurons, presenting with progressive distal muscle weakness followed by muscular atrophy.

Autosomal-dominant optic atrophy (ADOA) is a neuropathy linked to mitochondrial fusion (Delettre et al., 2000). Opa1 is the gene primarily responsible for this neuropathy (Alexander et al., 2000; Delettre et al., 2000). ADOA is characterized by progressive loss of visual acuity, with wide ranges in both expressivity and penetrance. Through histopathology, it appears there is primary degeneration of the retinal ganglion cells which proceeds to atrophy of the optic nerve. At least 83 different opa1 mutations have been identified (Ferre, Amati-bonneau, Tourmen, Malthie, & Reynier, 2005), most are truncations with the majority found within the GTPase domain. Though it is not clear exactly how opa1 mutations are affecting mitochondrial function in retinal ganglion cells, studies have shown clumped mitochondria in monocytes of affected patients indicating that mitochondrial morphology and/or localization is altered (Delettre et al., 2000).
b. Role of Mitochondrial Dynamics in Neurodegeneration

Mitochondrial impairments have been implicated in a causal role in the majority of neurodegenerative diseases (H. Chen & Chan, 2009). These impairments include disruption to the fission/fusion balance as well as in trafficking and mitophagy. Prominent changes in the expression of mitochondrial fission and fusion proteins have been observed in Alzheimer’s disease, suggesting an imbalance in the fusion-fission process (Xinglong Wang et al., 2009). While the mechanisms remain unclear, these changes may represent the direct effects of amyloid β (Aβ) as increased Aβ fragments mitochondria and decreases mitochondrial mass in neurites in cultured neurons (Barsoum et al., 2006). This process depends on the S-nitrosylation-mediated stimulation of Drp1 activity (D.-H. Cho, Nakamura, & Lipton, 2010). The mitochondrial fragmentation seen in AD may also be due to increases in phosphorylation of Drp1 leading to its recruitment to the surface of mitochondria (Bossy et al., 2010). Moreover, this increased mitochondrial localization may also be due to increases in Drp1 expression levels and its interactions with Aβ and phosphorylated tau in AD patients (Manczak, Calkins, & Reddy, 2011; Manczak, Mao, Calkins, Cornea, & Reddy, 2010; Manczak & Reddy, 2012). Further investigations are necessary to clarify the mechanisms at play.

Several mechanisms may be involved implicating alterations to mitochondrial dynamics in Parkinson’s disease (PD). One form of familial PD results from mutations in proteins PINK1 and Parkin, leading to an autosomal recessive PD. As discussed earlier, Parkin and PINK1 are important proteins in the regulation of mitophagy, and thus the impairments observed might be due to alterations to mitochondrial dynamics through their role in mitophagy. However, Parkin and PINK1 might also affect mitochondrial distribution by regulation of mitochondrial transport. Recently, PINK1 and Parkin have been shown to prevent mitochondrial movement in axons. When recruited to the surface of a dysfunctional mitochondria, PINK1 phosphorylates miro,
which is an adaptor protein that connects mitochondria to the microtubule motor kinesin (Y. Wang, Pan, Price, & Martin, 2011). Miro is then ubiquitinated by Parkin and degraded by proteasomes, leading to the inhibition of movement of these dysfunctional organelles. Affecting a larger proportion of PD patients, the sporadic LRRK2 leads to mitochondrial fragmentation which may occur through effects on Drp1 (Xinglong Wang et al., 2012). Additionally, mitochondrial fragmentation appears to occur through a direct interaction between synuclein and mitochondrial membranes.

Mitochondrial dynamics have also been found to be changed in Huntington’s disease (HD). Increased amounts of Drp1 and decreased levels of Mfn and Opal are found in HD patients, suggesting that mitochondrial dynamics are shifted toward fission over fusion (Shirendeb et al., 2012). In patients and animal models of HD, mitochondria are fragmented and show decreased motility and respiration (Perkins, Bossy-Wetzel, & Ellisman, 2009). Htt aggregates are known to bind to many proteins. Mitochondria can be damaged as a secondary consequence of decreases in cellular health. Excessive activation of Drp1 also occurs in HD. Htt aggregates directly bind to Drp1 in vitro and stimulate its GTPase activity which may facilitate abnormal assembly of Drp1 oligomers, leading to increased mitochondrial fission (W. Song et al., 2011; H. Wang, Lim, Karbowiak, & Å, 2009). Further implicating a role for Drp1 in Htt pathology, mitochondrial fragmentation and increased cell death are induced by mutant Htt and can be rescued by introducing a dominant negative form of Drp1. It is possible that the increases in Drp1 activity are also due to indirect effects of increased cytosolic Ca²⁺ which in turn activate calcineurin, leading to the dephosphorylation and activation of Drp1 (Shirendeb et al., 2012).

HIV promotes mitochondrial-mediated apoptosis of T cells (Matarrese et al., 2003). However, the discovery that the function (Lehmann, Chen, Borzan, Mankowski, & Höke, 2011) and morphology of neuronal mitochondria in HAND subjects are altered (Valeria Avdoshina et
al., 2016; J. A. Fields et al., 2016) expanded the interest in mitochondrial research in the
neuroAIDS field as a possible explanation for causes of neuronal degeneration. The
mislocalization of mitochondria and perturbations to energy production are known to impair
neuronal function, and have been linked to neurodegenerative diseases (Burté et al., 2014),
including evidence that impaired mitochondrial dynamics/function as an important contributor for
HIV-induced neurotoxicity (Lecoeur et al., 2012). Tat exposure causes a biphasic increase in
mitochondrial membrane potential that is both concentration- and time-dependent (Norman,
Perry, Kasischke, Volsky, & Gelbard, 2007). This increase is linked to significant alterations in
synaptic activity, which was surprisingly not found to be due to disruption to mitochondrial
distribution, localization, or morphology. The effect of Tat upon ATP production has conflicting
data (Perry et al., 2005; Tiede, Cook, Morsey, & Fox, 2011; Villeneuve et al., 2016), with
indications of both increases and decreases in ATP production, in all instances preceding cell
death. Nevertheless, the ability of Tat to cause a drastic inhibition of ATP synthase (Lecoeur et
al., 2012; Norman et al., 2007) points to a disruption of mitochondrial function. Changes in ETC
protein function lead to rapid dissipation of the mitochondrial membrane potential, inhibition of
mitochondrial Ca^{2+} uptake, and release of cytochrome c, a water-soluble component of ETC
located within the inner membrane of mitochondria. The release of cytochrome c is a strong
indication that apoptosis is activated. Indeed, mitochondria are also involved in the cytochrome
c or caspase-dependent death pathway (Xuesong Liu, Kim, Yang, Jemmerson, & Wang, 1996).
Regardless of whether Tat decreases ATP formation, when Tat-mediated mitochondrial
membrane potential changes are blocked, neurons can be spared from undergoing apoptosis
(Lecoeur et al., 2012; Turchan et al., 2001). This consideration indicates that the integrity of
mitochondrial function is the key to explaining the neurotoxic properties of Tat and that keeping
this function intact could be a therapeutic target to improve neuronal survival following HIV
infection.
D. Human Immunodeficiency Virus

a. Discovery, Structure, and Infectious Cycle

Arising from several zoonotic transmissions of simian immunodeficiency viruses (SIV) (Korber et al., 2000) the human immunodeficiency virus (HIV) was first described as the causative factor leading to the acquired immunodeficiency syndrome (AIDS) in the early 1980s by two groups lead by Luc Montagnier and Robert Gallo (Barre-Sinoussi et al., 1983; Gallo, Sarin, Gelmann, Robert-Guroff, & Richardson, 1983) In the ensuing decades, HIV has become a worldwide pandemic, infecting more than 30 million people across the globe. HIV is a Lentivirus, belonging to the family Retroviridae, a family that consists of single-stranded RNA viruses that typically have long incubation periods. Two viral strains exist, HIV-1 and HIV-2, with HIV-1 having the predominant infection rate throughout the world, and HIV-2 localized within Western African countries (Nyamweya et al., 2009). HIV is able to persistently infect humans through subversion of both adaptive and innate immune cells.

The HIV-1 genome consists of two single strands of RNA, coding for nine genes (gag, pol, env, tat, rev, nef, vif, vpr, vpu) (Frankel, Francisco, & Young, 1998). The virus also contains the enzymes necessary to carry out the replicative process: reverse transcriptase, integrase, proteases, and ribonuclease. Infection and the viral replication process is initiated by viral entry, which occurs following the binding of envelope glycoproteins gp41 and gp120 to cellular receptors CD4 and either co-receptor CCR5 or CXCR4 (Arenzana-seisdedos & Parmentier, 2006; Dragic et al., 1996). Selective binding to co-receptors is contingent upon the tropism of the virus, such that T-tropic viruses bind to CXCR4 co-receptor (Coakley, Petropoulos, & Whitcomb, 2005), whereas M-tropic viruses bind to CCR5 co-receptor. In some instances, HIV-1 is dual-tropic, with the ability to bind either co-receptor to gain entry into the host cell. Binding
to the receptors allows for membrane fusion between the virus and the host cell. Following
entry, the virus core is uncoated, freeing viral RNA and allowing for reverse transcription to take
place. The provirus DNA is then transferred to the cell nucleus where it is integrated into the
host genome. Upon activation, transcription of proviral DNA into messenger RNA (mRNA)
occurs. The transcription process initially results in the early synthesis of regulatory HIV-1
proteins such as Tat and Rev. Tat binds to the TAR site at the beginning of the HIV-1 RNA in
the nucleus and stimulates the transcription and the formation of longer RNA transcripts. Rev
transports the unspliced and incompletely spliced mRNA encoding structural proteins from the
nucleus to the cytoplasm. Once viral mRNA migrates into the cytoplasm, structural proteins of
new virions are synthesized. The proteins coded by pol and gag genes form the nucleus of the
maturing HIV particle; the gene products coded for by the env gene form the glycoprotein spikes
(gp41 and gp120) of the viral envelope. Once formed, the immature viral particle migrates
towards the cell surface where the large precursor molecules are cleaved by the HIV-1
protease, resulting in new infectious viral particles, which bud through the host cell membrane.

b. Highly Active Antiretroviral Therapy

The development of highly active antiretroviral therapy (HAART) was first introduced in
the mid-1990s and has since been an effective system of suppressing HIV replication. Since its
introduction, HAART has resulted in a 50% decline in the rate of deaths from AIDS, significant
reductions in rates of maternal-infant transmission, reduced incidence of opportunistic
infections, and a 40-50% decrease in the incidence of HIV-associated dementia (HAD)
(Maschke, Kastrup, Esser, Ross, & Hengge, 2000). HAART is a combinatorial drug therapy that
is individually tailored with three or more antiretroviral drugs in an effort to block the HIV
replication cycle at different steps. Initiation of this drug regimen can result in immune
reconstitution, undetectable viral load, and lowers the overall progression to AIDS and AIDS associated deaths (Boyd, 2009). The current recommendation in the care of HIV+ individuals is to begin HAART as soon as a patient is willing to commit to this lifelong therapy, regardless of CD4+ T cell count (DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents, 2015).

Antiretroviral drugs fall into four general classes, each targeting a different step along the viral replication cycle. These steps are: viral entry, reverse transcription, viral DNA integration, and the proteolytic processing by the viral protease. HIV reverse transcriptase (RT) is targeted by three classes of inhibitors: nucleoside RT inhibitors (NRTIs), nucleotide RT inhibitors (NtRTIs), and non-nucleoside RT inhibitors (NNRTIs). The NRTIs and NtRTIs interact with the catalytic site of the enzyme, whereas the NNRTIs interact with an allosteric site located near the catalytic site (De Clercq, 2009). Protease inhibitors (PIs), such as saquinavir or ritonavir, contain a hydroxyethylene scaffold which mimics the normal peptide linkage that is cleaved by the HIV protease. This scaffold is unable to be cleaved and thus prevents the HIV protease from carrying out its normal function (De Clercq, 2009). Viral entry is inhibited through the use of several types of drugs, those that interact with the viral particle itself as well as those that bind to cell receptors used by the virus. Fusion inhibitors (FIs) interact with the viral particle to prevent binding. Currently, one FI, enfuvirtide, is available for treatment of HIV infections. This polypeptide is homologous to a region of the viral glycoprotein gp41 and engages the protein, interfering with the fusion of the virus particle and the outer cell membrane. Co-receptor inhibitors (CRIs) interact with the co-receptors CCR5 or CXCR4 to block the binding of M-tropic and T-tropic HIV strains, respectively. Maraviroc, which targets the CCR5 receptor, is currently the only approved CRI available (Westby et al., 2007). Finally, integrase inhibitors (INIs) target HIV integrase by inhibiting its two important catalytic functions: 3'-processing and strand transfer.

Taken together, these drugs have been highly successful in limiting viral load in patients who adhere to the daily regimen. While these drugs have been paramount in prolonging the
lives and increasing the quality of life of HIV infected individuals, these drugs are not efficient at preventing the entry of HIV into the central nervous system (CNS). The incidence of neurological complications caused by HIV continues to expand, despite the effectiveness of HAART in the periphery (Mcarthur et al., 2003). This is due to the poor blood brain barrier permeability of many of the drugs within the HAART regimen (Kramer-Hämmerle, Rothenaigner, Wolff, Bell, & Brack-Werner, 2005). Thus, the CNS acts as a reservoir for active viral replication, leading to neurological complications.

c. HIV-Associated Neurocognitive Disorders

Between 15 and 55% of HIV infected individuals will develop some form of neurocognitive impairment (Heaton et al., 2010). Together these impairments are classified as HIV associated neurocognitive disorders (HAND). This spectrum disorder can be categorized into three levels of severity: asymptomatic neurocognitive impairment (ANI), the least severe form, mild cognitive disorder (MND), and HIV associated dementia (HAD), the most severe form (Saylor et al., 2016). The symptoms of HAND include a range of deficiencies in cognition, motor function, and altered behavioral states. Typically, HAND presents with executive dysfunction and memory impairment with prominent disruption of attention, multitasking, impulse control, judgement and memory encoding and retrieval. Motor dysfunction seen in HAND includes bradykinesia, loss of coordination, and gait imbalance. Prior to the widespread use of HAART, motor skills and psychomotor speed were more common. However, today deficits in learning and/or memory and executive function are more prevalent (Heaton et al., 2010). As one might expect given these symptoms, HIV exacerbates age-associated cognitive decline. Many of those who are affected in middle age experience cognitive decline similar to that observed in adults of a much older age. Furthermore, HIV and age appear to have a synergistic effect upon cognitive decline
observed in patients (Cohen, Seider, & Navia, 2015). Older adults with HIV showed a significant memory decline after one year whereas no decline was seen in younger adults with HIV or uninfected controls of the same age (Seider et al., 2014).

As of yet, it is unclear as to why some HIV+ individuals develop cognitive impairments whereas others remain free of such impairments. Risk factors include advanced age, female sex, intravenous drug use, as well as low body weight (Saylor et al., 2016). Much like HIV in the periphery, HAND has been transformed throughout the past two decades. With the prevalence of HAART use among infected individuals, an increasing number of those experiencing cognitive impairments are suffering from the least severe forms, compared to pre-HAART (Saylor et al., 2016).

HIV enters the CNS within weeks of infection (Ho et al., 1985) as HIV proteins can be observed in the brain tissue and cerebrospinal fluid (CSF) at this early time point. HIV entry into the CNS is termed the Trojan horse hypothesis, wherein infected monocytes from the periphery cross the blood brain barrier and spread the virus to CNS resident cells (Gartner, 2000). This peripheral invasion of the CNS has been linked to the chemokine CCL2 (E. A. Eugenin et al., 2006). Once within the CNS, a number of maladaptive changes occur, including the development of multinucleated giant cells, microgliosis, and astrocytosis (Glass, Fedor, Wesselingh, & McArthur, 1995), all contributing to axonal and dendritic damage that is most correlative to the neurocognitive impairments observed in HAND (Masliah et al., 1997).

The mechanisms by which HIV causes neuronal damage are numerous. Neurons are unable to be infected due to the lack of CD4 expression. However, neurons do sustain significant damage during the course of the HIV infection which leads to the cognitive, motor, and behavioral impairments observed in HAND. In general, there are two pathways by which neurons are impaired or killed in HIV infection: the indirect and direct pathways (Marcus Kaul & Lipton, 2006). The indirect pathway consists of impairments caused by the release of factors
from neighboring infected glial cells, including excitatory amino acids (Donnell et al., 2006), ROS (Elbim et al., 1999), and matrix metalloproteinases (MMPs) (Suyang Li et al., 2013), which impair neuronal viability. HIV infection activates CNS glial cells, including microglia and astrocytes, both of which are cell types able to be productively infected by HIV (Churchill et al., 2006). The activation of microglia and astrocytes results in the release of pro-inflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Wesselingh et al., 1997). Moreover, aberrant glial functions can decrease the expression of glutamate transporters leading to heightened glutamate in the extracellular space resulting in excitotoxic conditions (Xing, Hayakawa, & Gelpi, 2009). Finally, HIV decreases trophic support including brain derived neurotrophic factor (BDNF) (Valeriya Avdoshina et al., 2011; Bachis, Avdoshina, Zecca, Parsadanian, & Mocchetti, 2012).

The direct pathway consists of viral proteins shed from infected cells or free virus acting directly upon the neuron, leading to neuronal dysregulation and ultimately cell death. The two most toxic viral proteins related to this pathway are Tat (W. Li et al., 2008a) and gp120 (Toggas et al., 1994). Each of these proteins is able to confer damage upon neurons through interactions with cellular receptors, including NMDA receptors (N J Haughey, Nath, Mattson, Slevin, & Geiger, 2001) and low-density lipoprotein receptor related proteins (LRP) (Hudson et al., 2000) in the case of Tat and CXCR4 or CCR5 in the case of gp120 (M Kaul, Ma, Medders, Desai, & Lipton, 2007). These interactions lead to the rise of intracellular Ca\(^{2+}\), which can activate pathways ultimately contributing to the activation of caspase-3 dependent apoptosis (M Kaul et al., 2007). Additionally, these viral proteins are able to be taken up by neurons through both receptor-dependent and receptor-independent manners (Wenzel et al., 2017), further conferring toxicity upon the neuron (Cardarelli, Serresi, Bizzarri, Giacca, & Beltram, 2007; Y. Liu et al., 2000). Each is also able to be passed along anatomical pathways throughout the brain (Bachis, Aden, Nosheny, Andrews, & Mocchetti, 2006; Bruce-Keller et al., 2003; Chauhan et al., 2003).
Overall, the direct and indirect pathways are not mutually exclusive and it is well accepted that both mechanisms contribute to the overall pathogenesis of HAND. This dissertation focuses upon the direct neurotoxic mechanisms of Tat.

d. Trans-Activator of Transcription

As stated previously, the gene that encodes for trans-activator of transcription, Tat, is expressed very early after infection and promotes the expression of other HIV genes. Tat, a 101-amino acid protein, regulates HIV transcriptional elongation rather than transcriptional initiation (Kao, Calman, Luciw, & Peterlin, 1987). As mentioned briefly in this introduction, Tat is required for the efficient regulation of HIV transcription.

Similar to other retroviruses, the long terminal repeat (LTR) of HIV acts as the viral promoter. The first evidence that gene expression in HIV-1 also requires viral transactivating factors came from experiments by Fisher and colleagues (1986) who noted that the expression of reporter genes placed under control of the LTR was dependent on a transactivating factor, which they named Tat. Through a series of deletion experiments, it was determined that Tat activity required the TAR, a regulatory element located downstream from the initiation site for transcription. While viral transcription could proceed without Tat activity, only short, spliced mRNA were produced instead of the long, unspliced mRNA coding for structural proteins. Tat is able to specifically recognize the TAR RNA (Dingwall et al., 1989). Tat recruits cellular protein positive transcription elongation factor b (P-TEFb) to the TAR element present in viral transcripts (Bieniasz & Cullen, 1998; Taube, Fujinaga, Wimmer, Barboric, & Peterlin, 1999), which is required for Tat-mediated transactivation (Tahirov et al., 2010). Tat and P-TEFb are able to stimulate HIV-1 transcription both through the removal of blocks to elongation imposed
by NELF and DSIF and by the enhancement of RNAP II processivity through the phosphorylation of Spt5 and the RNAP II CTD.

i. Tat Neurotoxicity

Beyond its normal viral function, HIV Tat is also a known neurotoxin (W. Li et al., 2008a). When shed from infected cells, Tat confers toxicity upon neurons through a variety of actions. Currently there is no effective treatment that blocks Tat activity and because it is produced by infected cells once the proviral DNA is formed, even the current antiretroviral drugs are unable to prevent its production. The presence of Tat has been detected in the brains of patients with HIV encephalitis by immunostaining (Hofman, Dohadwala, Wright, Hinton, & Walker, 1994; Hudson et al., 2000; Kruman, Nath, & Mattson, 1998; Y. Liu et al., 2000). Supporting an important role for Tat in neurodegeneration and HIV, Tat levels positively correlate with cognitive alterations in HAND patients (A. Nath & Steiner, 2014). As a viral transcriptional regulator, Tat localizes mainly to the nucleus (Hudson et al., 2000; Stauber & Pavlakis, 1998), however it is known to exert its toxic effects throughout the cell as well as through interactions with receptors, namely binding to portions of the NMDA receptor on neurons (W. Li et al., 2008a). Tat has been shown to be released from infected lymphoid (Ensoli et al., 1993), monocytic cells (Turchan et al., 2001), and glial cells (Tardieu, Hery, Peudenier, Boespflug, & Montagnier, 1992) in vitro. Tat has been shown to be internalized into neurons through low-density lipoprotein receptor-related proteins (LRP) (Y. Liu et al., 2000) as well as having the ability to cross the cell membrane in a receptor independent manner (Cardarelli et al., 2007). Additionally, Tat is transported along anatomical pathways once inside a neuron, where it can confer toxicity upon distal sites (Bruce-Keller et al., 2003). The mechanisms by which Tat contributes to neuronal injury are many, including overactivation of NMDA receptors (J E King,
Eugenin, Buckner, & Berman, 2006; Jessie E King, Eugenin, Hazleton, Morgello, & Berman, 2010), synaptic and dendritic pruning (H. J. Kim, Martemyanov, & Thayer, 2008), induction of apoptotic cascades (Aksenov, Aksenova, Mactutus, & Booze, 2009; Aksenova, Aksenov, Adams, Mactutus, & Booze, 2009), Ca\(^{2+}\) dysregulation (Self, Mulholland, Nath, Harris, & Prendergast, 2004), oxidative stress (Aksenov et al., 2003), dopaminergic system dysfunction (Ferris, Frederick-Duus, Fadel, Mactutus, & Booze, 2010; Jun Zhu, Mactutus, Wallace, & Booze, 2009), and suppression of long-term potentiation (LTP) (Sheng-tian Li, Matsushita, Moriwaki, & Saheki, 2004). Recently, Tat-induced mitochondrial impairments have been increasingly identified as significant, causal factors of neurotoxicity observed in HAND. However, the mechanism by which this mitochondria-mediated toxicity occurs is still obscured.

E. Summary

Due to the significant role mitochondrial impairments are found to play in a broad range of neurodegenerative diseases as well as the numerous mitochondrial impairments observed both in post-mortem tissue of HAND patients and in *in vitro* investigations of mechanisms of HAND neurotoxicity, I hypothesized that Tat-induced impairments to mitochondrial dynamics play a significant role in Tat-induced neurotoxicity. To evaluate this hypothesis I first asked three main questions: 1) Does Tat impair neuronal mitochondrial integrity? 2) Does Tat alter mitochondrial fusion and/or fission? and 3) Does Tat alter mitochondrial dynamics in a calcineurin-dependent manner? To answer these questions, I utilized an *in vitro* neuronal cell culture system so as to best isolate the neuron-specific impairments following Tat exposure. Using a combination of approaches, we report that exposure of HIV Tat to neuronal cultures induces mitochondrial destabilization, rapid Ca\(^{2+}\) influx through both NMDA and non-NMDAR, followed by increased mitochondrial fragmentation. Additionally, we observed an increase in
mitochondrial fission protein, Drp1, as well as changes in its activity in a timeframe corresponding to mitochondrial fragmentation. These alterations in activity and mitochondrial morphology appear to be driven by the increased activity of calcineurin following Tat exposure, likely activated by the rise in intracellular Ca\(^ {2+} \) and activation of calmodulin by Tat. Intriguingly, these results suggest a novel mechanism through which Tat causes neurotoxicity, giving us better insight into how to best target neuron-specific impairments observed in HAND.
Chapter II: Materials and Methods

A. Reagents

HIV-1 Tat was purchased from Immunodiagnostics Inc (Woburn, MA) and used at a concentration of 100nM. Upon analysis, it consisted of roughly equal portions of monomeric and dimeric Tat (Figure II.1). Drp1 inhibitor Mdivi-1 was purchased from Sigma-Aldrich (St Louis, MO). Prior to each experiment, Mdivi-1 was dissolved into a solution by dimethyl sulfoxide (DMSO) and used at a concentration of 10 μM. Calcineurin inhibitor Cyclosporine A was purchased from Sigma-Aldrich and prepared prior to each experiment at a concentration of 10μM in DMSO. PACAP27 and PACAP38 were synthesized in the Department of Medical Chemistry, Szeged University, Hungary according to previous descriptions (Jozsa et al. 2005) or were purchased from R&D (Minneapolis, MN). FCCP was purchased from Sigma-Aldrich (St. Louis, MO) and aliquoted at 1mg/mL in cell culture grade 100% ethanol. It was diluted to a working concentration of 0.5 μM in culture media immediately prior to use. Anti-Tat antibody (#1102, Immunodiagnostics) was used at a concentration of 1:50 in culture media as a neutralizing antibody against Tat.
**Figure II.1** HIV-1 Tat western blot and semi-quantification. Two bands are depicted at the molecular weights of monomeric (~12kDa) and dimeric (~24kDa) Tat. The Tat utilized in all experiments consists of roughly equal portions of monomeric and dimeric Tat.

**B. Animals**

All studies were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by Georgetown University and University of California Animal Care and Use Committee.

**C. Generation of Inducible Tat Transgenic Mice and Doxycycline Treatment**

As previously described (B. O. Kim, Liu, Ruan, & He, 2003), inducible Tat transgenic mouse colonies were obtained by generation of two separate transgenic lines, Teton-GFAP mice (G-tg) and TRE-Tat86 mice (T-tg), and then cross-breeding these two lines of transgenic mice. Briefly,
a DNA fragment (2238 bp) containing the Teton-GFAP gene, along with downstream simian virus 40 splicing and polyadenylation sequences, was released by XhoI and Pvull digestion of the pTeton-GFAP plasmid and purified by agarose gel electrophoresis and microinjected into fertilized eggs of F1 females obtained from mating between C3HeB and FeJ mice (The Jackson Laboratory). Founder transgenic animals were crossed with C57BL/6 mice to generate stable G-tg transgenic lines. Similarly, T-tg transgenic lines were obtained using a DNA fragment (1189 bp) released by XhoI and Pvull digestion of the pTRE-Tat86 plasmid. Founder animals and progeny carrying the transgenes were identified by PCR analysis of genomic DNA, which was extracted from mouse tail clippings (0.5–1 cm long) using the Wizard genomic DNA isolation kit (Promega). With this construct, mice express Tat upon doxycycline (DOX) treatment. For these experiments a total of \(n = 8\) Non tg mice and \(n = 16\) GFAP-Tat tg mice were used (7–8 months old). The GFAP-Tat tg mice were divided into two groups, and the first was not treated with DOX; however, the other group \((n = 8)\) were treated with DOX at 80 mg/kg for 2 weeks and then killed immediately after (week 2). This treatment regimen has been shown to induce strong Tat expression.

D. Primary Cultures

a. Cortical neuronal cultures were prepared from the cortex of embryonic (E17–18) Sprague–Dawley rats (Taconic, Derwood, MD) following an established protocol (Dichter, 1983) with minor modifications. In brief, cortices were cleaned from blood vessels in Krebs-Ringers bicarbonate buffer containing 0.3% bovine serum albumin (BSA); hippocampi were dissected and disposed of. Cortices were minced and dissociated in the same buffer with 1,800 U/mL trypsin at 37°C for 15 min. Trypsin was inactivated by the addition of soybean trypsin inhibitor and DNase. The combined supernatants from dissociated cortices were centrifuged
through a 4% BSA layer, and the cell pellet was re-suspended in Neurobasal medium containing 2% B-27 supplement, 25 mM glutamate, 0.5 mM L-glutamine and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). Cells were seeded at density of 0.5 X 10^6 cells per mL onto poly-L-lysine pre-coated plates for protein analysis and RNA extraction or 0.25 X 10^5 cells per mL for imaging. Cultures were grown for 7 days at 37°C in 5% CO2/95% air. Cultures contained ~10 % of non-neuronal cells (Figure II.2).

Figure II.2 Representative images of cortical neuronal cultures. Primary cortical neurons were stained (DIV7) with MAP2 to label processes and DAPI to identify cell bodies.

b. Astrocytes were prepared from the cerebral cortex of 1- to 2-day-old SD rats according to an established protocol (Jakovcevski et al., 2007) with some modifications. In brief, the cortex was dissected and cleaned from the meninges. The tissue was mechanically dissociated by trituration. Cells were seeded on poly-L-lysine precoated tissue culture flasks in Dulbecco’s Modified Eagle Medium (DMEM)/F12 containing 10% fetal bovine serum, 2% antibiotic-antimycotic (Invitrogen, Carlsbad, CA) and grown at 37°C in 5% CO2/95% relative atmosphere. The culture medium was replaced twice a week. To grow astrocytes, fresh DMEM/F12 was added to the flasks which then were continually
shaken for 4 more days. Cells were then trypsinized for 5 min, collected and centrifuged at 400g for 8 min. Pellet was resuspended in the proper amount of DMEM/F12 containing 10% fetal bovine serum, 2% antibiotic-antimycotic. Cells were then plated and grown as described above for three more days. For experiments, cells were seeded at density of 0.25 X 10^6 cells per mL onto poly-L-lysine pre-coated plates for protein analysis or 0.125 X 10^5 cells per mL for imaging and grown at 37°C in 5% CO2/95% air for 24h prior to experimental treatment. Figure II.3 depicts representative images of prepared astrocyte cultures.

**Figure II.3** Representative images of astrocyte cultures. Primary astrocyte cultures were stained (DIV7) with GFAP to label processes and DAPI to identify cell bodies.
### E. Treatments

Experimental treatments were conducted as outlined in Table II.1

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Working Concentration</th>
<th>Vehicle*</th>
<th>Treatment Timing**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>100 nM</td>
<td>0.1% BSA</td>
<td>-</td>
</tr>
<tr>
<td>Heat inactivated Tat</td>
<td>100 nM</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>PACAP27</td>
<td>100 nM</td>
<td></td>
<td>15 min prior</td>
</tr>
<tr>
<td>PACAP38</td>
<td>100 nM</td>
<td></td>
<td>15 min prior</td>
</tr>
<tr>
<td>CsA</td>
<td>10 μM</td>
<td>DMSO</td>
<td>15 min prior</td>
</tr>
<tr>
<td>Mdivi-1</td>
<td>10 μM</td>
<td>DMSO</td>
<td>15 min prior</td>
</tr>
<tr>
<td>FCCP</td>
<td>0.5 μM</td>
<td>100% cell culture grade ethanol</td>
<td>concurrent</td>
</tr>
<tr>
<td>anti-Tat (mAb)</td>
<td>1:50</td>
<td>N/A</td>
<td>concurrent</td>
</tr>
<tr>
<td>NMDA</td>
<td>60 μM</td>
<td>dH₂O</td>
<td>-</td>
</tr>
<tr>
<td>MK801</td>
<td>100 μM</td>
<td>DMSO</td>
<td>15 min prior</td>
</tr>
</tbody>
</table>

**Table II.1 Table of Reagents and Experimental Treatments**

* If reagent was received as a lyophilized powder.

**Treatment timing is relative to treatment of cultures with HIV-1 Tat.
F. Immunocytochemistry

Cells grown on coverslips were fixed in 4% paraformaldehyde and 4% sucrose for 15 min at room temperature on a shaker. Cells were incubated for 15 min in blocking solution (0.03% Triton-X, 5% non-fat milk or BSA in PBS or TBS) at room temperature. Primary antibodies were used at the following concentrations: TOM20 (1:1000), pDrp1 (S637) (1:500), pDrp1 (S616) (1:1000), Drp1 (1:1000), MAP2 (1:1000), GFAP (1:1000) was used as a neuronal marker. Incubation with primary antibodies was carried out overnight at 4°C. DAPI was used to label nuclei. Coverslips were washed with PBS or TBS and then incubated for 2h at RT with the corresponding fluorescent secondary antibody. Immunofluorescence was analyzed with a Leica SP8 confocal microscope system.

G. Immunoprecipitation

Beads (Pierce® Protein A/G Magnetic Beads, Thermo Scientific, Rockford, IL) were washed 2X in TBS with 0.2% Tween and then blocked for 1h at room temperature in 1% BSA in TBS on a rotator. Supernatant was removed and beads were rinsed once with TBS. Following treatments, neuronal lysates were collected using 1X RIPA plus protease and phosphatase inhibitors, and 1% Triton-X 100. Protein levels were measured using Pierce BCA Assay (product #23225). Sample volumes with 200μg protein were added to respective microcentrifuge tubes containing 40μL washed and blocked beads. Antibody of interest (ms anti Drp1) was added to each sample tube (1:2000). A sample tube containing an IgG control antibody (ms IgG, Abcam #190475) was also prepared. dH2O was added to each sample to bring final volume to 500μL. Labeled sample tubes were placed on a rotator at 4°C, overnight. The next morning, beads were isolated using a magnetic tube stand and the supernatant was removed. Beads were washed 3X with ice cold TBS-T, vortexing with each wash. Washed beads were resuspended in 50μL of 1X loading buffer and boiled for 5 minutes. Again, a magnetic tube stand was used to separate the beads
from the supernatant. Supernatant was removed and saved for immunoblot. Gels were loaded with 25µL of each sample and run at 200V using MOPS running buffer. Proteins were transferred to Nitrocellulose membranes using iBlot2 transfer system. Protein levels were checked with Ponceau following transfer before probe. Blots were blocked with 5% BSA in TBS-T. Primary antibody (ms anti pSer, Abcam, Boston, MA) was prepared 1:1000 in 10% BSA in TBS-T and incubated on membranes for 1.5h at room temperature. Secondary antibody (HRP anti ms) was prepared 1:2000 in 5% BSA in TBS-T and incubated on membranes for 2h at room temperature. Immunoblot was developed using West Pico and GE Amersham Imager 600. Densitometry was performed using ImageJ.

**H. Western Blots**

Neuronal lysates were collected with 1X RIPA plus protease and phosphatase inhibitors, sonicated, and spun at 10,000rpm at 4°C for 10 min. Supernatant was collected and protein levels were measured (Pierce® BCA Assay, Thermo Scientific). Samples were loaded into 4-12% Bis-Tris gel and run at 200V then transferred to a nitrocellulose membrane using iBlot 2 (Program P0, Invitrogen, Carlsbad, CA). Membranes were blocked for 30 min (5% non-fat milk in PBS) at room temperature. Primary antibodies against Drp1 (Cell Signaling, Danvers, MA), TOM20 (Santa Cruz Biotechnology), and Mfn2 (Cell Signaling, Danvers, MA) were used at 1:1000 dilution. Anti-beta actin (1:10,000, Sigma-Aldrich) was used a loading control. Corresponding secondary antibodies conjugated to horseradish peroxidase were utilized and incubated for 2 hours at room temperature or overnight at 4°C. Densitometry was performed using ImageJ (NIH).
I. Image Analysis

Immunofluorescence images were captured using a Leica SP8 confocal microscope system. Analysis of mitochondria (area, perimeter, number), Drp1/pDrp1 (puncta number) were performed on these captured images as follows.

a. Mitochondria Size and Count Analysis: The ImageJ macro “Mito-Morphology” (Ruben K. Dagda, University of Pittsburgh, 2010) was utilized to isolate individual mitochondria within each image for measurement and counting. This macro allows you to measure mitochondrial interconnectivity and elongation from epifluorescence micrographs of cells immunostained for mitochondria. The macro allows you to threshold mitochondria in the cell to be analyzed and gives you measurements of mitochondrial count, mitochondrial area, and mitochondria perimeter. In brief, the macro proceeds as follows: 1) Select a single neuron (by outline) for analysis, 2) threshold mitochondria within selected neuron, 3) measure the mitochondria. The individual measurements for organelles within each analyzed image were used for the final calculations which were done manually so as to normalize the quantifications across images and treatment conditions. Identified organelles were excluded from the analysis if the outlined organelle was clearly the overlapping of two or more organelles that were not fused (determined by visual comparison with immunofluorescent image). This parameter eliminated the analysis of the majority of mitochondria within the soma as the density was too high for accurate measurements.

b. Drp1/pDrp1 Puncta Analysis: The ImageJ counting function was utilized to quantify both Drp1 and pDrp1 puncta from obtained confocal images. In brief,
after opening image in ImageJ, convert to 8 bit and threshold image. A single neuron was outlined for analysis before the image was watershed so as to separate any adjacent but non-overlapping puncta, followed by particle analysis.

J. Stochastic Optical Reconstruction Microscopy (STORM)

STORM was performed using a Nikon A1 confocal microscope with CFI SR Apochromat TIRF 100x oil objective and Andor Technology iXon3 897 EMCCD camera. Samples were treated according to the manufacturer’s protocol. Rabbit anti-Tom20 (1:25; Santa Cruz, CA), mouse anti-Drp1 (1:25; Abcam, Boston, MA) and mouse anti-MAP2 (1:200; Abcam, Boston, MA) were used overnight to label mitochondria, Drp1, and cytoskeleton, respectively.

K. Electron Microscopy (EM)

Vibratome sections from GFAP-Tat tg mouse brains were, as previously described (Fields et al., 2016), fixed, embedded, and sectioned with the ultramicrotome. To analyze the relative changes in average diameter of mitochondria, a total of 25 cells were analyzed per condition. Cells were randomly acquired from three grids. Grids were analyzed with a Zeiss OM 10 electron microscope as previously described (Fields et al., 2016). Electron micrographs were obtained at a magnification of ×25,000. All the analyses of images were conducted on blind-coded samples. After the results were obtained, the code was broken, and data were analyzed with the StatView program (SAS Institute, Inc., Cary, NC).

L. Neutral Comet Assay

The Comet Assay® kit (Trevigen, Gaithersburg, MD) was used with some modifications. Cells in 12-well plates were rinsed with ice-cold phosphate-buffered saline (PBS, Ca2+/ Mg2+-free), gently scraped and transferred to a centrifuge tube where they were pelleted. Pellets were then
washed in ice-cold PBS and cells were resuspended at 1×105 cells/ml in ice-cold PBS. Cells were combined with molten low melting-point agarose at 37°C (LMAgarose, Trevigen) at a ratio of 1:10 (v/v), and 50 μl of the cells/LMAgarose mixture was spread onto Comet Slides (Trevigen). After cooling at 4°C for 10 min to allow LMAgarose to solidify, slides were placed in lysis buffer overnight. Following lysis, slides were washed with 1× TBE buffer (Cellgro Mediatech, Manassas, VA) and subjected to electrophoresis in TBE buffer. Electrophoresis was conducted at 1 V/cm for 30 min at 4°C. The slides were washed twice with ddH2O for 10 min and dehydrated with 70% EtOH (Sigma-Aldrich) for 5 min. Slides were placed in a dry oven at 45°C until dry (~15 min). Subsequently, cells were stained with SYBR Green (Trevigen) for 10 min, air-dried and stored in the dark with desiccating material until imaging. Images taken with Nikon eclipse Ni microscope were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Tail moment, the length from the center of the head of the comet to the end of the tail, was quantified as a measure of DNA double-strand breaks (DSBs).

M. Hoechst 33258/Propidium Iodide

The viability of primary cortical neurons was estimated by Hoechst 33258 and propidium iodide (Hoechst/PI; Sigma-Alrich) co-staining and visualized using a fluorescence microscope. Briefly, cultures were incubated simultaneously with Hoechst 33258 and PI (both 5 μg/ml) for 30 min at 37 °C. Neurons were imaged in four microscopic fields in each well (three wells/treatment). Reaction was visualized with an Olympus IX71 (Tokyo, Japan) inverted fluorescence microscope. Hoechst/PI-positive cells were then counted using ImageJ and expressed as a percentage of the total number of neurons.
N. Enzyme-Linked Immunosorbent Assay

Levels of CCL5 were determined in the culture medium using the DuoSet enzyme-linked immunosorbent assay (ELISA) Development System Kits (R&D, Minneapolis, MN), according to the manufacturer’s instructions.

O. Calcineurin Activity Assay

The calcineurin cellular activity assay kit (BML-AK816-0001, Enzo Life Sciences, NY, USA) was used to prepare neuronal lysates and test for calcineurin activity, following manufacturer’s instructions. In brief, following experimental treatment, neurons were rinsed once with ice-cold TBS. All the following steps were performed on ice, unless otherwise noted. Lysis buffer (BML-LI135) with protease inhibitors was added to each well at a volume of 10 million cells per mL (150 μL per well of a 6 well plate). Cells were then scraped, collected by micropipette, and placed in labeled microcentrifuge tubes to further trituration (30 up/down passages through a 10 μL pipette). Samples were then frozen at -80°C for later analysis or immediately passed to the following step. Samples were desalted in rehydrated desalting column resin in a 50 mL conical tube following equilibration with lysis buffer by centrifuging at 800 x g for 3 minutes. The flow through was saved and either frozen for later analysis or immediately passed to the following step. All samples were tested qualitatively for effective removal of phosphate/nucleotides by adding 100 μL BIOMOL® GREEN reagent to 1 μL sample extract and a separate sample of 1 μL of dH₂O. Any samples with a visible green color present over a time period of 30 min at room temperature would undergo the desalting step again. During these experiments, no samples needed to undergo a repeat desaling. To complete the BIOMOL® GREEN phosphatase assay, the phosphate standard curve sample wells were prepared in serial dilutions to include concentrations of 2, 1, 0.5, 0.25, 0.125, 0.063, and 0.031 nmol PO₄. The calcineurin activity assay sample wells were prepared to include the following, in duplicate, for each sample:
background (no substrate), total phosphatase activity, EGTA buffer (Ca\textsuperscript{2+}/CaM free), OA (okadaic acid), OA+EGTA, and positive control (calcineurin enzyme). 10 μL of phosphopeptide substrate was added to each calcineurin sample well except the “Background” control. Plates were then equilibrated to room temperature for 10 min. Following equilibration, 5 μL sample extract or diluted calcineurin (8U/μL) were added to the appropriate wells and incubated for 30 min at room temperature. Reactions were terminated by adding 100 μL BIOMOL® GREEN reagent to all wells and color was allowed to develop for 20 min before reading the plate at OD\textsubscript{620nm} on a microplate reader. The standard curve was plotted to obtain a line of best fit, which was then used to calculate the amount of phosphate released in each sample, with the contribution of calcineurin determined through the subtraction of PO\textsubscript{4egta} from PO\textsubscript{4total}. All values were normalized to the values from control animals and plotted. One-way ANOVA was performed for statistical significance using Prism 7.0 software.

P. 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The conversion of the yellow tetrazolium salt (MTT) to the purple formazan dye is dependent on the activity of mitochondrial dehydrogenases and is, therefore, reflective of the viability of the cell and the cytotoxicity of glutamate. The assay was performed according to the specifications of the manufacturer (MTT Cell Grow Assay Kit, Millipore, Temecula, CA). Briefly, neurons were cultured on 96-well plates, following experiment treatment, 0.01 mL AB Solution (MTT) was added to each well containing neurons in 100 μL of medium, and the plate was incubated for 4 hr in a humidified atmosphere. After the incubation, 0.01 mL isopropanol with 0.04 N HCl was added to each well and mixed thoroughly by repeated pipetting with a multichannel pipettor. Within an hour, the absorbance of the samples was measured at a wavelength of 570 and 630 nm (reference wavelength).
**Q. Reactive Oxygen Species (ROS) Levels**

Intracellular accumulation of ROS was determined with H2DCF-DA (Sigma-Aldrich, St. Louis, MO). This nonfluorescent compound accumulates within cells upon deacetylation. H2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF). Following exposure to experimental compounds, cells were loaded with H2DCF-DA (5 μg/ml) at 37°C for 45 min in a humidified 5%CO₂/95% air incubator. The free dye was washed away by several medium changes and fluorescence was measured with an excitation wavelength of 488 nm and emission wavelength of 525 nm (Synergy H4 hybrid reader, Biotek, Winooski, VT).

**R. Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR)**

Total RNA was extracted using RNeasy Plus Mini Kit and reverse transcribed using SuperScript IV VILO Master Mix with ezDNase Enzyme (Invitrogen) according to manufacturer’s specifications. The qPCR reaction mixture contained the Power SYBR Green qPCR SuperMix (Invitrogen), cDNA and primers. Primers for Drp1 and GAPDH are listed in Table of Primers. GAPDH was used as housekeeping genes to normalize for total mRNA in each sample. Primers for housekeeping gene were obtained from RealTimePrimers.com. All primer pairs were selected that displayed linear amplification. The specificity of the qPCR was determined by the analysis of the dissociation curves. The 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used. Data were analyzed using SDS v2.3 software (Applied Biosystem).
### Table II.2 Table of Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drp1</td>
<td>5’- ATTTGCTAGATGTGCCAGTTCC-3’</td>
<td>5’- TACTGCCTTTGGGACGCTGTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’- CTGCCGTCCCTAGAGCTAT-3’</td>
<td>5’- AGACAGCCGCATCTTTCTTG-3’</td>
</tr>
</tbody>
</table>

#### S. Calcium Imaging

Neurons grown on glass coverslips (DIV7) were loaded with 1 μM Fluo4-AM (Molecular Probes) for 20 minutes and washed for a further 10-20 min before recording. The dye was excited at 488 +/- 15nm. Emitted fluorescence was filtered with 535 +/- 25 nm bandpass filter, captured by a SPOT RT digital camera (Diagnostic Instruments) and read into a computer. Analysis was performed offline by using Simple PCI Software (Compix Inc.).

#### T. Tetramethylrhodamine, Ethyl Ester (TMRE) Mitochondrial Membrane Potential Assay

Neurons grown on glass coverslips (DIV7) were loaded with mitochondrial membrane potential-dependent dye, TMRE (5 mM), after exposure to Tat, FCCP (0.5 μM, carbonilcyanide p-trifluoromethoxyphenylhydrazone), and control media. After 30 min incubation at 37°C, TMRE was washed from cells three times with 1X PBS warmed to 37°C. Coverslips were transferred to glass slides for immediate imaging and subsequent fluorescence intensity measurement.
Mean fluorescent intensity was measured using ImageJ (NIH). In brief, area of interest was selected. Within the Analyze menu, measurements were set to: area integrated intensity and mean grey value. Image selection was measured. An area of background was selected and measured similarly. Results were copied into a spreadsheet where the corrected total cell fluorescence (CTCF) was calculated by subtracting the product of the area of the selected cell and the mean fluorescence of the background readings from the integrated density of the selected cell. All values were normalized to control within each experiment for final analysis.

U. Statistics

The results of no less than three independent experiments were compiled, and they were analyzed using Student’s t test or one-way analysis of variance followed by post hoc tests (GraphPad Prism 7, La Jolla, CA). Data are shown as the mean ± S.E. Statistical significance was determined with *, p <0.05; **, p<0.01.
Chapter III: PACAP27 is Neuroprotective Against HIV-1 Tat-Induced Mitochondrial Impairments

A. Introduction

Human immunodeficiency virus type-1 (HIV) causes HIV associated neurocognitive disorders (HAND) in nearly one third of individuals (Heaton et al., 2010). Postmortem brains from subjects with the most severe form of HAND, HIV-associated dementia (HAD), exhibit neuronal loss accompanied by synaptic simplification, dendritic pruning, loss of spines, degradation of synaptic proteins (Crews, Patrick, Achim, Everall, & Masliah, 2009a), and neuronal apoptosis (Garden et al., 2002; H. J. James et al., 1999). These neurotoxic properties of HIV have been attributed to the combined effect of host cell-derived factors, including cytokines and glutamate, and other neurotoxins produced by activated microglia/macrophages (M Kaul, Garden, & Lipton, 2001). Moreover, different viral proteins have been shown to directly cause this type of neuronal degeneration including transactivator of transcription (Tat) a 101 amino acid protein that regulates transcription from the HIV promoter (Bruce-Keller et al., 2003; P. Chen, Mayne, Power, & Nath, 1997; N J Haughey et al., 2001). In infected individuals, Tat is actively secreted from infected astrocytes, microglia, and macrophages and can be rapidly internalized by a variety of cell types, including neurons (Y. Liu et al., 2000). This internalization has been reported to promote trimming of neurites, mitochondrial dysfunction, and cell death in neurons (Pocernich, Sultana, Mohmmad-Abdul, Nath, & Butterfield, 2005), all of which correlate with the neurological and cognitive decline more highly than cell death or viral load (Ances & Ellis, 2007). At present, there are no therapies that target Tat.

Tat-induced synaptic loss has been observed to differ both temporally and mechanistically from neuronal cell death (H. J. Kim et al., 2008); thus, synapse loss is not
necessarily a step on the path to apoptosis. Additionally, Tat-induced synaptic loss has been observed to be reversible in vitro (H. J. Kim et al., 2008), suggesting the amelioration of Tat-induced toxicity may be a target for adjunct therapies and the reduction of cognitive deficits. Significant neurological improvement accompanies initiation of HAART in patients with HAD (Bellizzi, Lu, & Gelbard, 2006), consistent with the idea that cognitive impairment is due at least in part to reversible actions of the virus. Determining the specific mechanisms leading to neuronal dysfunction will enable the identification of an effective mechanism for prevention of the neurocognitive decline observed in most cases of HIV. Thus, a protective agent acting upstream of the neurotoxic Tat pathway, before synaptic loss and cell death events are activated, is necessary in order to improve both neuronal survival and connectivity. Previous explorations of compounds to protect against Tat toxicity have failed as viable options in part due to their widespread antagonistic properties (Pocernich et al., 2005). Thus, a new compound lacking broad adverse effects is necessary for adjunct therapeutic potential.

Pituitary adenylate cyclase-activating peptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily. PACAP is an endogenous peptide synthesized by all tissues in the body including the CNS and is expressed in two bioactive isoforms, the pituitary adenylate cyclase-activating polypeptides PACAP27 and PACAP38, differing only in amino acid length (David Vaudry et al., 2009). PACAPs signal through two G protein-coupled receptor subtypes, the low-affinity VPAC receptors (VPAC1 and VPAC2), which they share with VIP, and the high-affinity PACAP-specific receptor, PAC1R (May et al., 2010). Binding of the peptide to PAC1R initiates signaling through adenylyl cyclase and cAMP as well as, to a lesser extent, phosphatidylinositol 4,5-bisphosphate. Additionally, PACAP is able to cross the plasma membrane in a receptor independent manner, initiating signaling in this fashion (Doan et al., 2012). Through these signaling cascades, PACAP supports a number of neuroprotective roles, protecting against excitotoxicity, stabilizing
mitochondrial membrane potential, and reducing reactive oxygen species (ROS) production (David Vaudry et al., 2009) and even preventing cortical neuron death through anti-inflammatory properties (Sanchez, Rao, & Grammas, 2009). In the present study we had two aims: 1) to establish a time course and gain preliminary data on the mechanism of Tat toxicity and 2) to investigate the protective effects of PACAP on Tat-induced neurotoxicity. Because Tat is known to lead to mitochondrial distress in other model systems, we used two mitochondrial measures to assess early markers of toxicity. Additionally, because oxidative stress commonly results from Tat exposure, we wanted to see if there were any downstream effects upon DNA. We demonstrated that the first signs of Tat toxicity occur within hours of exposure, preceding cell death by over 24 h and that PACAP27 is able to mitigate the toxic effects of Tat that are believed to contribute to neuronal loss.

B. Results

a. PACAP27 Prevents Tat-induced Mitochondrial Destabilization

The neurotoxic effect of Tat and the potential neuroprotection of PACAP were first analyzed by the MTT colorimetric assay in rat cortical neurons. As shown in Figure III.1, Tat (100 nM) significantly decreased MTT when compared to control (medium containing heat-inactivated Tat in 0.1 % BSA), suggesting that Tat promotes mitochondrial damage. The neurotoxic effect of Tat was not seen using a lower concentration (10 nM), whereas a higher concentration (1 μM) elicited a quantitative effect similar to that obtained with 100 nM concentration (data not shown). Both PACAP38 (100 nM) and PACAP27 (100 nM) alone did not significantly change MTT (Figure III.1). However, PACAP27 pretreatment was able to prevent Tat toxicity (Figure III.1). PACAP38 was significantly less potent than PACAP27 (Figure III.1).
Therefore, for the continuation of this study, we used PACAP27. Mitochondrial damage leads to the increased production and release of reactive oxygen species (ROS) into the cytoplasm and extracellular space. To further assess the toxic effects of Tat and the neuroprotective effect of PACAP27, we quantified ROS accumulation in neuronal cultures. Congruent with the results observed in the mitochondrial viability assay, ROS levels were significantly increased in neurons following a 15-min exposure to Tat (Figure III.2). PACAP27 pretreatment attenuated this increase in ROS accumulation (Figure III.2), confirming the ability of this neuropeptide to prevent mitochondrial destabilization.

Figure III.1 Tat-induced mitochondrial impairment is attenuated by PACAP27. Neurons were exposed to control medium (heat-inactivated Tat in 0.1 % BSA) or medium containing Tat (100 nM), PACAP27 (100 nM) or PACAP38 (100 nM) alone or in combination. Cell viability was determined 24 h later by MTT assay. Data, expressed as arbitrary units (A.U.) are the mean±SEM of three independent experiments (n=24). *p<0.01 vs. control; #p<0.05 vs. control
Figure III.2 Tat-induced ROS release is attenuated by PACAP27. Cortical neurons were exposed to control medium, Tat (100 nM) or PACAP27 (100 nM) alone or in combination for 15 min. ROS production was determined by H2DCF-dA fluorescence as described in “Materials and Methods” section. Data, expressed as percent of control, are the mean± SEM from three independent experiments (n=2 each experiment). #p<0.05 vs. control

b. Tat, PACAP27, and DNA Damage

The overproduction of free radicals can induce oxidation of DNA bases, and consequently, DNA damage including DNA double-strand breaks (DSBs), the most severe type of DNA damage. If not repaired properly, DSB damage can lead to long-term neuronal injury. DSBs can be quantified using single-cell gel electrophoresis (Neutral Comet Assay). Neurons exposed to Tat for varying time points exhibited a greater number of DNA fragments migrating out of the nuclei to form the “comet tail” than control (heat-inactivated Tat in 0.1%BSA) neurons, indicating a significantly greater number of DNA DSBs (Figure III.3). This effect was seen as early as 15 min after Tat exposure. PACAP27 prevented Tat-induced DNA DSBs. In fact,
cultures exposed to PACAP27 15 min prior to Tat exhibited significantly fewer DNA DSBs than Tat alone (Figure III.3), suggesting that PACAP27 may either prevent DNA DSB damage or facilitate the DSB repair process. Astrocytes are more resistant than neurons to Tat-induced toxicity (E. a Eugenin et al., 2007; Pocernich et al., 2005). To determine whether DNA DSBs underlie Tat neurotoxicity, we assessed DNA DSBs in cultured astrocytes following exposure to Tat. This viral protein did not cause a significant change in DNA DSBs in astrocytes (Figure III.4), indicating that this mechanism of Tat-induced damage may be neuron specific.

**Figure III.3** PACAP27 pretreatment protects against Tat-induced DNA DSBs in neurons. (A) Representative images of neurons exposed to control medium (heat-inactivated Tat in 0.1 % BSA), Tat (100 nM), PACAP27 (100 nM) alone or in combination for 15min showing tail moment. Insets are enlargements to show “tails.” (B) Quantification of tail moment representing DNA DSBs. Data, expressed as arbitrary units, are the mean±SEM of 100 cells/treatment randomly selected from four fields. The experiment was repeated four times. *p<0.01 vs. control
Figure III.4 Tat does not induce DNA DSBs in astrocytes. Cortical astrocytes were prepared as described in “Materials and Methods” section. (A) Representative images of astrocytes exposed to Tat (100 nM), PACAP27 (100 nM) alone or in combination for 15 min showing tail moment. Tail moment was quantified as described in “Materials and Methods” section. Insets are enlargements to show “tails.” (B) Quantification of tail moment representing DNA DSBs. Data, expressed as arbitrary units, are the mean±SEM of 100 cells/treatment from four fields (from two independent experiments).

c. PACAP27 Inhibits Tat-Induced Neuronal Cell Death

Mitochondrial health and DNA damage are acceptable proxies for neuronal health and viability; nevertheless, our data so far has failed to demonstrate the causal relationship between mitochondrial alteration and neuronal death. Thus, we assessed cell death at time points beyond the observed mitochondrial impairment and DNA DSB accumulation by using
Hoechst/PI. Neurons were exposed to Tat for several time points up to 72 h. Control cells were exposed to heat inactivated Tat in 0.1 % BSA for the same time points. While cell death was not significantly different from control at 24 and 48 h after Tat exposure, there was a significant increase in cell death 72 h after exposure (Figure III.5). Cultures exposed to PACAP27 before Tat had a similar proportion of cell death, as compared to those treated with PACAP27 alone and untreated controls (Figure III.5), indicating PACAP27 is effective in protecting neurons from Tat-induced death.
Figure III.5 Tat-induced cell death is prevented by PACAP27. Neurons were exposed to control medium or Tat (100 nM) for the indicated time points. PACAP27 (100 nM) was added 15 min prior to Tat. Quantification of cell death was done using Hoechst and PI, as described in “Materials and Methods” section. Data are the mean±SEM from a total of 500 neurons from 12 randomly selected fields/treatment from two independent experiments. (A) 24h; (B) 48h; (C) 72h. *p<0.01 vs. control
d. Potential Mechanisms of Neuroprotection

PACAP has been shown to activate a number of neuroprotective pathways (G. Horvath et al., 2010). Relevant for Tat toxicity is the fact that PACAP38 and related neuropeptide VIP can also induce the release of CCL5 from astrocytes (Brenneman, Hauser, Spong, & Phillips, 2002). Released CCL5, in turn, is neuroprotective against neurotoxins (Sanchez et al., 2009) including the HIV viral protein gp120 (Valeriya Avdoshina, Biggio, Palchik, Campbell, & Mocchetti, 2010; Brenneman et al., 1988). Therefore, we tested whether PACAP27 releases CCL5 in our neuronal cultures. We observed that PACAP27 promotes the release of CCL5 from our cultures that contain ~10 % of astrocytes. However, the effect of PACAP on CCL5 release was significantly more robust in primary cultures of astrocytes (Figure III.6). Thus, we confirm previous data that PACAP enhances the release of CCL5 mainly from astrocytes (Brenneman et al., 2002). To establish whether CCL5 prevents Tat toxicity in our experimental system, cultures were then exposed to CCL5 (20 nM) 15 min prior to Tat. MTT assay revealed that CCL5 inhibits the mitochondrial impairment induced by Tat (Figure III.6). Thus, it appears that CCL5 prevents the toxic effect of not only gp120 but also Tat.
**Figure III.6** PACAP27 induces the release of CCL5 from astrocytes. Rat primary astrocytes were exposed to control medium, or medium containing PACAP27 (100 nM) for 15 min and 24 h. The medium was collected and an aliquot was used to determine CCL5 levels by ELISA. CCL5 levels in control cells at 24 h were 104±5 picograms per milliliter. Data are the mean±SEM of three independent samples at each time point. *p<0.05 vs. control

**C. Discussion**

The ability of Tat to induce neuronal damage and dysfunction in vitro and in vivo has been established (W. Li, Li, Steiner, & Nath, 2009). Several mechanisms have been suggested to underlie the neurotoxic effect of Tat. These include activation of NMDA receptors (E. a Eugenin et al., 2007; N J Haughey et al., 2001; W. Li et al., 2008b), impairment of mitochondria physiology (D. Chen, Wang, Zhou, & Zhou, 2002; Norman et al., 2007), and production of ROS (Kruman et al., 1998) which ultimately may result in apoptosis. Our study showed that Tat, in addition to the destabilization of mitochondria and production of ROS previously described (Hui
et al., 2012), promotes accumulation of DNA DSBs, which can be lethal to cells. These events start as early as 15 min after Tat exposure and they occur at least a couple of days before neuronal cell death. Additionally, Tat caused a significant increase in the number of DNA DSBs in neurons, but not astrocytes, which are “resistant” to the toxic effect of Tat. Importantly, all of these neurotoxic effects of Tat were lessened by a 15-min pretreatment with PACAP27.

### a. The Role of DNA Damage

In this study, we observed a significant increase in ROS production in neurons exposed to Tat concomitantly to DNA DSBs. In normal and pathological metabolism, most ROS are formed as toxic by-products of oxidative phosphorylation within mitochondria (Turrens, 2003; Wallace, 2000) during synthesis of ATP. Overproduction of ROS and other forms of oxidative stress is linked to a range of neurodegenerative disorders (Andersen, 2004). ROS can interact with a variety of cellular macromolecules, resulting in oxidative DNA damage, among other ill effects. As postmitotic cells, neurons are particularly vulnerable to DNA DSBs. However, DNA damage even within neurons does not immediately induce apoptosis. The accumulation of DNA damage that is not repaired or is incorrectly repaired will lead to errors in protein translation, resulting in misfolded proteins and eventual cellular dysfunction and cell death (Brasnjevic, Hof, Steinbusch, & Schmitz, 2008; Jeppesen, Bohr, & Stevnsner, 2011). The precise load of DNA damage any neuron can withstand remains unclear. Nevertheless, DNA damage has increasingly been observed in neurodegenerative diseases (Fishel, Vasko, & Kelley, 2007) including HAND, where a significant accumulation of DNA damage has been observed in the post-mortem brain tissue of HIV patients with cognitive deficits (Y. Zhang et al., 2012). Indeed, patients with HAND had an average of 45% 8-oxoG positive cells (the most common form of DNA DSBs caused by oxidative stress), compared to only 30% in HIV patients without HAND.
and 4% in controls (Y. Zhang et al., 2012). These results suggest that nuclear DNA damage exists at least in part due to the high levels of ROS, likely contributing to neuronal injury and cell death. In addition, Tat is known to decrease the expression of DNA-PKs, a critical component of the non-homologous end joining pathway (NHEJ) of DNA DSBs (S. Zhang et al., 2014), which would likely hamper the repair of these breaks.

The present study is the first implicating Tat in the development of DNA damage in neurons, preceding cell death. DNA damage, both nuclear and mitochondrial, is associated with accelerated aging (Schumacher, Garinis, & Hoeijmakers, 2007). Importantly, accelerated brain aging is a common observation and a topic of much current research in the HIV positive population, even among those undergoing consistent and long-term adherence to HAART. Abundant DNA damage was found in the brains of HIVE patients while less was found in infected and uninfected controls (Karbowski et al., 2002; Wiley et al., 2000). Just as we have found in our investigation, this damage was not closely associated with cells undergoing apoptosis, indicating that the damage was an event separate from and preceding this process. Additionally, DNA repair enzymes KU80 and poly ADP-ribose polymerase (PARP) were more abundant in neurons and glia of HIVE patients compared to infected and uninfected controls (Wiley et al., 2000). This abundance of DNA damage is likely linked to the accelerated brain aging and cognitive impairments observed in the HIV population. In fact, several recent investigations found HIV positive individuals to have accelerated brain aging (Cole et al., 2017; S. Horvath & Levine, 2015). This aging was also found to correlate highly with a decrease in cognitive function (Cole et al., 2017). The increasing lifespan of HIV infected individuals is only going to further implicate the effects of accelerated aging upon the cognitive impairments observed in HAND.

While the tool we utilized to assess DNA damage measured only breaks of nuclear DNA, mitochondrial DNA (mtDNA) is 10 to 100 times more sensitive than nuclear DNA to oxidative
stress (Yakes & Van Houten, 1997), making it very likely that the integrity of mtDNA is also significantly hampered following Tat exposure. The most commonly referenced mechanistic pathway of mitochondrial toxicity in neurodegeneration is the accumulation of oxidative stress, further emphasizing likely impairments of mtDNA following Tat exposure. Mitochondrial haplogroup has been found to influence the progression of HIV (Guzmán-fulgencio et al., 2013), likely due to differences in ATP production efficiency, though the vulnerability of mtDNA or the proteins it encodes are not excluded. Another found a strong association between mtDNA 8-oxo-dG and decreased brain volume in several regions of the brains of HIV positive patients, including regions surrounding the hippocampus, pallidum, and subcortical gray matter (Kallianpur et al., 2016). mtDNA impairments are likely to precede cognitive impairments, as Miro et al. (2004) found significant depletion of mtDNA in peripheral blood mononuclear cells (PBMCs) in asymptomatic HAART-naive HIV-infected patients.

While in our model system, inflammation and resultant oxidative stress triggered by activated microglia is eliminated, when looking at the HIV infected brain as a whole, these factors cannot be forgotten. It can be expected that inflammation of this sort will only exacerbate the DNA damage we have observed in an isolated cell culture system exposed to only Tat, further emphasizing the significance of these findings.

b. PACAP as an Adjunct Therapy

As detailed here, we have found PACAP27 to be neuroprotective against Tat-induced impairments (Figures III.1-III.3). PACAPs have been previously observed to provide neurotrophin-like protection to different populations of neurons, including against ischemia, trauma or exogenous toxic substances such as 6-hydroxy-dopamine (6-OHDA), MPTP and rotenone both in vivo and in neuronal cultures (Offen et al., 2000; Reglodi et al., 2001; Reglodi, 2002).
Tamas, Lubics, Szalontay, & Lengvari, 2004; Tamas et al., 2012; Tsuchikawa & Nakamachi, 2012). Most of these injuries result in the production of ROS; though it is not clear how PACAPs regulate this oxidative stress. At least some of the protective effects of PACAP are due to its anti-apoptotic effects, shown through a reduction of caspase 3, as well as up-regulation of BDNF and enhancement of its signal transduction mediated via phosphorylation of cyclic AMP response element-binding protein (CREB) (Frechilla, Garcia-Osta, Palacios, Cenarruzabeitia, & Del Rio, 2001; Lazarovici et al., 2012; Racz et al., 2006; Vale et al., 2011; D Vaudry et al., 2000). Some reports have raised the possibility of PACAPs' involvement in the production of antioxidants (Fabian, Reglodi, Mester, & Szabo, 2012). Also, particularly of interest to this work, PACAP has been found to increase the activity of the essential base-excision repair enzyme apurinic/apyrimidinic endonuclease 1 (APE1)/redox effector factor-1 in following injection into the hippocampus of ischemic mice (Stetler et al., 2010).

Our findings demonstrate for the first time that PACAP27 prevents Tat-induced neurotoxicity. The neuroprotective activity of PACAP27 on viral proteins is not surprising because previous studies have shown that both PACAP27 and PACAP38 attenuate neuronal death induced by the HIV envelope protein gp120 (Brenneman et al., 2002). Surprisingly, PACAP38 was unable to counteract the toxic effect of Tat. This appears to be a contradictory result because both PACAPs bind to same receptors and share a similar pharmacological profile. On the other hand, previous studies have shown that PACAP38 and PACAP27 have an opposite profile on the secretion of luteinizing hormone, most likely through a vasoactive intestinal peptide receptor, VPAC1 (Kantor, Molnar, Arimura, & Koves, 2000). Thus, different receptors may mediate the neuroprotective effect against Tat. As mentioned previously, PACAP signals through three receptors: PAC1 as well as VPAC1 and VPAC2.

On the other hand, PACAPs can also be neuroprotective by the activation of anti-apoptotic chemokines such as CCL5. CCL5 exerts neuroprotective activity against other viral
proteins such as gp120 (Valeriya Avdoshina et al., 2010; Campbell, Avdoshina, Day, Lim, & Mocchetti, 2015; M Kaul et al., 2007). Indeed, both PACAP38 (Brenneman et al., 2002) and PACAP27 promote the release of CCL5 from astrocytes. Intriguingly, Brenneman et al. (2002) have shown that the ability of PACAP38 to induce the release of CCL5 from astrocytes is biphasic and concentration-dependent, with the maximal activity on the release of CCL5 in the low picomolar range. Thus, we may have used a concentration of PACAP38 that does not release sufficient amount of CCL5 to prevent Tat toxicity. Further studies are needed to confirm this hypothesis. Hence, while we cannot point at a specific mechanism of PACAP27 neuroprotection we cannot exclude the hypothesis that PACAP27 may be neuroprotective against Tat because of its antioxidant property combined with its ability to release CCL5. Future studies, using CCL5 knock-out animals or a CCL5 blocking antibody, will prove or disprove this hypothesis.

Whereas CCL5 is one mechanism by which PACAP27 can be neuroprotective, other mechanisms may also be implicated including the activation of cAMP-protein kinase A (PKA) pathway which then activates cAMP-response element binding protein-mediated gene expression (Baxter, Martel, McMahon, Kind, & Hardingham, 2011). PACAPs have also been previously observed to provide neurotrophin-like protection to different populations of neurons (G. Wang, Qi, Fan, Zhou, & Chen, 2005). For instance, PACAP exhibits properties similar to nerve growth factor (NGF) in peripheral neurons (Lioudyno, Skoglosa, Takei, & Lindholm, 1998), and both PACAP forms activate TrkA and TrkB, the tyrosine kinase receptors for NGF and brain-derived neurotrophic factor (BDNF), respectively (F. S. Lee, Rajagopal, Kim, Chang, & Chao, 2002). TrkA is mostly localized in the basal forebrain; thus, TrkA most likely does not account for the neuroprotective effect of PACAP against Tat. Nevertheless, activation of TrkB could participate in fast-acting protection that we have observed in vitro. Recent studies of the CSF of HIV-infected patients demonstrate a strong correlation with increasing severity of HAND
and a decline in growth factors, particularly BDNF and NT-3 (Bachis et al., 2012; Meeker, Bragg, Poulton, & Hudson, 2012). Indeed, BDNF is particularly potent as neuroprotective compound against Tat (Ramirez et al., 2001) and gp120 (Bachis, Major, & Mocchetti, 2003). This would be in line with a recent study (S. Nath, Bachani, Harshavardhana, & Steiner, 2012) showing that flavonoids, alkaloids present in many plants, prevent Tat-mediated mitochondrial dysfunction and neuritic damage by up-regulating the expression of BDNF. Thus, the ability of PACAP to activate TrkB signaling pathways could provide an additional neurotrophic effect against Tat. BDNF has long been considered as an adjunct therapy for HAND, but has run into issues because it needs to be directly administered to the brain as a recombinant protein or via viral vectors (Kells, Henry, & Connor, 2008). Our results do not exclude that CCL5 works in concert with BDNF or other trophic factors. While the precise mechanism by which PACAP27 protects against Tat-induced neurotoxicity remains elusive, we have shown here that it is neuroprotective against Tat induced DNA damage and mitochondrial dysfunction. The activation of neuroprotective pathways, such as cAMP-PKA pathway (Shibuya et al., 1998; Tanaka et al., 1997), induction of transcriptional expression of BDNF (Frechilla et al., 2001), and reduction of proapoptotic factor Bax (Falluel-Morel et al., 2004), may all be contributing to protection against the toxic effects of Tat. In summary, our findings implicate PACAP27 as a potent neuroprotective peptide against Tat; however, more experiments are needed to further examine mechanisms underlying its neuroprotective effect. Such mechanisms may yield novel targets for preventing Tat-mediated neuronal injury and delay HAND.

While PACAP is neuroprotective in numerous capacities, PACAPs do not come without issues of their own. The physiochemical and pharmacokinetic properties of PACAP make it difficult to work with. PACAPs released in the blood circulation exhibit poor metabolic stability, with a half-life of 2 to 10 minutes in mice or humans (M. Li, Maderdrut, Lertora, & Batuman, 2007; L. Zhu et al., 2003). To combat this, metabolically stable PACAP analogs or derivatives
may represent promising drug candidates. A metabolically stable PACAP derivative, acetyl-[Ala15, Ala20]PACAP38-propylamide, which behaves as a super-agonist of the PAC1 receptor, is being developed (Bourgault et al., 2009).

Once administered, PACAPs are easily removed from the brain through an efflux component of peptide transport system-6 (PTS-6), which decreases brain uptake by approximately 10-fold (Banks, Kastin, Komaki, & Arimura, 1993). Inhibition of the efflux pumps would increase retention of the endogenous PACAP produced in the brain as well as increase brain retention of the circulating endogenous and peripherally administered PACAP that crosses the blood brain barrier. Dogrukol-Ak and colleagues (2009) were able to drastically increase PACAP27 uptake by the brain using enzymatically resistant antisense inhibition that selectively targeted the β-1 ATPase (the component of the PTS-6 efflux pump responsible for eliminating PACAP27 from the brain). Following peripheral administration of PACAP27, not only were PACAP27 levels within the brain increased by fourfold, but improved learning was also observed in a mouse model of AD and reduced infarct size was observed after middle cerebral artery occlusion in mice. Additionally, several pre-clinical evaluations of the intranasal administration of PACAP have provided promising results towards the application of PACAP or similar analogs in the treatment of neurodegenerative diseases such as Alzheimer’s disease (Nonaka et al., 2012) and neuromuscular diseases like spinobulbar muscular dystrophy (Polanco et al., 2016).

Perhaps the greatest obstacle to overcome in the development of PACAPs or metabolically stable analogs as therapies is the involvement of PACAP and its receptors in migraines. In a double-blind placebo controlled trial, the majority of both healthy subjects and migraine patients experienced mild to moderate headache following PACAP38 injections. Moreover, 50% of the migraine patients reported migraine-like attacks within several hours of the PACAP38 infusion. PACAP is known to induce vasodilation which is likely a factor in its
induction of headaches and migraines (Schytz, Olesen, & Ashina, 2010). In the majority of instances, migraine medication was capable of reducing symptoms. Despite these hurdles, further investigation in PACAP or metabolically stable analogs provide an intriguing possibility as an adjunct therapy for combatting the neurotoxic effects of Tat and other HIV proteins.

c. Tat and Mitochondrial Dysfunction

As shown here and by others, Tat induces ROS production, rapid loss of mitochondrial membrane potential, and increases mitochondrial uptake of intracellular Ca\(^{2+}\) (Norman et al., 2008). Tat injections into the frontal cortex of young adult mice lead to irregularly shaped mitochondria (Norman et al., 2008). This aberrant morphology mirrors the mitochondrial irregularities observed in the cortex of patients with HIV encephalitis (Zhang et al., 2012), indicating the relevance of mitochondrial impairment to disease progression. Mitochondria are vital for cell function, wherein they supply up to 95% of the required ATP and regulate intracellular Ca\(^{2+}\) homeostasis. In neurons, mitochondria must travel extreme distances (e.g., axons) and maintain energy homeostasis in these highly metabolically active cells. In fact, synaptic activity and mitochondrial motility are highly positively correlated processes (Sun, Qiao, Pan, Chen, & Sheng, 2013). Neuronal mitochondria are distributed to regions of high metabolic demand, including synapses, nodes of Ranvier, and myelination/demyelination interfaces. For these reasons, mitochondrial health is intimately tied with the functional status of neurons.

Recently, impairments to mitochondrial dynamics have been implicated in a causal role of neurodegenerative diseases including Parkinson’s, Alzheimer’s, and Huntington’s diseases (Eckmann et al., 2013; Itoh et al., 2013). The prevalence of neuronal diseases associated with general mitochondrial impairment underscores the important functional relationship between neurons and mitochondria. Thus taken with the knowledge that the early time points at which
these impairments occur in relation to cell death (providing a therapeutic window), in the second part of this dissertation, we continue our investigations further into understanding the mechanism behind Tat-induced mitochondrial impairment.
Chapter IV: Human Immunodeficiency Virus Tat Impairs Mitochondrial Fission in Neurons

A. Introduction

Human immunodeficiency virus type-1 (HIV) causes HIV-associated neurocognitive disorders (HAND) in nearly one third of individuals (Saylor et al., 2016). Synaptic simplification, dendritic pruning, loss of spines, degradation of synaptic proteins (Crews, Patrick, Achim, Everall, & Masliah, 2009b) and neuronal apoptosis (Garden et al., 2002; H. J. James et al., 1999) are all hallmark features of the most severe form of HAND. The neurotoxic properties of HIV have been attributed to the combined effect of host cell-derived factors, including cytokines and glutamate, and other neurotoxins produced by activated microglia/macrophages (M Kaul et al., 2001). Moreover, different viral proteins have been shown to directly cause this type of neuronal degeneration, including Tat (W. Li et al., 2009), a 101 amino acid protein that regulates transcription from the HIV promoter (Debaissieux, Rayne, Yezid, & Beaumelle, 2012). Tat is actively secreted from infected astrocytes, microglia, and macrophages and can be rapidly internalized by a variety of cell types, including neurons (Y. Liu et al., 2000). This internalization has been reported to promote trimming of neurites, mitochondrial dysfunction, and cell death in neurons (Pocernich et al., 2005).

As laid out in Chapter III, Tat exposure to rat primary neurons leads to rapid release of ROS and an increase in MTT, suggesting impaired mitochondrial activity (Figures III.1 and III.2). Both ROS and MTT are reliable and reproducible markers of mitochondrial activity and viability. These mitochondrial impairments precede Tat-induced neuronal cell death (Figure III.4). In addition, previous findings have indicated loss of mitochondrial membrane potential (Lecoeur et al., 2012) and increased mitochondrial uptake of intracellular Ca^{2+} (Kruman et al., 1998). Furthermore, Tat leads to morphologic and functional changes in mitochondria (Norman et al.,
This aberrant morphology mirrors the mitochondrial irregularities observed in the cortex of patients with HIV encephalitis (Valeria Avdoshina et al., 2016; J. A. Fields et al., 2016). Efficient mitochondrial function is essential for the health of highly energetic and polarized neurons. Inefficient function leading to the overproduction of cellular waste products and underproduction of ATP not only impairs neuronal function, but can contribute to neuronal cell death (Mattson, Gleichmann, & Cheng, 2009). These considerations underscore the important functional relationship between HIV, neurons, and mitochondria. However, to date, few investigations have detailed the mechanisms behind these impairments.

Regulation of mitochondrial health is tightly controlled by the dynamic processes of fusion and fission, which in turn, directly affect the size of the organelles and their abilities to be trafficked throughout sub-compartments of the neuron (Cagalinec et al., 2013; Sheng & Cai, 2012). Aberrations to any of these processes can contribute to organelle inefficiencies, impair cellular functions, and lead to cell death (Burté et al., 2014). Mitochondrial dynamics are processes mediated by large GTPases, including, in mammals, Drp1 for fission and Mfn1 and 2, and OPA1 for fusion (Bliek, Shen, & Kawajiri, 2017; Y. Lee et al., 2004). Drp1 is found throughout the neuronal cytoplasm in its inactive form. Upon post-translational modification, Drp1 translocates to the mitochondrial membrane where it oligomerizes, forms a band around the mitochondria, and promotes fission of the organelle (Pernas & Scorrrano, 2016). Acting in an opposite fashion are Mfn1 and Mfn2, which interact with the outer mitochondrial membrane of two adjacent organelles to induce mitochondrial fission, and OPA1, which is bound to and fuses the inner mitochondrial membrane (Kasahara & Scorrano, 2014). Mitochondria that accumulate defects in proteins and mitochondrial DNA (mtDNA) must be either repaired by fusion with healthy mitochondria or cleared from the cell by selective autophagy (Twig et al., 2008). In the cases of extreme damage and disrepair, damaged mitochondria might be transported back to the cell body to be replenished or degraded. Thus, in order to keep energy homeostasis and
maintain essential activities, neurons must precisely establish an adequate distribution of mitochondria and also efficiently sustain them in the periphery and clear them away when necessary.

Here, we have sought to determine how Tat impairs mitochondrial dynamics in neurons, contributing to cell death. We show that Tat impairs mitochondrial membrane potential shortly after exposure and subsequently leads to alterations in mitochondrial size and subcellular localization. This decrease in mitochondria size is due to an increase in active mitochondrial fission protein, Drp1, following an increase in the Ca$^{2+}$-dependent phosphatase activity of calcineurin.

B. Results

a. Mitochondria are Fragmented in Brains of Tat Mice.

Impaired mitochondrial metabolism (Bennett et al., 2015) and damaged mitochondria cristae (Valeria Avdoshina et al., 2016; J. A. Fields et al., 2016), have been observed in HIV positive subjects. These effects could be due to the toxicity of combined antiretroviral therapy (Opip et al., 2007) or soluble viral proteins, such as Tat. Mice overexpressing Tat in the brain develop neurodegeneration similar to the post mortem brains of HIV+ individuals that were diagnosed with HAND. Therefore, we used these mice to establish whether Tat alters the morphology of mitochondria similar to the alterations observed in brains from HAND decedents. Figure IV.1 shows that the brains of Tat animals exhibit significantly smaller mitochondria when compared to wild type littermates. In wild type mice, neuronal mitochondrial diameter averaged approximately 1.2 mm, while in Tat animals; neuronal mitochondrial diameter averaged approximately 0.8 mm (Figure IV.1).
Figure IV.1 Mitochondrial diameter is decreased and morphology is altered in neurons in GFAP-Tat tg mouse brains. Vibratome sections of wild type and GFAP-Tat tg mouse brains were analyzed for mitochondrial morphology by transmission electron microscopy. (A) Ultrastructure images by TEM of neuronal mitochondria from wild type and GFAP-Tat tg mouse brains. (B) Average quantification of diameter of neuronal mitochondria in Non tg and GFAP-Tat tg mouse brains. (*p<0.05 by student t-test; n= 16, 8 Non tg and 8 GFAP-gp120 tg; scale bar= 500nm)

b. Tat Impairs Mitochondrial Membrane Potential.

To examine whether Tat changes the mitochondrial membrane potential we determined the accumulation of TMRE, a permeant dye that is selectively taken up by mitochondria in relation to their membrane potential. A decrease of mitochondrial potential results in a lower TMRE uptake. We exposed cortical neurons to Tat for various time-points and determined
TMRE fluorescence. FCCP, an uncoupler of mitochondrial oxidative phosphorylation, was used as a positive control for the assay.

By 30 minutes, Tat-treated neurons exhibited a decreased fluorescent intensity compared to controls neurons (heat inactivated Tat 0.1% BSA) imaged under the same conditions (Figure IV.2). The decrease in fluorescence intensity was significant by 1 hr and up to 4 hr (Figure IV.2).

To better understand what molecular changes were happening at these early time points, we performed calcium imaging on cultured neurons exposed to Tat, NMDA, and MK801, alone or in combination. A rapid and robust rise in intracellular calcium was also observed following both Tat and NMDA (Figure IV.3). When paired with NMDA receptor antagonist, MK801, only the NMDA initiated calcium rise was blocked, indicating that the rise in calcium occurs through channels other than those associated with the NMDA receptor (Figure IV.3C and D).
Figure IV.2 Tat elicits a time-dependent decrease in mitochondrial membrane potential.

Cortical neurons (DIV 7) were exposed to boiled Tat (control), Tat (100nM) for the indicated times or to FCCP (0.5 µM) for 30 min. Cells were then loaded with mitochondrial membrane permeant and potential dependent dye, TMRE (5 nM) for 30 minutes in media. Cells were then rinsed in warmed (37°C) HBSS, placed on glass slides, and immediately imaged. The fluorescent intensity was quantified using ImageJ. Data are expressed as mean ±SEM (n=20 coverslips each time point). *p<0.001, **p<.0001 vs control (ANOVA and Tukey test)
Figure IV.3 Tat rapidly increases intracellular Ca\textsuperscript{2+} in a NMDA receptor independent manner. Cultured rat cortical neurons (DIV7) prepared as described in the Materials and Methods were exposed for up to 200 s to recombinant Tat (100 nM) or NMDA (100 μM) alone or in combination with the NMDA receptor antagonist MK801(60 μM). (A), (B) Representative Fluo-4 fluorescence images of cortical neurons captured before (baseline) and after application of Tat or NMDA in the presence of absence of MK801. (C), (D) Mean Fluo-4 fluorescence of a and b, respectively (n > 20 cells). Note that addition of Tat resulted in a significant increase in intracellular Ca\textsuperscript{2+}, which was not prevented when the NMDA receptor antagonist MK801 was added concurrently. Neurons were responsive to NMDA, which caused a rapid and robust rise in intracellular Ca\textsuperscript{2+}, prevented by the concurrent administration of MK801 (B) and (D).
c. Tat Causes Neuronal Mitochondrial Fragmentation.

Significant fluctuations in mitochondrial membrane potential indicate destabilization of the organelle which could be indicative of morphologic changes. To first visualize changes to mitochondrial morphology, we analyzed neurons with stochastic optical reconstruction microscopy (STORM), which enables visualization of individual organelles within cultured neurons. Mitochondria in cortical neurons exposed to Tat for 4h (Figures IV.4C and D) had visibly shorter mitochondria than those exposed to control media (Figures IV.4A and B).

To quantify these alterations as well as to assess subcellular distribution of the organelles, we next performed confocal microscopy. Beginning 2 hr after Tat exposure, mitochondrial fragmentation was observed as measured by both mitochondrial area (Figure IV.5B) as well as mitochondria perimeter (Figure IV.5C). Changes in mitochondrial size were not accompanied by alterations to the total number of mitochondria (Figure IV.5D). To determine if at least some of the fragmentation we observed involved mitochondrial fission, we utilized mitochondrial division inhibitor 1 (mdivi-1), which is a selective Drp1 inhibitor (Cassidy-stone et al., 2008). Mdivi-1 inhibited Tat-mediated mitochondrial fragmentation at any time point (Figure IV.6B and C) and had no effect on total mitochondrial number (Figure IV.6D). This fragmentation precedes Tat-mediated neuronal loss by at least 24 hr (Figure III.4). Moreover, beginning at 4 hr, mitochondria began to show altered subcellular distribution, such that fewer mitochondria occupy the processes and more of the mitochondrial volume can be seen near the soma (Figure IV.7). This altered distribution is exacerbated at 48 and 72 hours (data not shown).
Figure IV.4 Tat causes mitochondrial fragmentation. Cortical neurons (DIV 7) were fixed with 4% paraformaldehyde + 4% sucrose (pH 7.4) following exposure to control media (A and B) or Tat (100nM) (C and D) for 4 hours. Enlargements of each treatment are shown (B and D) for better detail. Cells were then stained for MAP2 (red) and TOM20 (green) to label microtubules and mitochondria, respectively. Images were acquired by STORM.
**Figure IV.5** Tat decreases mitochondrial size without altering mitochondria number. Cortical neurons were exposed to boiled Tat (control) or Tat (100 nM) for the specified time points. A. Cells were then fixed and stained for MAP2 (green) and TOM20 (red) to label neuronal microtubules and mitochondria, respectively. Scale bar, 10μM. Quantification of mitochondrial area (B), perimeter (C), and number (D) was then done as described in Materials and Methods. Data are the mean ± SEM of 20 neurons, normalized to control. *p<0.05, **p<0.01 vs control (ANOVA and Tukey Test).
Figure IV.6 Mdivi-1 prevents Tat-induced mitochondrial fragmentation. Cortical neurons were exposed to boiled Tat (control), Tat (100nM), Mdivi-1 (10 μM), or Mdivi-1 + Tat for the specified time points. (A) Cells were then fixed and stained for MAP2 (green) and TOM20 (red) to label neuronal microtubules and mitochondria, respectively. Quantification of mitochondrial area (B), perimeter (C), and number (D) was then done as described in Materials and Methods. Data are the mean ± SEM of 20 neurons, normalized to control. *p<0.05, **p<0.01 vs control (ANOVA and Tukey Test).
Figure IV.7 Tat causes the altered subcellular localization of mitochondria. Cortical neurons were exposed to boiled Tat (control) or Tat (100nM) for the specified time points. In control cells, mitochondria can be visualized throughout the neuronal processes and within the soma. By 4h after Tat exposure, mitochondria have mostly vacated the processes and are predominantly within the soma. Importantly, we do not observe significant cell loss until 72h after Tat exposure in vitro, indicating this is not a phenomenon directly related to the process of apoptosis.

d. Tat Exposure Leads to an Increase in Levels of Drp1 Protein.

The increase we observed in fragmented mitochondria can be due to either an increase in mitochondrial fission, a decrease in mitochondrial fusion, or a combination of the two altered processes. Perturbations to either of the oppositional forces controlling mitochondrial morphology can be detrimental to the health of the organelle and neuron. Thus, we examined whether Tat changes the levels of the proteins regulating fusion and fission. First, we determined the levels of Drp1, the large GTPase regulating mitochondrial fission (Y. Lee et al., 2004). To assess Drp1 levels, Western blots were performed on lysates collected from
neuronal cultures exposed to Tat for 30 min to 4 h and probed with antibodies against Drp1. Drp1 protein levels were increased compared to controls beginning 2 hr after Tat exposure and persisted for up to 4h (Figures IV.8A and B).

Next, to determine if proteins regulating mitochondrial fusion were also affected, we probed Western Blots with antibodies against Mfn2. Unlike the changes we observed following Tat exposure in Drp1 protein levels, Mfn2 levels were not significantly different than control (Figure IV.8C and D). Taken together, these results indicate that the increased fragmentation of mitochondria is likely due to an increase in fission activity and not a decrease in fusion.
Figure IV.8 Tat promotes a time-dependent increase in total Drp1, but not Mfn2. Cortical neurons were exposed to Tat (100nM) for the indicated times and levels of Drp1 and Mfn2 were determined by Western blot in cell lysates. (A) Representative Western blot analysis of cortical neuronal lysates probed with a Drp1 antibody. (B) Semi quantification of the 82kDa band (Drp1) by densitometric analysis with beta-Actin (42kDa) as a loading control. (C) Representative Western blot analysis of cortical neuronal lysates probed with a Mfn2 antibody. (D) Semi quantification of the 86kDa band (Mfn2) by densitometric analysis with beta-Actin (42kDa) as a loading control. Data are the mean ± SEM of three experiments, normalized to control. *p<0.05 vs control (ANOVA and Tukey Test).
e. Tat Exposure Leads to an Increase in the Association of Drp1 to Mitochondrial Membranes.

While increased mitochondria fragmentation and corresponding increase in Drp1 levels may prove to be related, the active form of Drp1 must be assessed to better understand the relationship. Drp1 is a protein that can be found throughout the neuronal cytoplasm in its inactive form. In neurons, Drp1 is most commonly dephosphorylated at Ser637 to produce the active form of Drp1 (Cereghetti et al., 2008). Upon dephosphorylation, Drp1 is able to translocate to the mitochondrial membrane, where it oligomerizes and initiates mitochondrial fission. To first assess if there were changes in Drp1 translocation to the mitochondrial membrane, we performed 3D-STORM on neurons exposed to Tat for 4 hours. These neurons were fixed and stained for TOM20, MAP2, and Drp1. In neurons exposed to Tat, we observed a significant increase in the accumulation of Drp1 on and around mitochondria (Figure IV.9). To quantify changes in the phosphorylation state of Drp1 following Tat exposure, we then performed an immunoprecipitation of Drp1 from neuronal cell cultures exposed to Tat for 30min up to 4h. Immunoblots were probed for pSer. We found a decrease in pSer levels in the immunoprecipitate of samples exposed to Tat for 2 and 4h, indicating a decrease in levels of pDrp1 (Figure IV.10).

Drp1 can be phosphorylated at several sites to regulate its activity (Dickey & Strack, 2011; Karbowski et al., 2002). Phosphorylation at S616 by Ca$^{2+}$/Calmodulin-dependent protein kinase Iα (CamKIIα) increases Drp1 activity whereas phosphorylation at S637 or S656 by cyclic-AMP dependent kinases, decreases activity. To discern where changes in pDrp1 were taking place, we next performed immunocytochemistry on neurons following Tat exposure, labeling pDrp1 S616 or S637. Further supporting our observations of increased mitochondrial fragmentation and decreased levels of pDrp1 by immunoprecipitation, we observed a decrease
in pDrp1 S637 puncta following Tat exposure whereas pDrp1 S616 puncta remain unchanged (Figure IV.11). Taken altogether, these data indicate that the dephosphorylation of Drp1 at residue serine 637 may be contributing to the increased fragmentation of mitochondria following Tat exposure in neurons.

**Figure IV.9** Tat promotes Drp1 localization near mitochondria. Cortical neurons (DIV 7) were exposed to control media or Tat (100nM) for 4 hours after which time they were fixed and stained for MAP2 (red), TOM20 (green), and Drp1 (blue) to label microtubules, mitochondria, and Drp1, respectively. Images were acquired by 3D-STORM.
Figure IV.10 Tat decreases pDrp1 in cortical neurons in a time-dependent manner. Cortical neurons were exposed to Tat for the indicated times and then levels of pDrp1 were determined by Western blot in cell lysates. (A) Representative Western blot analysis of cortical neuronal lysates immunoprecipitated with anti-Drp1 antibody. The blot was analyzed with a pSer antibody. (B) Semi quantification of the 81kDa band by densitometric analysis. Data expressed as mean ± SEM represent the average of three experiments. *p<0.001, **p<0.0001 vs control (ANOVA and Tukey test).
Figure IV.11 Tat decreases pDrp1 S637 puncta but not pDrp1 S616 puncta in a time-dependent manner. Cortical neurons were exposed to Tat for the indicated times, fixed and stained to label pDrp1 S637 or S616 puncta. Images were then analyzed for puncta quantity. (A) Quantification of pDrp1 S637 puncta. (B) Quantification of pDrp1 S616 puncta. Data expressed as mean ± SEM normalized to control represent an average of three experiments (n = 10 neurons each experiment). *p<0.05, ***p<0.001 vs control (ANOVA and Tukey test).
f. Tat Exposure Increases Calcineurin Activity.

The GTPase activity of Drp1 is regulated by phosphorylation at S637 or S656 by Ca^{2+}-dependent phosphatase calcineurin (Cereghetti et al., 2008). The regulatory subunit of calcineurin, in turn, is activated by calmodulin (Guerini, 1997). Tat causes a fast and robust Ca^{2+} rise in neurons (Figure IV.3; Eugenin et al., 2007; Haughey et al., 2001; Kruman et al., 1998), subsequently activating calmodulin (McQueen et al., 2011). Thus, it is plausible that neuronal exposure to Tat can cause the activation of Drp1 via a calcineurin-mediated mechanism. To determine whether Tat augments calcineurin activity, neurons were exposed to Tat for various time points from 30 min to 4h and calcineurin cellular activity assay was performed. Tat exposure caused a rapid rise in calcineurin activity that was maintained for up to 4h (Figure IV.12) and prevented by cyclosporine A (CsA).

To further establish a correlation between the calmodulin-dependent phosphatase calcineurin, Drp1 and mitochondrial fission, neurons were exposed to a calcineurin inhibitor CsA (Fruman, Klee, Bierer, & Burakoff, 1992; J. Liu et al., 1991) 15 min prior to Tat. We observed that in cultures pre-treated with CsA, Tat-induced mitochondrial fragmentation was partly mitigated (Figure IV.13). Together these results indicate that Tat-induced mitochondrial fragmentation is mediated in part by increased calcineurin activity in neurons.
Figure IV.12 Tat increases calcineurin activity in cortical neurons in a time-dependent manner. Cortical neurons were exposed to Tat for the indicated times or to Cyclosporine A (CsA) + Tat for 4 hr. Lysates were then collected and calcineurin activity was measured as described in Materials and Methods. Data expressed as mean ± SEM normalized to control represent an average of three experiments (n = 2 each experiment). **p<0.01, ***p<0.001 vs control (ANOVA and Tukey test).
**Figure IV.13** CsA prevents Tat-induced mitochondrial fragmentation. Cortical neurons were exposed to boiled Tat (control), Tat (100 nM), CsA (10 μM), or CsA + Tat for the specified time points. Cells were then fixed and stained for MAP2 (green) and TOM20 (red) to label neuronal microtubules and mitochondria, respectively. Quantification of mitochondrial area (B), perimeter (C), and number (D) was then done as described in Materials and Methods. Data are the mean ± SEM of 20 neurons, normalized to control. *p<0.05, **p<0.01, ***p<0.001 vs control (ANOVA and Tukey Test).
C. Discussion

The ability of Tat to induce neuronal damage and dysfunction in vitro and in vivo has been established (W. Li et al., 2009). Several mechanisms have been suggested to underlie the neurotoxic effect of Tat, including activation of NMDA receptors (E. a Eugenin et al., 2007; N J Haughey et al., 2001), impairment of mitochondria physiology (D. Chen et al., 2002; Norman et al., 2007), and DNA damage (Rozzi et al., 2014). These mechanisms may ultimately participate in inducing neuronal apoptosis. In this chapter, we investigated whether the neurotoxic mechanism of Tat is mediated through alterations of mitochondrial dynamics. Impaired mitochondrial dynamics, morphology, and distribution have been observed in postmortem brains of patients suffering from HAND (Valeria Avdoshina et al., 2016; J. A. Fields et al., 2016), further identifying mitochondria as critical features for the pathology of HAND. Our data show that Tat elicits time-dependent changes in mitochondrial dynamics starting with their depolarization, followed by increased fission, leaving a fragmented mitochondrial population. Under normal conditions, mitochondria can repair themselves through fusion, allowing for the mixing of healthy organelles with those that are damaged. However, following Tat exposure we observed that mitochondria begin to fragment and accumulate near the soma. At the same time, we observed an increase in total levels of Drp1, consistent with an increase in fission. Increased Drp1 activity has been shown to contribute to neuronal injury and cell death (Frank et al., 2001; Nakamura, Cieplak, Cho, Godzik, & Lipton, 2010). Moreover, the co-exposure of neurons to Tat and Drp1 inhibitor mdivi-1 prevented Tat-induced mitochondrial fragmentation (Figure IV.4), further indicating that Drp1 activation is a necessary step in this neurotoxic process. These changes in mitochondrial morphology and subcellular localization can both contribute to neuronal destabilization including the pruning of neurites and eventual cell death.
Mitochondria play a role in neuronal survival through a variety of mechanisms, including Ca\(^{2+}\) homeostasis (Levy et al. 2003), as well as control the production of ROS (Gleichmann & Mattson, 2011). Moreover, mitochondria regulate the synthesis of ATP, which is absolutely necessary for neuronal function (Berthet et al., 2014; Dickey & Strack, 2011). In fact, disruption of energy because of mitochondrial dysfunction has been linked to numerous neurodegenerative diseases (Burté et al., 2014). Thus, it is plausible to suggest that Tat-mediated mitochondrial damage is a key mechanism responsible for some of the neuronal impairments observed in HAND.

a. Mechanism of Tat-Induced Mitochondrial Toxicity

My data show that Tat promotes a time-dependent depolarization of mitochondria (Figure IV.2). This effect is likely due to the rapid rise of intracellular Ca\(^{2+}\) caused by Tat (Figure IV.3) through NMDA receptor activation (N J Haughey et al., 2001), ryanodine receptors (Norman et al., 2008), and/or L-type Ca\(^{2+}\) channels (Wayman et al., 2012). Excessive mitochondrial Ca\(^{2+}\) loading causes a severe reduction in mitochondrial membrane potential, in extreme cases triggering apoptosis (D. C. Chan, 2006). Concurrent with these fluctuations in membrane potential is the significant fragmentation of neuronal mitochondria as well as the accumulation near the soma (Figure IV.7). In the current study, we present evidence that Tat enhances mitochondrial fission associated with increased activity of mitochondrial fission protein Drp1 and Ca\(^{2+}\)-dependent phosphatase calcineurin. These events occur days prior to Tat-induced apoptosis (Figure III.5), indicating the events are not synonymous with mitochondrial fragmentation observed in apoptosis. In fact, while induction of mitochondrial fission is necessary, it is not sufficient to induce neuronal apoptosis (Meuer et al., 2007).
We have shown that Tat causes an influx of intracellular Ca\(^{2+}\) mediated by both NMDA and non-NMDA receptors (Figure IV.3) which precedes mitochondrial impairment (Figure IV.2 and Figure IV.3). While it has been known for decades that Tat causes such a Ca\(^{2+}\) influx (Magnuson, Jsnudsen, Geiger, Brownstone, & Nath, 1995), the entirety of the toxic effects remain to be elucidated. Here, we identify a new mechanism of toxicity through mitochondrial fragmentation. Inhibiting both NMDA and non-NMDA receptors as well as the removal of extracellular Ca\(^{2+}\) significantly reduces both the Ca\(^{2+}\) influx as well as neurotoxicity caused by Tat (Magnuson et al., 1995), indicating an important component of Tat-induced neurotoxicity. Intracellularly, Tat activates phosphatidylinositol 3-kinase, increase levels of IP\(_3\), releases Ca\(^{2+}\) from IP\(_3\)-sensitive ER stores, and increases activity of PKC isoforms α, ε, and ζ (Borgatti, Zauli, Cantley, & Capitani, 1998). Tat-induced dysregulation of intracellular Ca\(^{2+}\) from mitochondria has been demonstrated as well (Lecoeur et al., 2012; Norman et al., 2008). When compared with untreated and mutant Tat controls, Tat induced a rapid decrease in free mitochondrial Ca\(^{2+}\) (Figure IV.14). Like the ER, Tat-induced mitochondrial Ca\(^{2+}\) loss is mediated through ryanodine receptors (Norman et al., 2008). The loss of mitochondrial Ca\(^{2+}\) uptake is crucial not only for the maintenance of mitochondrial function (reviewed in Glancy & Balaban, 2012), but is also key in signaling dysregulation due to the loss of Ca\(^{2+}\) buffering in cellular microdomains (Fonteriz et al., 2016). While this Ca\(^{2+}\) release and other changes to mitochondrial membrane stability have been detailed by others as a mechanism for Tat toxicity (Kruman et al., 1998; Norman et al., 2007), we are the first to identify an imbalance to mitochondrial dynamics as a mechanism in Tat-induced mitochondrial dysfunction. Here, we identified that following Tat administration, calcineurin is activated, contributing at least in part to the Tat-induced mitochondrial fragmentation (Figure IV.4), which was validated through the use of CsA, a calcineurin inhibitor (Figure IV.13). Tat-induced calcineurin activation had been speculated about previously following Tat peptide-calmodulin binding studies (McQueen et al., 2011). Tat-peptides were
found to bind with high affinity to calmodulin in the presence of \( \text{Ca}^{2+} \). It is unsurprising that Tat is able to bind to host-cell machinery, as viruses encode only a limited number of proteins and therefore have limited biological machinery. In fact, other HIV-1 proteins, including gp120, have been previously found to bind calmodulin (K. Micoli et al., 2006; K. J. Micoli et al., 2000). A model of the proposed mechanism of toxicity is laid out in Figure IV.14. We propose that due to the \( \text{Ca}^{2+} \) influx mediated by Tat, Tat is able to bind calmodulin enabling it to then bind to the calcineurin A subunit, leading to phosphatase activity. Once activated, calcineurin can dephosphorylate Drp1 at Ser637, leading to the translocation of Drp1 to the mitochondria and initiating mitochondrial fission. This results in a population of mitochondria that is fragmented in comparison to those of untreated neurons. As calcineurin is not the sole target of calmodulin, the effects of Tat-calmodulin binding may be numerous and widespread. Other calmodulin targets already implicated in Tat-mediated toxicity include iNOS and IP₃ receptor, among others (O'Day, 2003).
**Figure IV.14 Mitochondrial calcium response to HIV-1 Tat treatment.** Application of 1 g/ml Tat (n=4; TX treatment) to transfected neurons initiated a loss of CFP:EYFP calmodulin fluorescence, indicating a loss in mitochondrial Ca\(^{2+}\) (*, p 0.05; #, p 0.001). Application of the positive control, ionomycin (2 M) and Ca\(^{2+}\) (10 mM), increased the CFP:EYFP calmodulin fluorescence. Permission to reproduce this figure from Norman et al. (2007), the Journal of Immunology, granted by the American Association of Immunologists.
Figure IV.15 Schematic depicting mechanism of Tat-induced mitochondrial fragmentation via Ca^{2+} influx, calcineurin activation, and Drp1 dephosphorylation. Tat interacts with receptors embedded within the cell membrane (i.e. NMDA and LRP) which can lead to a rapid rise in intracellular Ca^{2+} as well as Tat internalization. Tat is also able to cross the cell membrane in a receptor independent manner. Once internalized, the majority of Tat localizes to the nucleus, however, some remains in the cytoplasm. In combination with the rise in intracellular Ca^{2+}, Tat activates calmodulin (CaM) which can then go on to bind to the catalytic subunit of calcineurin (CnA). Calcineurin regulates Drp1 activity through the dephosphorylation
of serine residue 637. Drp1 is found throughout the neuronal cytoplasm in its inactive form. Upon activation (in this case, dephosphorylation by calcineurin), Drp1 translocates to the mitochondrial membrane where it oligomerizes and initiates mitochondrial fission. The resulting daughter mitochondria can either be equal in polarization or be asymmetrical products, yielding one depolarized mitochondria and one hyperpolarized mitochondria. The fate of these fission projects can be one of a few: 1) re-fuse with a nearby mitochondrion, 2) traffic to the soma for repair or recycling, 3) be marked for mitophagy in the processes, or 4) remain. Following Tat exposure, I observe 1) depolarized mitochondria population, 2) more fragmented mitochondria, and 3) no change in overall mitochondria number.
Translocation of Drp1 to mitochondria is regulated heavily by post-translational modifications, predominantly through phosphorylation and dephosphorylation. The phosphatase calcineurin plays a key role in these events. Indeed, calcineurin-dependent dephosphorylation of Drp1, at its conserved serine 637, regulates its translocation to mitochondria and increases GTPase activity as well (Cereghetti et al., 2008). In this investigation, I found that calcineurin activation corresponded with an increase in the active form of Drp1. Activation of calcineurin can lead to other detrimental effects on neurons, including cell cycle arrest in non-neuronal cells (Li et al., 1997) and interactions with transcription regulation (Jeang, Xiao, & Rich, 1999). Calcineurin has also recently been found to play a central role in protein misfolding in several neurodegenerative diseases (Shah, Hussain, Zhao, & Yang, 2017). Drp1 activity is also critical for neuronal survival. Excessive Drp1 activity is linked to neuronal death (Manczak & Reddy, 2015), while down-regulation of Drp1 leads to neuroprotection (Meuer et al., 2007). Our data show that Tat activates calcineurin and decreases the levels of pDrp1 S637 which leads to an increase in Drp1 activity. The immunosuppressant cyclosporine A, which has been used experimentally to prevent mitochondrial dysfunction (Sullivan, Thompson, & Scheff, 1999) and restore mitochondria-mediated synaptic plasticity (Levy, Faas, Saggau, Craigen, & Sweatt, 2003), blocks the effects of Tat both on calcineurin activation as well as decreased mitochondrial fragmentation, suggesting that Drp1 could play a role in Tat toxicity.

In addition to Drp1, mitochondrial fission is regulated in part by several small adaptor proteins (Figure V.3; Mff, MiD49, MiD51, Fis1) that bind directly to the outer mitochondrial membrane and recruit Drp1 in several ways (Elgass, Pakay, Ryan, & Palmer, 2013; Otera, Ishihara, & Mihara, 2013). Removal of these proteins with shRNA or blockade of function through mutation can obscure their ability to normally recruit Drp1 to the mitochondrial surface, thus indicating their significance in the regulation of mitochondrial fission. Alterations to the localization and activity of these proteins were also considered in the development of the
experiments detailed within this dissertation. However, none served to be ideal candidates for the changes I observed following Tat exposure in neurons due to their regulation. In particular, Mff has been shown to be functionally regulated by phosphorylation (Toyama et al., 2016). Recruitment of Drp1 by Mff to the outer mitochondrial membrane is sufficient to induce fragmentation of neuronal mitochondria in vivo and this effect requires phosphorylation of Ser155/172 by adenosine monophosphate-activated protein kinase (AMPK) (Toyama et al., 2016). Phosphorylation of Mff by AMPK occurred within 15 minutes of exposure to mitochondrial-destabilizing stimuli, indicating that this rapid induction of mitochondrial fission may serve as one way for the cell to prepare to initiate mitophagy of those mitochondrial fragments that have extensive damage (Youle & van der Bliek, 2012). While the timing of this activation makes Mff phosphorylation a reasonable target player in the mechanism mediating Tat-induced mitochondrial fragmentation, Tat inhibits SIRT1 resulting in AMPK inhibition (H. Zhang & Wu, 2009) which would result in precisely the opposite effect I observed. This further strengthens our finding that Drp1 regulation is the key event leading to mitochondrial fragmentation following Tat exposure.

b. What is the Fate of Fragmented Mitochondria Following Tat Exposure in Neurons?

Fission can yield uneven products, with one depolarized and one hyperpolarized daughter mitochondrion. While I have observed a significant increase in mitochondrial fragmentation (Figure IV.4) as well as a change in mitochondrial membrane potential at the scale of the whole cell (Figure IV.2), I have not investigated the distribution of individual depolarized mitochondria. The utilization of membrane potential dependent dyes during live-cell imaging would help to assess this question. In addition, depolarization of mitochondria is a pre-
requisite of mitophagy (reviewed in Kim, Rodriguez-enriquez, & Lemasters, 2007). Thus, further investigation into any alterations of mitophagy following Tat-exposure would be of interest. Recently, Tat has been found to increase autophagy (J. Fields et al., 2015), from which one could speculate that mitophagy would also be increased. However, this specific action has yet to be assessed in detail. An increase in mitophagy paired with uneven fission products would support our finding that while mitochondrial fission increased, mitochondria number did not. An increase in mitophagy could easily explain this outcome. Another explanation for this observation could be due to the altered subcellular localization of mitochondria following Tat exposure (Figure IV.7). Due to the imaging and analysis techniques utilized in the completion of this dissertation, mitochondria within the soma were unable to be quantified (count or size). However, in an investigation of mitochondria within Jurkat T cells overexpressing Tat (Jurkat-Tat101), a significant increase in mitochondrial number was found (Rodriguez-Mora et al., 2015), which would make it unsurprising if neurons did in fact have more mitochondria after Tat exposure. Thus, further assessment of this subpopulation of mitochondria would need to be assessed to determine if trafficking is playing a role in these changes.

c. Reconciling Conflicting Actions of HIV Proteins in Neurons

Several HIV proteins have been found to be mitotoxic, a number of them are contradictory in their morphological manifestation, leading me to need to reconcile the true impact of Tat-induced mitochondrial impairments when examined within the complete system of HIV infection and HAND. Namely, Tat-induced mitochondrial impairments result in the increases in fission protein Drp1 and the reduction in mitochondrial size, both in vitro and in Tat-tg animals (Figure IV.1 and Figure IV.4). However, post-mortem brains of HAND patients have shown decreased Drp1 protein levels along with increases in mitofusins and enlargement of
mitochondria (Fields et al., 2016). These contradictory results do not completely negate each other, but rather require us to further investigate the intricate mechanisms at play within this complex system. To support this notion, a study of Drp1 heterozygous knockout mice had no synaptic or mitochondrial deficiencies (Manczak & Reddy, 2012) and another showed that Drp1 inhibition attenuated neurotoxicity in mouse models of PD (Rappold et al., 2014). This suggests that the impact of Drp1 levels may be contextual.

On top of the context of protein level modifications, the simple upregulation of one process does not resolve the issues of the over activity of another, nor the converse, especially in the instances of fusion and fission (Xinglong Wang et al., 2008). Rather, the symptoms of these artificially rebalanced processes may appear to be fixed while continuing to generate long-term dysfunction within the cell. This has been documented numerous times within the literature through the use of inhibitors and promoters of fusion and fission (C. Chang & Blackstone, 2010; Xinglong Wang et al., 2008). The predominant mitochondrial phenotype observed in postmortem brains of HAND patients resembles that observed in gp120-tg animals (Fields et al., 2016), though mitochondrial fragmentation does not go unseen (Fields et al., unpublished data). This could be due to several reasons, none of which negate the significance of Tat-induced mitochondrial impairment. To begin, gp120 is a more abundant and potent neurotoxin (Bansal, Mactutus, Nath, & Maragos, 2000) (Jones, Bell, & Nath 2000) than Tat, which would enable it to manifest more abundant morphological impairments. Additionally, these changes have been observed in post-mortem tissues, giving us only a snapshot of the end-result of HAND, not a clear picture of the modifications and impairments occurring within the highly dynamic and complex living system. It is possible that the hyperfused state of mitochondria observed at the end of life is a self-protective response that also results in damage to the brain (Friedman & Nunnari, 2014). Each insult to the system is significant when considered as a feature of the whole. Furthermore, the question remains whether these
mitochondrial perturbations are the primary cause of, or a response to, neurodegeneration caused by HIV infection of the CNS.

In instances of either increased fusion or fission, the questions arise: is this increase in fusion an accumulation of damaged mitochondrial components in need of being disposed? Or is this increase in fission a sign of excessive (unnecessary) removal of crucial organelles? Or, in either instance, is the neuron acting in this manner as a buffering mechanism compensating for the damage induced by HIV proteins? These questions require further investigation to be answered.

d. How Can These Mitochondrial Impairments be Targeted with Therapy?

Mitochondrial dysfunction has been found to be causally linked to a number of neurodegenerative diseases (Lin & Beal, 2006), underscoring the significance of these impairments to brain health. Not only are mitochondria necessary for energy production within highly energetically active neurons, but they also play a crucial role in Ca\(^{2+}\) buffering. Ca\(^{2+}\) buffering is especially important in an environment more prone to excitotoxicity, like the brain in HAND. Numerous HIV proteins cause a dysregulation of excitatory amino acids (EAAs) handling by astrocytes (Z. Wang et al., 2003) as well as augmented activity of Ca\(^{2+}\) permeant channels and receptors on neurons, such as both non-NMDAR and NMDA receptors and L-type Ca\(^{2+}\) channels (Magnuson et al., 1995; L. Song, Nath, Geiger, Moore, & Hochman, 2003). HIV-1 Tat causes pathophysiological effects through the dysregulation of intracellular Ca\(^{2+}\) homeostasis and dysfunctional activity of neurons in certain brain regions most vulnerable to HIV (Hu, 2016). When mitochondria are unable to buffer Ca\(^{2+}\), whether due to subcellular localization or fragmentation, such as I observe in these studies, synaptic pruning may ensue (Sun et al., 2013). The depletion of synapses is linked to cognitive deficits seen in HAND,
among other neurodegenerative diseases (Saylor et al., 2016; Wishart, Parson, & Gillingwater, 2017). If homeostasis to mitochondrial dynamics is restored, then we may be closer to preventing some of the damage observed in HAND brains. In our studies, I was able to mitigate Tat-induced mitochondrial fragmentation through the administration of the Drp1-selective inhibitor, mdivi-1. Mdivi-1 was first found to be protective in the rat hippocampus against seizure-induced upregulation of Drp1 activity and resultant mitochondrial fragmentation (Xie et al., 2013). Mdivi-1 was further found the suppress mitochondrial fission and significantly attenuate oxidative stress and reduce neuronal loss in an epileptic model of rats (Qiu et al., 2013). The lipophilic nature of mdivi-1 enables the small molecular to penetrate the BBB, reaching its peak concentration in brain tissue 4h after IV injection (Cui, Ding, Chen, & Zhao, 2016). Mdivi-1 has good therapeutic potential because it is timely and reversible, as total inhibition of Drp1 activity entirely is neurotoxic. The use of a drug to increase fusion and/or decrease fission alone will not likely be sufficient in repairing the impairment I observe. However, pairing a partial promoter/inhibitor of these impaired processes with an antioxidant could prove to be an effective adjunct therapy.

In conclusion, alterations in mitochondrial dynamics are believed to initiate neurodegeneration because they negatively influence energy distribution within synapses (Pekkurnaz, Trinidad, Wang, Kong, & Schwarz, 2014). HIV infection of the CNS causes distinct mitochondrial alterations (Valeria Avdoshina et al., 2016; J. A. Fields et al., 2016). A similar scenario is induced by Tat. Remarkably, these effects occur even in the absence of the virus suggesting that this protein is sufficient to initiate an irreversible neurodegenerative process that may overlap with other endogenous neurotoxins or other pathophysiological insults. Our discovery provides new significant data that will help in the design of adjunct therapies against HAND.
Chapter V: Conclusion and Future Directions

The overarching goal of this dissertation was to elucidate the impacts HIV-1 Tat has upon neuronal mitochondria, ultimately contributing to neuronal dysregulation and eventual cell death. Additionally, I have identified a potential therapeutic compound that may be used in conjunction with current antiretrovirals to combat the neuronal impairments observed in HAND. The rationale for this work was based upon observations of mitochondrial irregularities following HIV viral protein administration in vitro, as well as the growing understanding of the implication of such irregularities in neuronal health and survival and neurodegeneration. I hypothesized that HIV-1 Tat would cause an imbalance in the mitochondrial fusion/fission dynamics through signaling cascades emanating from a rise in intracellular Ca\(^{2+}\). In the first part of this dissertation, I 1) established a time course of Tat toxicity, 2) identified early mitochondrial dysfunction following Tat exposure, and 3) identified a protective compound against Tat-induced toxicity. To further elucidate the mechanism behind Tat-induced mitochondrial toxicity, I utilized a pure neuronal culture system as to isolate the direct, neuron-specific Tat-induced effects. Our main finding was that Tat induces mitochondrial fragmentation following the activation of Drp1 via calcineurin (Figure IV.10). My results, herein, demonstrate a novel mechanism by which Tat impairs neuronal function through the dysregulation of mitochondrial dynamics. These findings support morphological characteristics of mitochondria within the brains of mice expressing a Tat transgene as well as many structural abnormalities observed in mitochondria in post-mortem brains of patients with HAND. Additionally, I identified PACAP27 as a protective compound against Tat-induced neurotoxicity. Because of the ability of PACAP27 to cross the blood brain barrier, the abundance of receptors throughout brain tissue, and the reversible nature of its effects, PACAP27 or a metabolically stable analog have intriguing potential as an adjunct therapeutic.
As is the case for most research endeavors, additional questions beyond the scope of this dissertation were raised. The results of the experiments presented here open the door to new, unasked, and unanswered questions.

As shown here, Tat causes changes in mitochondrial morphology, resulting in smaller mitochondria. With the consistent alterations to the fission regulatory protein Drp1, these alterations are concluded to be due to an increase in fission events. However, without visualizing the specific event it remains an open question. The utilization of live imaging and dual fluorescent probes to label mitochondria will allow us to more directly clarify this question. Relatedly, examining the potential protective effects of partial inhibitors of fragmentation upon neurons following Tat exposure would be illuminating and help to lead toward the development of adjunct therapies for the neurotoxicity observed in HAND.

Numerous investigators, including us, have found Tat to cause a rapid and robust rise in intracellular Ca\(^{2+}\) which precedes many toxic events (Haughey & Mattson, 2002). Throughout this dissertation I have cited Ca\(^{2+}\) with a causative role in our key finding of mitochondrial fragmentation. Mitigating these Ca\(^{2+}\) currents could better clarify the significance of this role as well as identify which Ca\(^{2+}\) source is most critical.

As I am the first to show that Tat activates calcineurin, it would be fascinating to determine which other downstream effectors are being targeted by this Tat-induced activation. Calcineurin and its regulator calmodulin have far reaches in cellular regulation, including numerous transcription factors (Stull, 2001), and thus could be playing a much wider role in Tat- and HIV-induced neuronal impairment.

The culture system utilized in these investigations was highly enriched in neurons, containing less than 10% glia. This system was used for the purpose of being able to identify neuron-specific impairments of Tat. While this is necessary in order to best isolate the cellular mechanisms at work, it removes the model system distantly from the effects that are seen in an
animal. We know that mitochondria in Tat-tg mice are reduced in size (Figure IV.1), thus the use of fission inhibitors in these animals may be interesting in understanding the impacts of such drugs on the Tat-impaired system.

I have mentioned throughout this dissertation that HIV envelope protein gp120 also causes significant mitochondrial impairments through mitochondrial dynamics (Avdoshina et al., 2016; J. A. Fields et al., 2016). In addition, mitochondrial abnormalities were observed in the post-mortem brain tissue of patients with HAND (Avdoshina et al., 2016), where all components of HIV and the resultant inflammation and pathology manifest. It would be interesting to better understand the specific interactions of Tat and gp120 upon neuronal mitochondria by utilizing an isolated culture system co-treated with these viral proteins. While determining the most physiologic doses of each protein in relation to one another would be difficult considering the wide variance in detection in the infected system, these experiments would still allow us to gain insight into the interactions between the two most potent neurotoxic proteins found in HIV. I propose determining the lowest combined toxic dose of each that best mimics the ultimate mitochondrial pathology observed in post-mortem HAND brains. Experiments can then be performed along a time course of exposure giving us snapshots of interactions. Similarly, experiments utilizing double-transgenic animals (Tat and gp120) would add further understanding to the mechanisms and interactions at hand.

Lastly, it would be fascinating to know if PACAP is able to protect neurons from Tat-induced dysregulation of mitochondrial dynamics. As mentioned in Chapter III, PACAP binds to PAC1 with high affinity as well as VPAC1 and VPAC2, which is shares with VIP, with low affinity. The PAC1 receptor is a G protein-coupled receptor signaling with Gαs/Gαq. PAC1 receptor Gαq stimulates cAMP mechanisms to promote sustained ERK activation, as well as activation of PKA (Vaudry et al., 1998). In addition, PAC1 Gαq activates phospholipase/PKC pathways. Drp1 can be regulated by phosphorylation at serine residue 616 by PKA (Chang &
Blackstone, 2010) which decreases fission activity. Previous investigations have shown that the mitochondria of both neurons and non-neuronal cells can be reshaped into an interconnected network following cAMP elevation (Merrill et al., 2011). However, Drp1 is also regulated through PKC pathways (Licci, Scorrano, & Frank, 2012; Qi et al., 2011). Activation of PKCδ leads to phosphorylation of Drp1 at serine 579, and induces aberrant mitochondrial fragmentation and impaired mitochondrial function in cultured SH-SY5Y cells as well as in a rat model of hypertension-induced encephalopathy (Qi et al., 2011). Given the competing regulation of Drp1 by each pathway, it is hard to know whether PACAP would prove to be protective. I hypothesize that the combined neurotrophin-like effect elicited by PACAP in combination with robust cAMP/PKA signaling would result in at least partial protection against Tat-mediated mitochondrial fragmentation.

In summary, the results put forth in this dissertation identify a novel mechanism by which HIV-1 Tat induces neuronal dysfunction through dysregulation of mitochondrial dynamics. Further investigations should aim to better understand the wider impacts of this dysregulation and means to mitigate such changes so as to develop adjunct therapies for HAND.
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