The Dynamics Of Life And Death: Mitochondrial Fragmentation, Mitophagy, And Swelling During Neuronal Ischemia/reperfusion Injury

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DEDICATION

I dedicate this to my wife Emily. As it hasn’t been easy for me, I know it also hasn’t been easy for you. Thank you so much for all of your love and unrelenting support. Thank you for your understanding with countless late nights in the lab and for helping me deal with all of the stressors that come with being a graduate student. You are my best friend and partner in life.
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CHAPTER 1: MITOCHONDRIAL QUALITY CONTROL AND DISEASE: INSIGHTS INTO ISCHEMIA-REPERFUSION INJURY

(This Chapter contains previously published material. See Appendix B)

1. Introduction: Mitophagic Balance in Acute and Chronic Disease

Cardiovascular and neurologic diseases are leading causes of morbidity and mortality in the U.S.[1]. Cardiovascular disease can result in acute injuries sustained by both the heart and the brain in the form of acute myocardial infarction (AMI) and stroke. AMI and stroke are induced by a cessation of blood flow (ischemia), caused by blockage of one or more of the coronary or cerebral arteries that supply the heart or brain. This cessation of blood flow will subsequently lead to tissue hypoxia or anoxia and, ultimately, necrotic cell death (characterized by cellular swelling and membrane rupture due to energy failure). It is well-known that although restoration of blood flow (reperfusion) is essential to salvage ischemic tissue, this can also, paradoxically, exacerbate damage from several cellular alterations including excessive reactive oxygen species (ROS) production from mitochondria [2-4]. ROS production will lead to mitochondrial damage and, ultimately, mitochondrial failure and programmed cell death (regulated by mitochondrial permeability transition or outer membrane permeabilization) [5, 6]. Ultimately, cell death observed during ischemia and reperfusion in both heart and brain occurs over a broad spectrum of cell death phenotypes depending on the duration and severity of the ischemic insult. This process occurring at the level of the tissue has been termed lethal ischemia/reperfusion (I/R) injury.

In addition to acute injuries such as stroke or AMI, the incidence of chronic neuropathologies (including, for example, Parkinson’s Disease (PD) and Alzheimer’s Disease (AD)) are on the rise as the average lifespan continues to increase [7, 8]. PD and AD are neurodegenerative diseases that affect different parts of the brain and are
typically seen in the elderly, although inherited mutations may also lead to disease in younger patients. PD is a movement disorder and, while many forms of hereditary and acquired PD have unique mechanistic causes, all result in a pathologic dysfunction and death of dopaminergic neurons. AD is a neurodegenerative disorder that affects the elderly and causes memory loss and declined cognitive function. The pathological factors of the disease consist of the presence of amyloid-β plaques and neurofibrillary tangles in the brain [9]. Although I/R injury, Parkinson’s Disease, and Alzheimer’s Disease differ in terms of their etiology, the literature suggests that loss of mitochondrial integrity play a central role in each of these disease processes.

It is well-established that mitochondria are key regulators of cell fate, controlling survival (via the production of ATP that fuels cellular processes) and, conversely, death (via the induction of apoptosis). Indeed, mitochondrial dysfunction has been well-characterized as a precursor to cell death [10-13]. Therefore, it is essential to have stringent control mechanisms regulating the quality of mitochondria to avoid the pathologic effects of dysfunctional mitochondria on the cell.

While the negative roles of mitochondrial failure and apoptosis are well-documented, much less is known of the causal role of mitochondrial quality control in disease and the potentially nuanced role for these mechanisms in different disease settings. Importantly, evidence suggests a divergent role of mitochondrial quality control in acute vs. chronic disease. Defects in the mechanisms that regulate quality of mitochondria are recognized to play a large role in chronic diseases such as PD and AD, but, to date, their role in the acute setting of I/R injury is poorly understood. In contrast, evidence supports a salutary role for mitochondrial quality control in acute cardiac and neurologic injury, suggesting quality control mechanisms can serve both survival and
death functions depending on the nature of the disease. This Chapter aims to: (i) discuss the molecular mechanisms involved in mitochondrial quality control including mitochondrial dynamics and mitophagy (Fig. 1-1), (ii) detail the role that mitochondrial quality control plays in chronic and acute neurodegenerative and cardiovascular diseases, and (iii) provide a better understanding of the intricacies and balance of this process in the progression of acute vs chronic diseases.

2. The Axes of Mitochondrial Quality Control

2.1 Mitochondrial Dynamics: To Divide or Not to Divide?

Fig. 1-1. The mitochondrial quality control cycle. The mitochondrial quality control cycle involves a dynamic process of fission, fusion, mitophagy, and biogenesis. When mitochondria become depolarized or dysfunctional, they are marked for degradation. Once marked, the unhealthy component of the mitochondria will undergo fission from the healthy mitochondrial network. Certain damaged mitochondria can fuse with other healthy mitochondria in an attempt to salvage that mitochondrion, but typically dysfunctional mitochondria will undergo mitophagy. When the dysfunctional mitochondria are segregated from the healthy mitochondrial network, mitochondria will accumulate mitophagy markers that will recruit the phagophore or endosome. The phagophore or endosome will fuse with the lysosome to degrade the mitochondria. Once degraded, the cell will recycle the amino acids and fatty acids to enable the remaining healthy mitochondrial network to grow and divide through biogenesis.
Within the cell, mitochondria exist in an ever-changing dynamic state, where mitochondrial networks are constantly elongating and dividing (i.e., mitochondrial fusion and fission, respectively). The balance of these two events provides an equilibrium of small fragmented mitochondria and long interconnected mitochondrial networks, and is thought to be essential for mitochondrial homeostasis, cell stability and cell survival (1) [14, 15]. Fission plays a role in segregating dysfunctional mitochondria that contain damaged proteins, destabilized membranes, and mutated or damaged mitochondrial DNA (mtDNA) [16-20]. Fusion, in contrast, has been shown to aid in equilibration of matrix metabolites, intact mtDNA, and even membrane components such as Complex I of the electron transport chain [16, 21-24]. Fission and fusion are both regulated by a family of dynamin-related proteins (DRPs). These proteins are unique in that they are large self-assembling GTPases that also possess the capability of assembly stimulated GTP hydrolysis [25]. Through the work of DRPs, the mitochondrial network can be in constant communication to ensure a healthy connected network, while at the same time allowing the distribution of mitochondria to specific sites of the cell via transport on actin or microtubule networks [26, 27].

2.1.1 Mitochondrial Fission

The primary mediator of fission is dynamin related protein 1 (Drp1), which has been shown to be essential for noncytokinetic mitochondrial division [16, 28]. Drp1 is distributed diffusely throughout the cytosol and, when activated through post-translational modifications (predominantly phosphorylation/dephosphorylation), translocates to the outer mitochondrial membrane via actin and microtubule mechanisms [29-33]. These post-translational modifications, described in detail below, include phosphorylation/dephosphorylation, ubiquitination, and sumoylation, in a cell-specific
manner [34, 35]. Once positioned on the outer mitochondrial membrane, Drp1 interacts with four mitochondrial-bound proteins that serve as Drp1 receptors (mitochondrial dynamic proteins of 49 and 51 kDa (Mid49 and Mid51), mitochondrial fission protein 1 (Fis1), and mitochondrial fission factor (Mff), where it constricts and cleaves the mitochondria (Fig. 1-2A) [36-38]. Fis1 is an 18-kDa adaptor protein anchored to the outer mitochondrial membrane and has been implicated in recruiting Drp1, as well as modulating the assembly of the fission complex [39, 40] Fis1 is thought to be required for mitochondrial fission, although this remains controversial as other groups have found it to be dispensable in the fission process [38, 41, 42].

Fission is regulated by numerous post-translational modifications of Drp1 as well as endoplasmic reticulum (ER)-mitochondrial contact sites. Phosphorylation/dephosphorylation is one the main regulators of Drp1 and is carried out at two different serine sites located 20 amino acids apart (Ser616 and Ser637) [43]. Phosphorylation of Ser616 is associated with Drp1 activation and is phosphorylated by cyclin dependent

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**Fig. 1-2. Mitochondrial dynamics.** A) Fission is mediated by a family of dynamin related proteins (Drp). Activated Drp1 translocates to the mitochondrial membrane where it interacts with Drp1 receptors (Mid49, Mid51, Mff) and Fis1 to create the fission complex. Drp1 oligomers constrict and divide the mitochondria. B) Fusion is mediated through the mitofusins (Mfn1/2) and optic atrophy 1 (Opa1). The mitofusins mediate the fusion of the outer mitochondrial membrane, while Opa1 mediates fusion of the inner mitochondrial membrane. Mitofusins are anchored to the outer mitochondrial membrane and interact with each other and form a hemifusion stalk. The stalk then grows into a lipidic hole and finally reestablishes membrane continuity. Opa1 forms a fusion pore for the inner mitochondrial membrane via its cardiolipin binding domain.
kinase 1 (CDK1), extracellular signal-regulated kinase (ERK1/2), and protein kinase C delta (PKCδ) [44-46]. CDK1 induces mitochondrial fragmentation during mitosis [44]. ERK1/2 and PKCδ induces Drp1-mediated mitochondrial fission via increases in ROS production during hyperglycemic conditions and hypertensive neuroencephalopathy respectively [45, 46]. Ser637 is phosphorylated by protein kinase A (PKA), calcium/calmodulin-dependent protein kinase 1 alpha (CAMK1α), and the Rho-associated coil-containing protein kinase 1 (ROCK1) [34, 35, 47]. PKA-phosphorylated Drp1 has been shown to have decreased GTPase activity and result in decreased fission during starvation, stress, or exercise. Studies in neurons and cardiac tissue exposed to oxygen-glucose deprivation (OGD) and ischemia-reperfusion respectively demonstrate calcineurin-mediated dephosphorylation of Ser637, subsequently leading to Drp1 activation, mitochondrial fission, and apoptosis [33, 48, 49]. Conversely, CAMK1α – phosphorylation of Ser637 results in enhanced fission during conditions of high extracellular K+ (inducing Ca2+ influx) in primary rat hippocampal neurons [33, 35, 47]. Additionally, ROCK1-phosphorylation of Ser637 has been demonstrated to induce mitochondrial fission in podocytes and endothelial cells of mice with metabolic syndrome and diabetes [34]. Phosphorylation of the same residue leading to opposite effects points to the complexity of Drp1 regulation that is likely dependent on cell type, extracellular conditions, as well as intracellular status.

Recent literature also suggests a role for the ER in mitochondrial fission. Studies conducted in both yeast and mammalian cells have shown that ER tubules will wrap around the mitochondria and mediate constriction before Drp1 recruitment via the ER-localized inverted formin 2 (INF2) mechanisms [50, 51]. INF2 is thought to drive initial mitochondrial constriction that provides sites for subsequent Drp1 recruitment and
secondary constriction [51]. The multiple mechanisms involved in the regulation of fission underscore the complexity of this process and may provide insight into potential mechanisms by which dysregulated mitochondrial dynamics may interact with disease processes.

2.1.2 Mitochondrial Fusion

Fusion is mediated by three different GTPases: Optic Atrophy 1 (Opa1), Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) [24]. Both Mfn1 and Mfn2 mediate fusion of the outer mitochondrial membranes, while Opa1 mediates the fusion of the inner mitochondrial membrane, along with its role in maintaining normal inner membrane cristae structure [52-54]. Mitofusins, which are required for fusion, are anchored to the outer mitochondrial membrane where they interact and form a hemifusion stalk to initiate the joining of two mitochondrial membranes [16, 55]. The stalk then grows and creates a lipidic hole as well as a hemifusion diaphragm to reestablish membrane continuity. Finally, a fusion pore is made for inner membrane fusion via the lipid binding domain in Opa1 that is specific for cardiolipin [55, 56] (Fig. 1-2B).

Fusion of the inner and outer mitochondrial membrane is mediated mainly through proteolytic cleavage and ubiquitination respectively. Opa1, in mammals, consists of eight different isoforms generated by alternative splicing of three of the 30 Opa1 exons [57, 58]. Membrane-bound long (L)-Opa1 can be further processed via two proteolytic cleavage sites (S1 and S2), generating short (S)-Opa1 forms [54]. Proteolytic processing is carried out predominantly through two intermembrane space AAA-proteases (ATPases associated with diverse cellular activities): (i) overlapping with m-AAA (OMA1) cleaving at the S1 site and (ii) yeast mitochondrial DNA escape 1-like (YME1L) cleaving at the S2 site [59, 60]. Every (L)-Opa1 isoform contains a S1 cleavage site, while about half of the
(L)-Opa1 isoforms contain both S1 and S2 cleavage sites [61, 62]. Under normal physiological conditions S1 and S2 are constitutively cleaved to produce a 50/50 ratio of (L)-Opa1 and (S)-Opa1. The balance in Opa1 isoforms is thought to mediate the balance between mitochondrial fission and fusion [63]. Under pathophysiologic conditions, such as membrane depolarization, low levels of ATP, or dysfunctional quality control mechanisms, the balance is tipped and the remaining (L)-Opa1 are cleaved by Oma1 resulting in mitochondrial fragmentation [64-68]. Mitofusins are regulated mainly by ubiquitin-mediated degradation, specifically through the PTEN-induced kinase (PINK1) and Parkin-mediated ubiquitination pathway during mitophagy [69]. Abnormalities in proteolytic cleavage of Opa1 or ubiquitination of the mitofusins result in impaired fusion, changes in cristae architecture, and favor a fragmented mitochondrial phenotype.

### 2.2 Mitophagy: Out with the Old, In with the New

Mitophagy is thought to be carried out via two different mechanisms: autophagosomal mitophagy and endosomal-mediated mitophagy (Fig. 1-3). Autophagy is the catabolic process of cellular components including cytosolic protein aggregates and organelles such as mitochondria that are sequestered in a double-membrane structure called an autophagosome [70, 71]. There are three distinct subtypes of autophagy [72].

**Macro-autophagy** (typically referred to as autophagy) is the process of taking damaged proteins and organelles from the cytoplasm to the lysosome for degradation via an intermediate vesicle termed the autophagosome (summarized in Fig. 1-4). Macro-autophagy typically involves the degradation of large cellular components such as organelles through both selective and non-selective mechanisms. In contrast, in so-called **micro-autophagy**, particles are directly taken up by the lysosome (no intermediate vesicle) by direct engulfment where they are degraded. Lastly, **chaperone-mediated autophagy**
involves targeting dysfunctional proteins to be taken across the lysosomal membrane with the aid of the cytosolic chaperone heat shock cognate 70 kDa protein (HSC-70). The protein-chaperone protein complex then interacts with a specific lysosomal membrane receptor, lysosomal-associated membrane protein 2A (LAMP-2A), resulting in their degradation [73, 74].

Endosomes are membrane bound organelles which are responsible for sorting both extracellular and intracellular proteins, lipids, and solute [75]. There are three types of endosomes which can be distinguished by morphology and protein markers such as the Rab-GTPase proteins [75, 76]. Early endosomes (EEs) receive extracellular cargo from several endocytic pathways and represent the main sorting compartment [77, 78]. They consist of highly dynamic tubular-vesicular networks and are mainly enriched with Rab5, which plays a dual role in mediating the fusion of other EEs as well as maturation of EEs to late endosomes via the recruitment of Rab7 [76, 77]. EEs sort extracellular content within their tubular networks and generate vesicle carriers targeted to two distinct fates: recycling to the plasma membrane or degradation via the lysosome [77, 79]. Recycling endosomes (REs) will shuttle the majority of extracellular content back to the plasma membrane and are marked mainly with Rab4 and Rab35 (rapid recycling) or by Rab11 (slow recycling) [80-86]. Late endosomes (LEs) are responsible for delivering cargo targeted for degradation to the lysosome and appear spherical. The maturation of EEs to LEs involve changes including the switch from Rab5 to Rab7 enrichment, acidification, and the formation of intraluminal vesicles (ILVs) [77, 87-92]. ILVs are responsible for the recognition and sequestration of ubiquitinated proteins while Rab7 enables the fusion of LEs to the lysosome [77, 93].
Mitochondrial degradation through macroautophagy or endosomal-mediated
degradation has been termed mitophagy. Mitophagy occurs through several different
pathways (summarized in 1-6) that all involve: (i) detection of dysfunctional mitochondria,
(ii) segregation from the healthy mitochondrial network, (iii) recruitment of the phagophore
or early endosome, and (iv) degradation through fusion lysosomal processes. Mitophagy,
in concert with mitochondrial biogenesis, ensures a healthy mitochondrial network
through mitochondrial turnover. Clearance of dysfunctional mitochondria is critical to limit
cellular damage via ROS production and subsequent apoptosis. Mitophagic proteins,
specifically Parkin, are critical for eliminating mitochondria with deleterious mtDNA
mutations via mitophagy [20]. This suggests that mitophagy is selective and plays pivotal
role in the maintaining a functional population of mitochondria.

2.3 Autophagy: The Four Phases

There are four phases in the development of an autophagosome: (i) nucleation, (ii)
elongation, (iii) sequestration and maturation, followed by (iv) fusion and degradation (Fig. 1-4) [94, 95].

*Nucleation* of the isolation membrane is initiated first through the phosphorylation of the Unc-51 Like Autophagy Activating kinase 1 (ULK1) complex, typically by 5’AMP-activated protein kinase (AMPK) [96]. Once phosphorylated, the ULK1 complex will recruit several different autophagosome-related proteins (Atg) to the autophagosome formation site. In addition, ULK1 also phosphorylates Beclin1 (Atg6) which, in turn, initiates the activity of the class III phosphatidylinositol 3-kinase (PI3K) complex (Beclin1 and vacuolar proteins sorting 34 and 15 (VPS34, and VPS15)) for nucleation of the phagophore [96-98].

*Elongation* of the phagophore is mediated by two ubiquitin-like systems (ULS) [96]. In the Atg5-Atg12 conjugation system (first of the two ULS), Atg12 is activated by Atg7 (an E1-like enzyme) and is then transferred to Atg10 (an E2-like enzyme) that is on the target protein Atg5. This Atg5-Atg12 will form a dimeric complex with Atg16, which will target the phagophore membrane. The second ULS involves the cleavage of Atg8 by Atg4 (cysteine protease) and, subsequently, the processing by the ubiquitin-like enzymes Atg7 and Atg3 [96]. Together, these ULS, along with light chain 3 (LC3), extend the autophagosome membrane.

*Sequestration* is the process by which the isolation membrane encircles the damaged organelle. This is mediated through the binding of LC3 to a variety of different proteins/receptors that detect damaged organelles [97, 99, 100]. LC3 is processed by a cysteine protease to its cytosolic form, LC3I. Cytosolic LC3I then conjugates with phosphatidylethanolamine (PE) associated with the inner and outer membrane of the phagophore to form LC3II [101]. The phagophore will then continue to elongate until it
completely engulfs its cargo and matures into an autophagosome.

In the *degradation* phase, the autophagosome fuses with the lysosome, resulting in the degradation of its cargo via acid hydrolase enzymes [94, 95]. In the fusion process, soluble NSF attachment protein receptor (SNARE) proteins, endosomal coating proteins (COPs), the endosomal sorting complex require for transport (ESCRT III) complex, the homotypic fusion and protein sorting (HOPS) complex, LAMP proteins, GTPase Rab proteins, the Beclin 1 binding protein Rubicon, and chaperon HSP70 family proteins have all been implicated to play contributing roles [102-109]. More specifically, Chen et al. reported that tectonin beta-propeller repeat-containing protein 1 (TECPR1) of the lysosome binds phosphatidylinositol 3-phosphate upon conjugation of Atg12-Atg5 to promote autophagosome-lysosome fusion [110]. After degradation, the cargo’s amino acid...
acids and lipids can then be reused for synthesis of new organelles.

2.4 Endosomal-Mediated Degradation: Another Avenue for Degradation

Endosomal-mediated degradation is a cellular degradation process that sequesters cellular components into ILVs through three distinct steps: i) identification and recruitment to the endosome, ii) sequestration, and iii) degradation (Fig. 1-5) [77, 111]. The endosomal sorting complex required for transport (ESCRT) complexes play critical roles in this degradation pathway and are named in order of their recruitment: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III.

Identification and recruitment to the endosome occurs through the interaction and recruitment of ESCRT-0, ESCRT-I, and ESCRT-II to ubiquitinated proteins [111, 112]. ESCRT-0, which is composed of two subunits in mammalian cells, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal transducing adaptor molecule1/2 (STAM1/2), will i) bind to ubiquitinated proteins, ii) bind to phosphatidylinositol 3-phosphate (PtdIns(3)P), an endosomal lipid, to recruit the entire complex to the endosome, and iii) recruit ESCRT-I to the endosome. The ESCRT pathway is carried out in three phases: identification and recruitment, sequestration, and degradation. Identification of ubiquitinated cargo is mediated by ESCT-0. ESCRT-0 recruits ESCRT-I to the ubiquitinated cargo where it binds to ubiquitin and recruits ESCRT-II. Recruitment of the cargo to early endosomes is mediated by ESCRT-0 and ESCRT-II through their PtdIns(3)P binding domains. ESCRT-II recruits and activates ESCRT-III to induce sequestration. ESCRT-III oligomerizes around the ubiquitinated cargo, recruits Vsp3/Vta1, and in a concerted fashion mediate pore formation, ESCRT disassembly, and vesicle scission. In the degradation phase, lysosomes will fuse with late endosomes, releasing acid hydrolase enzymes that degrade the contents.

![Endosome pathway diagram](image)
endosome [111-115]. ESCRT-I, composed of tumor susceptibility gene 101 (Tsg101), multivesicular body 12 (Mvb12), vacuolar sorting protein 28 (Vps28), and Vps 37 in mammalian cells, will help bind ubiquitinated proteins and recruit ESCRT-2 [116-121]. Like ESCRT-0, ESCRT-II, composed of ELL-associated protein of 30kDa (EAP30), EAP45, and EAP20, can also bind to ubiquitin as well as PtdIns(3)P, which help provide endosomal localization [122-126]. In addition, ESCRT-2 plays a critical role in initiating ESCRT-III assembly [111].

-Sequestration- is mediated by ESCRT-III and consists of four subunits of the charged multivesicular body proteins (CHMPs): CHMP6, CHMP4, CHMP3, and CHMP2 [111, 127]. Once activated by ESCRT-II, CHMP6 nucleates the oligomerization of two CHMP4 filaments around the ubiquitinated cargo in either a parallel or branched fashion [128, 129]. CHMP3 provides the cap for the two CHMP4 filaments and recruits CHMP2 to complete ESCRT-III assembly [111, 128]. Once assembled, CHMP2 recruits Vps4, a class I ATPase associated with various cellular activities (AAA), and vesicle trafficking1 (Vta1), which in a concerted process mediate pore formation, ESCRT disassembly, and vesicle scission [111, 130, 131].

Similar to autophagy, degradation is marked by the fusion of LEs to lysosomes and the resulting degradation of its cargo with acidic hydrolases [132, 133]. Along with SNARE protein interaction, Rab interacting lysosomal protein (RILP) interacts with the HOPS complex in a Rab7 dependent manner to mediate fusion of the lysosome [132, 134]. In addition, the interaction of RILP and Rab7 help regulate the assembly and function of V-ATPase for acidification [135, 136]. Similar to autophagy, following degradation, the cargo’s amino acids and lipids can be reused for synthesis of new organelles.
2.5 Identification of Dysfunctional Mitochondria: The Pathways of Mitophagy

Four pathways have been identified that detect dysfunctional mitochondria and recruit autophagosomes for degradation (summarized in Fig. 1-6). The most well-known pathway is *PINK1/Parkin-mediated mitophagy* [137, 138], named for its role in the pathogenesis of Parkinson's Disease. PINK1 contains a mitochondrial targeting domain such that, in healthy mitochondria, it is: (i) transported into the inter-membrane space (IMS) through the translocase of the outer mitochondrial membrane (TOM), then (ii) integrated into the inner mitochondrial membrane via insertion into the translocase of the inner mitochondrial membrane (TIM) [139], and (iii) rapidly processed and degraded by the mitochondrial membrane peptidase and presinilin-associated rhomboid-like protease (PARL). Under healthy conditions, this rapid degradation serves to keep the mitochondrial...
concentration of PINK1 low [140]. However, TIM-mediated import of protein relies on a steady mitochondrial membrane potential. When mitochondria are depolarized, PINK1 can no longer be inserted into the mitochondria, inhibiting its proteolytic cleavage and subsequent degradation [141]. PINK1 then accumulates on depolarized mitochondria, where it phosphorylates and activates a myriad of proteins including Parkin, ubiquitin, and TANK binding kinase 1 (TBK1) [137, 142-146] (Fig. 1-6A).

Parkin is an E3 ubiquitin ligase that is activated by phosphorylation by PINK at Ser65 within its ubiquitin like domain [147, 148]. In addition, Parkin has also been reported to be activated by PINK1-dependent phosphorylation of ubiquitin at Ser65 [144, 146, 149]. When activated, Parkin will ubiquitinate numerous outer mitochondrial membrane proteins including mitofusins and voltage dependent anion channel (VDAC) [69, 150, 151]. Interestingly, the ubiquitin chains generated by Parkin are major targets of PINK1 phosphorylation, allowing Parkin retention on mitochondria, providing a feed-forward mechanism to promote mitophagy [143, 149]. Concurrently, PINK1 also phosphorylates TBK1 at Ser172, promoting the phosphorylation of three different autophagy adaptor proteins: p62 (also known as sequestrome 1 (SQSTM1)), optineurin (OPTN), and nuclear dot protein (NDP52) [152-154]. The aggregation of dysfunctional mitochondria is mediated via p62, while OPTN and NDP52 serves as receptors for the phagophore via ubiquitin and LC3 binding domains in autophagosomal mitophagy[153-158] (Fig. 1-6A). Histone deacetylase 6 (HDAC6) will also translocate upon ubiquitination of outer mitochondrial membrane proteins and has been shown to enhance fusion of the autophagosome and lysosome [159, 160]. Likewise, in endosomal-mediated mitophagy, ESCRT complexes on EE will recognize and capture Parkin ubiquitinated mitochondria [161, 162]. ESCRT machinery then internalizes the captured mitochondria via
invagination and subsequent scission of the endosome membrane [161, 162]. EEs containing mitochondria mature into LEs and fuse with lysosomes to finalize degradation. Additionally, recent evidence suggests that TBK1 may play a role in promoting PINK1-Parkin endosomal-mediated mitophagy through the direct phosphorylation of Rab7a [163]. Whether TBK1 phosphorylation of Rab7a drives mitophagy or is a downstream effector of mitophagic processes still remains to be elucidated.

Mitophagy can also be regulated in a receptor-mediated fashion. One of these pathways is through BNIP3/NIX, which are B-cell CLL/Lymphoma 2 (BCL-2) related proteins. These proteins play a dual role by both: (i) inducing mitochondrial apoptosis, and (ii) localizing to the mitochondrial membrane and acting as autophagy receptors where they can directly bind to LC3 [164-168] (Fig. 1-6B). This pathway is distinct from the PINK1/Parkin-mediated mitophagy in that PINK1/Parkin requires depolarized mitochondria to initiate mitophagy, whereas BNIP3 can activate mitophagy in mitochondria that have a stable membrane potential [137, 138, 169]. BNIP3 does recruit Parkin to mitochondria, and it has been shown that Parkin-deficient myocytes display a reduction in mitophagy despite overexpression of BNIP3 [170]. Although these proteins have dual function in activating mitophagy or inducing apoptosis, it is unclear how they are recruited for each divergent role.

FUNDC1 (FUN14 domain containing 1) is an outer mitochondrial membrane protein that mediates mitophagy through receptor binding with LC3 and has been implicated to play a role in hypoxia-mediated mitophagy [171] (Fig. 1-6C). FUNDC1 is regulated by (casein kinase 2 (CK2) and the mitochondrial serine/threonine protein phosphatase PGAM5 [172]: specifically, CK2 phosphorylates FUNDC1 to inhibit its function while, during hypoxia, PGAM5 phosphatase dephosphorylates FUNDC1 to
activate its binding to LC3 and thus promote mitophagy. This pathway has been shown to be related to both PINK1/Parkin and BNIP3 primarily through PGAM5. PGAM5 phosphatase activity is required for PINK1 stabilization as well as PINK1/Parkin-mediated mitophagy, and PGAM5 deficient mice develop Parkinson’s Disease [173]. BNIP3 has also been shown to be activated in hypoxia and induce mitophagy [174]. Whether or not these three pathways communicate with one another is still a question that needs further investigation.

Cardiolipin is a lipid predominantly localized to the inner mitochondrial membrane, is involved in mitochondrial metabolism and, interestingly, has also recently been implicated in receptor-mediated mitophagy [175, 176]. When oxidized, cardiolipin undergoes redistribution and externalization to the surface of damaged mitochondria where it is recognized by LC3 [176] (Fig. 1-6D). Nucleoside-diphosphate kinase-D (NDPK-D (NM23-H4)), a hexameric intermembrane space protein, mediates the externalization of cardiolipin in artificially depolarized mitochondria [177]. How this process may interact with the PINK1/Parkin pathway is still unknown, but may provide novel insight to a potential role for cardiolipin signaling in pathologies involving mitochondrial membrane depolarization that occurs during I/R injury.

2.6 Mitochondrial Biogenesis

Mitochondrial biogenesis refers to the growth and division of pre-existing mitochondria. After mitochondria are degraded, the existing mitochondrial pool needs to continue growing to keep pace with energy demands of the cell. The increase in mitochondrial content involves an array of processes that include protein and lipid synthesis driven by both nuclear and mtDNA transcription. The double-stranded circular mtDNA is about 16.5 kb in length and contains 37 genes that encode for 13 proteins
(subunits of electron transport chain complexes), 22 transfer RNAs, and 2 ribosomal RNAs necessary for translation [178]. Similarly, lipids such as phosphatidylethanolamine, phosphatidylglycerine, and cardiolipin are synthesized within the mitochondria from ER-derived phospholipids [179]. The rest of the ~1000 proteins and lipids come from the nucleus and ER, respectively. This coordinated import and synthesis of proteins and lipids are essential for healthy mitochondrial biogenesis.

Peroxisome proliferator-activated receptor co-activator (PGC-1α) is considered to be the primary regulator of mitochondrial biogenesis [180]. PGC-1α is induced under conditions of increased energy demand such as fasting, cold, and exercise where it increases the expression of, and co-activates, a variety of transcription factors [180-183]. These transcription factors include the nuclear respiratory factors (NRF1/2), peroxisome proliferator-activated receptor (PPAR), as well as estrogen-related receptors (ERR) [180]. NRF1 and NRF2 promote the expression of the nuclear encoded mitochondrial transcription factor A (Tfam), which is responsible for the transcription of mtDNA [184]. As described previously, mtDNA gives rise to 13 subunits of the electron transport chain as well as the 22 tRNAs and 2rRNAs. The nuclear proteins come from the transcriptional activity of PPARs and ERRs, which are involved in regulating the expression of proteins and enzymes that control multiple aspects of mitochondrial oxidative metabolism ranging from fatty acid transport and oxidation, glucose utilization, the TCA cycle, to oxidative phosphorylation [185]. Once transcribed in the nucleus, the mRNA is then translated in the cytosol complete with a mitochondrial localization signal. The proteins are subsequently transported in an unfolded fashion with the aid of molecular chaperones such as Hsp70 and inserted into the mitochondria through protein translocases, including TOM and TIM (both involved in the translocation of PINK1), as well as presequence
translocase-associated motor (PAM), and sorting and assembly machinery (SAM) [186].

Lipids, on the other hand, are primarily synthesized in the ER and transported to the mitochondria during biogenesis [187]. The transfer of primarily phospholipids [188] from the ER to mitochondria has been thought to be mediated via ER-mitochondrial contacts, effectively termed the mitochondria-associated membranes (MAMs) [189]. The MAMs are purportedly comprised of a variety of proteins including: (i) the IP3 receptor and VDAC1, through Grp75, that play a role in calcium signaling, (ii) the mitofusins, expressed both on mitochondria and ER membranes, that play a role in tethering and modulating mitochondrial dynamics, (iii) the ER stress sensor PERK that initiates signaling in response to ER stress, and (iv) many more [190-192]. Although it remains unclear how lipids are transported in MAMs, in yeast it is thought that ER-mitochondrial encounter structure (ERMES) are responsible [193]. ERMES are composed of the structural components maintenance of mitochondrial morphology 1 (MMM1), mitochondrial distribution and morphology 34 (Mdm34), Mdm12, and Mdm10 as well as a the regulatory subunit GTPase EF-hand protein of mitochondria (Gem1) [194]. The ERMES complex possesses a synaptotagmin-like mitochondrial-lipid-binding (SMP) domain that harbors an elongated hydrophobic groove in which different lipids can bind and possibly be transported [195]. Once the lipids are transported from the ER, mitochondrial enzymes can then synthesize the lipids critical for mitochondrial function.

2.7 The Interplay Between Mitochondrial Dynamics and Mitophagy

Mitochondrial dynamics and mitophagy have been thoroughly examined individually, but investigations aimed at elucidating the interplay between these two components of mitochondrial quality control have been limited. It has been shown that fission can trigger mitophagy and govern mitochondrial clearance [18, 196]. In this regard, multiple studies
have demonstrated that alterations to pro-fusion or pro-fission proteins can affect mitophagy: i.e., inhibition of Fis1 in insulin secreting (INS1) cells resulted in a 70% reduction of mitophagy, while overexpression of Drp1 in HeLa cells was accompanied by a 70% decrease in mitochondrial mass [196, 197]. Further evidence that fission and mitophagy are intimately associated is that the Drp1-dependent mediator of fission, endophilin B1, colocalizes with autophagic markers LC3, Atg5, and Atg9 specifically in response to nutrient starvation [198, 199]. Conversely, proteins associated with mitophagy (in particular, excessive PINK1 in depolarized mitochondria) may also play a role in fission by mechanisms that are, at present, unclear. It has been proposed that when PINK1 accumulates and recruits Parkin, Parkin ubiquitinates mitofusins to inhibit fusion [17]. Accordingly, in a state where all mitochondria are depolarized with PINK1 accumulation and mitofusin ubiquitination, the only path for mitochondria would be fission. However, although fission is apparently necessary for mitophagy, mitophagy is not necessary for fission [69, 137, 170, 196, 200, 201].

3. Mitochondrial Quality Control in Disease

As discussed in the previous sections, mitochondrial dynamics and mitophagy are essential regulators of mitochondrial quality control and play a role in maintaining mitochondrial homeostasis in healthy cells. Defects in mitochondrial quality control have also been implicated to contribute to both chronic and acute neurological and cardiovascular diseases; however, little is known about how mitochondrial dynamics and mitophagy interact/communicate with each other under pathophysiological conditions.

3.1 Chronic Diseases: Parkinson’s and Alzheimer’s Disease

3.1.1 Parkinson’s Disease

The pathologic signature of PD is the accumulation of damaged protein aggregates
such as α-synuclein (SNCA) and ubiquitin into intracytoplasmic inclusions termed Lewy bodies. PD has been associated with mutations, sporadic or hereditary, in at least 6 genes that are responsible for generating mutations that encode the following proteins: SNCA, Parkin, β-glucocerebrosidase (GBA), PINK1, the protein deglycase DJ1, and leucine-rich repeat kinase 2 (LRRK2) [202]. Interestingly, these genes give rise to proteins that are associated with mitochondria or located within mitochondria, thereby implicating mitochondria as key players in PD [203-206]. Evidence of mitochondrial abnormalities in PC (including reduced complex I activity, reduced mitochondrial membrane potential, increased ROS production, altered mitochondrial dynamics, impaired mitochondrial trafficking, and increases in mtDNA mutations) underscore this association [207-211].

To maintain a healthy mitochondrial network, cells must undergo mitophagy to dispose of damaged and dysfunctional mitochondria and produce new healthy mitochondria via mitochondrial biogenesis. In PD, patients with PINK1 and Parkin mutations display impaired mitophagy. Mutations and defects in PINK1: (i) have the potential to diminish both the mitochondrial translocation and activation of Parkin, (ii) can result in the failure to segregate dysfunctional mitochondria for mitophagy via fission [212], and (iii) have been associated with a decrease in phospho-Drp1 levels and an increase in Drp1 GTPase activity, suggesting a direct role of PINK1 to induce fission [213]. PINK1 deficiency has also been shown to be associated with dysfunctional Na⁺/Ca²⁺ exchangers in the inner mitochondrial membrane that cause unbalanced mitochondrial calcium homeostasis [208]. This impairment of calcium efflux from the mitochondria results in reduced respiration from ROS-stimulated inhibition of glucose uptake. Finally, and not surprisingly based on the aforementioned associations, mutations in PINK1
reportedly increase the sensitivity of cells to stress-induced cell death. Studies have shown that PINK1 is necessary for long-term survival of cells [203, 214].

Mutations in Parkin, on the other hand, can lead to impaired ubiquitination of outer mitochondrial membrane proteins, which has been shown to play a role in recognition by the autophagosome [215]. Interestingly, Parkin was first linked to the mitochondria by evidence that the protein prevented mitochondrial swelling and cytochrome C release in cells treated with ceramide [216]. In addition to this purported neuroprotective role, Parkin was found to protect mtDNA from oxidative stress and stimulate mtDNA repair systems [217], while, in strains of Parkin knockout mice, neurons in the ventral midbrain displayed severe mitochondrial damage and decreases in complexes I and IV, despite being devoid of the phenotypical motor impairment characteristic of PD [218, 219]. It remains unclear how deficiencies in Parkin lead to severe mitochondrial damage and PD. Mitochondria do undergo a basal level of “wear and tear” via mtDNA mutations as well as oxidation of lipids and proteins. As discussed previously, under normal conditions, these damaged mitochondria would be sequestered and undergo proteolytic degradation. If, in PD, defects in PINK1 and Parkin compromise the ability of the cell to degrade and dispose of proteins or damaged mitochondria, the accrual of damaged organelles would, in all likelihood, ultimately lead to cell death.

3.1.2 Alzheimer’s Disease

AD currently affects 1.5 million Americans, with the associated memory loss and decline in cognitive function attributed to the accumulation of amyloid-β plaques and phosphorylated tau [220]. Sporadic and hereditary AD are attributed to mutations in several genes, as well as accumulation of mtDNA mutations that generally lead to an increase in β-amyloid levels in the brain [221, 222]. Although the underlying mechanisms
are unclear, accumulation of amyloid-β in neurons and formation of plaques has been attributed to excessive cleavage of amyloid precursor protein (APP: a transmembrane glycoprotein) or mutations in the apolipoprotein APOE4, which, under normal conditions, contributes to the breakdown of amyloid-β [223] [224].

Early in the pathogenesis of AD, mitochondrial abnormalities are also common, including defective glucose metabolism, a reduction in enzyme activity, mitochondrial DNA mutations, defected gene expression, and aberrant mitochondrial dynamics [225]. Mitochondria in AD patients have been observed to reveal significant structural damage together with decreases in mitochondrial fusion proteins, increases in Fis1, and increases in Ser616 phosphorylated Drp1 (despite decreases in total Drp1) – all of which favor excessive fragmentation [226, 227]. In vitro studies corroborated this concept: i.e., overexpression of APP in M17 cells was associated with mitochondrial fragmentation, reduced neurite growth, abnormal mitochondrial distribution, and modulation of mitochondrial fission/fusion proteins [228]. Similar findings were obtained in primary neurons of transgenic mice expressing the human APP Swedish mutation [229]. The interactions between amyloid-β and Drp1 are still unknown, but limited data have proposed GSK3β may be the mediator in Drp1 phosphorylation via the association of amyloid-β with NMDA receptors and the Wnt signaling pathway [230]. With excessive fission, healthy mitochondria are cleaved unnecessarily, thereby disrupting the equilibration of mitochondrial matrix metabolites (required for efficient production of ATP) and making the mitochondria more vulnerable to injury. In addition, this excessive fragmentation could potentially lead to an upregulation in mitophagic pathways.
4. Insights into I/R Injury

4.1 Fission, Fusion and Cell Fate

There has been growing interest in mitochondrial dynamics, and its potential association with apoptosis, in the setting of I/R injury [231-234]. Several studies have uncovered excessive mitochondrial fission or fragmentation during both ischemia and I/R injury [235-238]. Using a 6-hour OGD model to simulate ischemia, Kim et al. observed a massive mitochondrial fragmentation profile during OGD in H9C2 cells [235]. This mitochondrial fission profile was confirmed in vivo using a 24-hour left anterior descending permanent ligation model in mice [235]. Disatnik et al. and Ong et al. observed mitochondrial fragmentation during reoxygenation in OGD/reoxygenation models using neonatal primary cardiomyocytes and HL1 cells respectively [237, 238]. In the brain, Tang et al. also demonstrated a highly fragmented mitochondrial profile in mouse N2a neuroblastoma cells following OGD/reoxygenation [236]. Previous studies from our lab, conducted using both primary rat neurons and HT22 cells, revealed evidence of mitochondrial fission during OGD and reoxygenation [239]. Moreover, mitochondrial fragmentation was accompanied by Opa1 processing and concomitant release of cytochrome C [239]. Using an in vitro real time imaging model of OGD/reoxygenation, we further observed complex temporal alterations in mitochondrial morphology [240]. Using HT22 cells transfected with a plasmid containing a GFP-marker, two distinct phases of fragmentation were detected: the first phase of fission occurred during OGD, while reintroduction of oxygen triggered initial fusion followed by complete and massive fragmentation after late reoxygenation. The massive fragmentation observed during late reoxygenation was confirmed in vivo in CA1 hippocampal neurons of rats exposed to global brain ischemia/reperfusion [240].
The aforementioned studies suggest that mitochondrial fragmentation is a pathophysiological consequence of I/R injury and that inhibition of mitochondrial fragmentation may reverse this. Indeed, in support of this concept, there is evidence that, after exposure to apoptotic stimuli, Drp1 inhibition or overexpression of a dominant negative Drp1 blocked the induction of apoptosis [241]. Moreover, inhibition of Drp1 was found to be neuroprotective in response to OGD *in vitro* and transient focal ischemia *in vivo* [242], and cardioprotective in cultured HL-1 cardiomyocytes subjected to OGD and reoxygenation [243]. However, in the latter study, cardioprotection was only seen when inhibition of Drp1 was initiated as a pretreatment; cell death was paradoxically exacerbated when treatment was administered during reoxygenation [243]. This points to the complexity of mitochondrial dynamics and its effects on cell death or survival – an issue that is highlighted by observations that fusion (presumably favoring survival) involves the formation of lipidic pores that may contribute to mitochondrial permeabilization and compromise cell viability [244], while fission (as discussed above, associated with cell death) is necessary for mitophagy and governs clearance of dysfunctional mitochondria [18, 196]. Thus, despite strong evidence to suggest that mitochondrial fragmentation can be detrimental to the cell during stress conditions, collectively these results reveal a complex dynamic nature of mitochondria that requires further study to understand (i) why fission occurs during these stress states (ii) why inhibition of mitochondrial fission is only cardioprotective when initiated before the OGD/ischemic event and (iii) why fusion is not possible after reoxygenation/reperfusion.

### 4.2 Mitophagy and I/R injury

I/R injury has been shown to activate mitophagy pathways through multiple signals. During the ischemic phase when ATP production halts, AMPK pathways are
upregulated to initiate autophagy [245]. AMPK activates ULK1 via phosphorylation, which will activate the class III PI3K complex (Beclin1, VPS34, and VPS15) that initiate nucleation of the phagophore [246]. Interestingly, ULK1 may have a redundant role in activating mitophagy: i.e., has also been shown to translocate to mitochondria and activated the FUNDC1 receptor [247]. During the reperfusion phase, ROS serves as a signaling molecule to inhibit the mechanistic target of rapamycin (mTOR) pathways, thereby contributing to the initiation and nucleation of the autophagosome [248]. ROS has also been shown to activate mitophagy via BNIP3 although, as stated previously, high levels of BNIP3 can induce apoptosis [249, 250]. Overexpression of BNIP3 in HL-1 myocytes was reported to increase cell death in response to simulated I/R injury by facilitating mPTP opening through the activation of Bcl-2-asscoaited X protein (Bax) [164, 250]. Moreover, BNIP3−/− mice subjected to 1 hour coronary artery occlusion and 3 week reperfusion exhibited preserved left ventricular (LV) systolic function and diminished LV dilation, while conditional overexpression of BNIP3 reversed these effects, resulting in increased apoptosis and infarct size [251]. Collectively, these results demonstrate a threshold for BNIP3, as increases or overexpression will inevitably lead to increased apoptosis. Given the dual “life-or-death” role of mitochondria, together with reports of the strong association between mitophagic proteins (ie. BNIP3 and Drp1) and programmed cell death, this raises the question of whether mitophagy is beneficial or detrimental to cell fate in response to I/R injury.

4.2.1 Heart

During ischemia, up-regulation of mitophagy is agreed to confer protection [252, 253]. The most compelling evidence is provided by Kubli et al. Using an in vivo mouse model, the investigators demonstrated that Parkin deficient mice are more sensitive to
myocardial infarction [253]. Following permanent left anterior descending coronary artery occlusion, Parkin deficient mice displayed accumulation of swollen and dysfunctional mitochondria due to impaired mitophagy, which resulted in larger infarcts and reduced survival rates [253]. Moreover, the investigators observed up-regulation of mitophagy with increased expression of Parkin at the border zone of the infarct in wild-type mice [253]. In vitro studies corroborated this concept: i.e., overexpression of Parkin in isolated cardiomyocytes subjected to hypoxia-mediated cell death was associated with increased Parkin translocation to the mitochondria and increased cell viability, while cardiomyocytes expressing Parkinson disease-associated mutants of Parkin failed to reduce hypoxia-mediated cell death [253]. In accordance with this concept, evidence in the in vivo mouse model of permanent coronary ligation revealed that the up-regulation of mitophagy via the genetic deletion of two molecular inhibitors, p53 and TP53-induced glycolysis and apoptosis regulator (TIGAR), attenuated apoptotic cell death and provided resistance to subsequent remodeling [252]. Moreover, cardioprotection was reversed in p53-/- and TIGAR-/- mice following permanent myocardial infarction with administration of chloroquine, an autophagy inhibitor, an effect characterized by the accumulation of abnormal mitochondria in the ischemic myocardium [252]. Interestingly, the up-regulation of mitophagy via inhibition of p53 and TIGAR was induced through an increase in ROS production followed by BNIP3 activation [252]. In this case BNIP3 activation was necessary and beneficial in attenuating cardiac I/R injury.

In contrast to ischemic injury, the role of mitophagy in I/R injury remains controversial. A considerable body of evidence suggest that an up-regulation of mitophagy during myocardial I/R injury is protective [166, 254-256]. It was first described that upregulation of autophagy in HL-1 cells protected against simulated
ischemia/reperfusion by Hamacher-Brady et al [256]. The investigators observations revealed that autophagosomal engulfment of mitochondria was a prominent response in their model. Subsequent studies using HL-1 cells demonstrated an upregulation of BNIP3-regulated mitophagy following simulated I/R [166]. Purportedly, overexpression of BNIP3 during simulated I/R induces mitochondrial damage via ROS production, leading to an up-regulation of mitophagy [166]. Expression of ATG5K130R, a dominant negative of ATG5 shown to suppress vacuole formation, significantly reduced mitophagy and increased BNIP3-induced cell death [166]. Together, these data suggest that up-regulation of mitophagy occurs following BNIP3 induced mitochondrial damage as a cellular response to remove damaged mitochondria during I/R [166]. More recently, using Langendorff heart I/R model, Lu et al. observed that PGAM5 deficient mice had exacerbated necroptosis in response to 25 minutes of ischemia followed by 90 minutes of reperfusion [254]. Data in their mouse embryonic fibroblasts (MEF) model of ROS-dependent necroptosis revealed impaired autophagic removal of LC-3II as well as impaired mitochondrial clearance following 24 hours of TNF-α cycloheximide and z-VAD-fmk (TCZ) stimulation. Finally, up-regulation of mitophagy has been reported to play a role in the gold standard of cardioprotection, ischemic preconditioning (IPC) [255]. In both Langendorff perfused rat hearts and in vivo mice subjected to regional IPC, Parkin and P62 translocate to the mitochondria and mediate mitophagy [255]. Moreover, IPC is abolished in Parkin-deficient mice, suggesting a critical role for Parkin in IPC [255]. The investigators propose that selective mitophagy of mitochondria that have the lowest threshold for mPTP opening during IPC would leave behind mitochondria that are more equipped to handle sustained ischemic insults [255].

In contrast to the aforementioned studies, there is some evidence to suggest that
the suppression of mitophagy may protect the heart from I/R [257]. In a rat model of left anterior descending coronary artery occlusion, pre-treatment with mitochondrial aldehyde dehydrogenase 2 (ALDH2), an allosteric tetrameric enzyme responsible for the metabolism or detoxification of toxic aldehydes, conferred cardioprotection via attenuation of apoptotic cell death [257]. *In vitro* studies corroborated this concept using H9C2 cells subjected to 2 hours of hypoxia (1% oxygen) and 1 hour of reoxygenation [257]. Pre-treatment of ALDH2 increased cell viability through the suppression of mitophagy [257]. Mitophagy was measured through co-localization of PINK1, Parkin, and the mitochondrial electron transport chain protein cytochrome C oxidase subunit IV (COXIV) [257]. However, given that mitophagy is in constant flux and mitochondria are degraded in autophagolysosomes, mitophagy proteins such as PINK1 and Parkin that are associated with dysfunctional mitochondria would also be degraded. Therefore, evaluation of the mitophagic flux would aid in confirming that ALDH2 is suppressing mitophagy as opposed to enhancing mitophagy and subsequently breakdown of mitophagy proteins.

4.2.2 Brain

There are notable differences between brain and heart in that controversy lies with respect to the role of mitophagy in ischemia as well as I/R (beneficial or detrimental). Cerebral ischemic preconditioning has been associated with increased autophagosome formation and confers neuroprotection in rats subjected to permanent middle cerebral artery occlusion (pMCAO) [258]. More recent evidence in the same model of pMCAO confirmed that the up-regulation of autophagy during ischemia includes mitophagy, revealed by increased autophagic vacuoles containing mitochondria and LC3 co-localization with COXIV [259]. Interestingly, the up-regulation of mitophagy was mediated
through Drp1 as treatment with Mdivi, a pharmacological inhibitor of Drp1, prevented mitophagy and resulted in decreased LC3 and COXIV co-localization, increased levels of mitochondrial proteins, and the accumulation of damaged mitochondria following 1 hour of pMCAO [259]. Moreover, Drp1 inhibition exacerbated mitochondrial-mediated brain injury to an even greater extent compared to the inhibition of autophagy using 3-MA following 24 hours of pMCAO [259]. Conversely, in mice subjected to pMCAO, administration of 3-MA conferred neuroprotection via a reduction in infarct size along with a dose-dependent increase in cell viability following exposure of 4 hour OGD in rat primary cortical neurons [260]. Knockdown of Atg7 with siRNA reinforced this concept, resulting in increased cell viability in response to ischemia-induced neuronal injury [259]. Interestingly, the investigators observed no change in infarct volume with administration of mdivi in both in vitro and in vivo models of ischemia [260]. The lack of observable change could be a result of when the inhibitor was administered, i.e. at the point of artery occlusion or the beginning of OGD. Results from our lab demonstrate that mitochondria undergo massive fragmentation within 20 minutes of ischemia [240]. In this case, mitochondrial fragmentation could have occurred before the onset of inhibition with mdivi, resulting in segregated mitochondria ready to be recruited for mitophagy.

In cerebral I/R, there is a consequential amount of literature that suggests an up-regulation of mitophagy confers neuroprotection [254, 260-262]. The strongest evidence to support this concept is provided by Zhang et al [260]. Using pharmacological and genetic suppression of autophagy (i.e. 3-MA treatment, Bafilomycin A1 (BafA) treatment, Atg5 knockout, and Atg7 knockdown), primary cortical neurons subjected to 2 hours of OGD followed by a 24-hour reperfusion had significantly decreased cell viability as compared to controls [260]. These results were affirmed in an in vivo transient MCAO
model, where inhibition of autophagy via 3-MA treatment and Atg7 knockdown exacerbated I/R injury [260]. Moreover, addition of rapamycin, a known enhancer of mitophagy that attenuates mitochondrial dysfunction following cerebral ischemia [261], partly reversed the deleterious effects of 3-MA treated primary neurons subjected to OGD-reperfusion [260]. Similar to heart, PGAM-/- mice subject to transient MCAO displayed significantly higher infarct sizes as compared to wild type mice after 72 hours of reperfusion [254].

At variance with the preceding studies, evidence provided by Shi et al. suggests that excessive mitophagy following cerebral I/R results in cell death [263]. Using an adapted Rice-Vanucci model of neonatal stroke or hypoxia-ischemia encephalopathy (HIE), the investigators observed that pups deficient of BNIP3 had a decrease in mitophagy that resulted in significantly smaller infarct sizes in response to neonatal stroke and 7 days of reperfusion [263]. Interestingly, the infarct volume in BNIP3-/- pups was significantly larger after 1 day of reperfusion, but then recovered while the infarct volume in wild type pups was exacerbated after 3 to 7 days of reperfusion [263]. The investigators also demonstrated in wild type pups, a dramatic increase in BNIP3 mitochondrial-localized homodimer expression in a time-dependent manner following neonatal stroke, accompanied by a significant decrease in mitochondrial proteins from isolated cortical neurons following 6 hours of OGD [263]. Although these data were collected in a neonatal model, it suggests that i) BNIP3 induces excessive mitophagy following I/R, which ultimately exacerbates cerebral I/R injury and ii) underscores the balance of mitophagy required to prevent pro-apoptotic proteins such as BNIP3 to surpass the “death signal” threshold.
4.3 Beyond ‘Good’ Versus ‘Evil’: A Question of Balance?

As reviewed in the preceding sections, there is continued controversy regarding whether upregulation of mitophagy in the setting of I/R is ‘good’ or ‘bad’. It may, however, be more appropriate to consider mitophagy (and mitochondrial quality control as a whole) as a balancing act (Fig. 1-7). For example, it has been proposed that, following reperfusion, mitophagy is essential to clear dysfunctional mitochondria [260]. However, excessive mitophagy coupled with inhibited mitochondrial biogenesis and a global decrease in protein synthesis [264, 265] will result in a decrease in mitochondrial mass and, subsequently, a deficit in ATP production that may fail to meet the demands of the cell. This energy imbalance could eventually cause energy deprivation and cell death. With the other extreme – that is, with minimal mitophagy – damaged mitochondria will not be

**Fig. 1-7. Finding a balance of mitochondrial quality control.** During I/R injury there is excessive mitochondrial fragmentation, favoring an increase in mitophagy. Degradation of dysfunctional ROS-producing mitochondria is critical for survival, however, mitochondrial content decrease would compromise ATP production. Insufficient ATP production paired with inhibited biogenesis will ultimately lead to cell death. On the other hand, if fission were completely inhibited, mitochondrial content would be maintained, but damaged mitochondria would not be segregated and could lead to accumulated mitochondrial dysfunction. The increase in mitochondrial damage would ultimately cause cell death. Therefore, a balance in mitochondrial quality control (i.e., an equilibrium between retaining adequate mitochondrial content for sufficient ATP production versus disposal of dysfunctional mitochondria) is optimal for cell survival after I/R injury.
eliminated and the overall ROS burden would increase. This excessive ROS formation could further induce mitochondrial dysfunction, leading to a feed-forward cycle of ROS production and ultimately cell death (Fig. 1-7).

Accordingly, there is a fundamental need for balance in mitochondrial quality control, and further investigation is needed to define this threshold. If this threshold can be identified, modulation of mitophagy may represent a valuable therapeutic option, with the goal of eliminating dysfunctional mitochondria while still providing sufficient energy to repair cellular damage, restore protein translation, and ultimately return to homeostasis. PINK1 and Parkin (rather than BNIP3 and NIX) may yield the greatest promise as effective targets for manipulation of mitophagy, given their reported favorable association with cell survival together with a lack of involvement in apoptotic pathways [253, 255, 260].

5. Conclusions and Challenges

Mitochondrial quality control is critical for the homeostasis of the mitochondrial network, and a constant balance needed between mitochondrial fission/fusion as well as mitophagy and biogenesis. Disruption of mitochondrial quality control has been proposed to contribute to the pathogenesis of acute and chronic diseases, including Parkinson’s Disease, Alzheimer’s Disease, and ischemia-reperfusion-induced cell death in brain and heart. Accordingly, targeted modulation of one or more of the molecular components involved in mitochondrial quality control provide opportunities for the design of novel therapies. However, to capitalize on this potential opportunity, a greater mechanistic understanding of mitochondrial fission/fusion, mitophagy and mitochondrial quality control – together with the development of improved molecular tools to investigate these complex phenomena [266] – will be required. To overcome these challenges, we have utilized
novel mouse lines such as the mitoQC reporter mice to help visualize mitochondria undergoing degradation in real time. In addition, we have developed a highly specific and sensitive mitochondrial morphology classification system to precisely measure mitochondrial dynamics through the duration of *in vitro* I/R injury. Together, this dissertation aims to further elucidate the role of mitochondrial quality control in the context of cerebral I/R injury.
CHAPTER 2: METHODS

1. Animals (Chapters 4-6)

All experimental procedures were performed in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee under protocol #IACUC-18-08-0767 at Wayne State University and under protocol #PRO00009531 at the University of Michigan. Mitochondrial quality control (mitoQC) reporter transgenic mice (C57BL/6-Gt(ROSA)26Sortm1(CAG-mCherry/GFP)Ganl/J) were provided by Dr. Ian Ganley, University of Dundee, Scotland, UK [267]. Drp1 floxed (Drp1fl/fl) mice (Dnm1flm1.1Hise) were provided by Hiromi Sesaki, Johns Hopkins, Baltimore, MD [268]. Wild Type (WT) mice (B57BL/6Crl) were purchased from Charles River Laboratories.

2. Primary Cortical Neuron Isolation and Culture (Chapters 4-6)

Mouse pups were sacrificed by decapitation on postnatal day 0-2 (P0-P2). Heads were soaked in ethanol for 30 seconds then brains were dissected in ice cold Dulbecco’s phosphate buffered saline (DPBS). A midline incision was made to allow access to the skull using a #10 stainless steel scalpel. The skull was resected away from the midline, and the brain was extracted to isolate the cortex, a midline incision was made along the the corpus callosum to isolate the cortex. Cortices were transferred to a 35 mm dish containing hibernate complete medium (1X Hibernate-A medium (Gibco, A1247501), 2% B27 supplement (ThermoFisher, 17504044), 0.5 mM Glutamax Supplement (Gibco, 35050061), and 1% Penn/Strep (Gibco, SV30010)). Brains were isolated, processed, and seeded separately for individually biological replicates. Cortices were rolled over filter paper to remove the meninges. Cortices were then cut into ~1-2mm pieces and transferred into a 15 ml Falcon tube. Tissue was digested with enzyme digestion solution
(1x hibernate complete medium, 0.06 mg/ml L-cysteine (Sigma, 778672), 1.4X10^-4 N NaOH (sigma, 43617), 10 ng/ml APV (2-Amino-5-phosphonopentanoic acid, Sigma, A-5282), 50 ul of Papain (Worthington, LS 03126)) and incubated at 37°C for 30 minutes. After 30 minutes, the tissue was washed with DPBS twice and replaced with hibernate complete medium and the tissue was dissociated by pipetting up and down 25 times to generate a cell suspension. Cell suspension was passed through a 40-micron strainer to separate tissue debris. Cells were stained with a 1:1 mixture of cell suspension and Trypan Blue and counted with a hemocytometer. Cells were seeded at 190,000 cells/cm² and incubated for 30 min at 37°C in 5%CO₂. After 30 min, media was replaced with neurobasal complete medium (1X Neurobasal Plus medium (Gibco, A3582901), 2% B27 plus (Gibco, A3653401), 0.5 mM Glutamax Supplement (Gibco, 35050061), and 1% Penn/Strep (Gibco, SV30010)) and cultured at 37°C in 5% CO₂. Half-media changes occurred every 3-4 days with neurobasal complete medium.

3. Oxygen Glucose Deprivation and Reoxygenation (OGD/R) (Chapters 4-6)

OGD was achieved utilizing the O₂ Control InVitro Glove Box from Coy Lab Products. The hypoxic chamber was maintained at 0.1% O₂ and 5% CO₂. OGD media (0.20 g/L CaCl₂ (Spectrum, CA138500GM), 0.4 g/L KCl (Fisher Chemical, P217-500), 0.097 g/L MgSO₄ (Fisher Chemical, M65-500), 6.8 g/L NaCl (Fisher Chemical, S2711), 2.2 NaHCO₃ (Acros Organics, AC447102500), 0.14 g/L NaH₂PO₄-H₂O (Fisher Chemical, S369-500, and 0.01 g/L Phenol red (Fisher Chemical, P74-10)) was bubbled with 95% N₂/5% CO₂ inside of the of the hypoxic chamber for 60 min. Cells were transferred into the hypoxic chamber, washed 2X with OGD media, and then incubated with OGD media inside the hypoxic chamber for 180 min. After OGD, cells were taken out of the hypoxic chamber, media was replaced with Neurobasal medium without antioxidants (complete
neurobasal medium with B27-AO (Gibco, 10889038)), and incubated in 37°C 5% CO₂ for 6 h.

4. Lentiviral Transduction (Chapter 6)

Cells were infected with lentivirus on DIV 7. Experiments were performed 7 days post-transduction (DIV14). Lentiviral plasmids and Lentivirus were generated by the vector core at the University of Michigan (Thomas Lanigan, PhD., Director): Lenti-EF1a-Cre-VSVG, Lenti-EF1a-VSVG, and Lenti-EF1a-GFP-VSVG. The EF1-alpha promoter, possessing a broad host range, demonstrated robust, constitutive, and long-term expression of both Cre-recombinase and GFP in our primary cortical neuron cultures (see Chapter 6). Vesicular stomatitis Indiana virus G-protein (VSVG) envelope was used to enhance viral entry. Viruses were made in a concentrated 10X form in Neurobasal Medium. Titration curves were performed with Lenti-EF1a-VSVG and Lenti-EF1a-GFP-VSVG to evaluate toxicity and transduction efficiency respectively. Toxicity was tested at 0.5X, 1X, 2X, 4X, and 5X concentrations. Transduction efficiency was tested at 0.5X and 1X concentrations.

5. MTT Assay (Chapter 6)

Viability was assessed after 2.5h of OGD followed by 6h of reoxygenation by Thiazolyl Blue Tetrazolium Bromide (MTT) assay. MTT (5mg/ml in PBS, Acros Organics, AC158992500) was added to cultures for a final concentration of 0.5 mg/ml and cells incubated in 37°C 5%CO₂ for 2h. Following the 2h incubation, media was removed and replace with MTT solubilization solution (10% Triton-X100 and 12mM hydrochloric acid (Fisher Chemical, A144SI-212) in Isopropanol (Fisher Chemical, A419-4)) and placed on a shaker at room temperature for 15 min. 100 ul of each sample was then pipetted into a 96-well plate and analyzed for absorbance at 570 nm utilizing a PerkinElmer Multimode
Plate Reader containing Enspire interface software.

6. Western Blot (Chapters 5-6)

6.1 Mitochondrial Isolation

To obtain mitochondria, primary cortical neurons were collected in homogenization-A buffer and homogenized utilizing a Teflon Potter-Elvehjem homogenizer (10 strokes at 300 rpm) (Thomas Scientific, 3432S90). Cell homogenates were collected into Eppendorf tubes and centrifuged at 1,000 X g for 10 min at 4°C. The supernatant (containing mitochondria and cytosol) was removed and placed into an Eppendorf tube and centrifuged at 10,000 X g for 10 min at 4°C. The cytosolic supernatant was removed, and the mitochondrial pellet was washed once with homogenization-A buffer followed by another centrifugation at 10,000 X g for 10 min at 4°C. Mitochondrial pellets were resuspended with fresh homogenization-A buffer and sonicated. Whole cell pellets were saved and sonicated to be used as whole cell lysate samples.

6.2 Western Blot – General Methods

Protein homogenates were collected using homogenization-A buffer (10 mM HEPES (pH7.5) (Sigma, H4034), 1 mM EDTA (Sigma, E6758), 1mM EGTA (Sigma, 03777), 100mM KCl (Sigma, P9333), 210 mM Mannitol (Sigma, M4125), 70 mM Sucrose (Sigma, S0389), and 1X Halt™ protease and phosphatase inhibitor cocktail (ThermoFisher, 78440)). Homogenates for evaluating mitochondrial ubiquitination used homogenization-A buffer with the addition of the proteasome inhibitor MG132 (5 uM, Sigma, M7449). Cells were scraped off the plate and collected into a 1.5 mL Eppendorf tube and sonicated. Protein concentration was measured via Bradford Plus Assay Reagent (ThermoFisher, PI23236). Whole cell lysates were loaded with 10 ug of protein, while mitochondrial samples were loaded with 5 ug of protein. Proteins were separated
on polyacrylamide gels (10% 29:1 polyacrylamide/bisacrylamide (Fisher BioReagents, BP1408-1), 375 mM Tris pH 8.8 (Fisher BioReagents, BP152-1), 0.1% sodium dodecyl sulfate (SDS, Sigma, L3771), 0.1% ammonium persulfate (APS, Sigma, #A3678), and 0.1% TEMED (GE Healthcare, # 45-000-226)) and transferred to nitrocellulose membranes at 100V for 90 min. Membranes were incubated with indicated primary antibodies at 4°C overnight. Membranes were then washed 3X, 5 min each wash, with Tris-Buffered Saline with 0.1% Tween (TBST, Fisher Scientific, BP337500) and incubated with the indicated secondary antibodies for 60 minutes at room temperature. Membranes were washed 3X with TBST, then incubated in SuperSignal West Pico Plus Chemiluminescent Substrate (ThermoFisher, 34577) for 5 minutes. The membranes were then imaged utilizing a BioRad ChemiDoc XRS + imager. Images were quantified by densitometry using ImageJ software.

6.3 Western Blot – Antibodies

Dynamin like protein 1 (Dlp1/Drp1) (Clone 8) Mouse antibody (BD Transduction Laboratories, BD611112, 1:1,000, 2% milk), Mono- and polyubiquitinylated conjugates mouse monoclonal antibody clone FK2 (Enzo, BML-PW8810, 1:1,000, 5% milk), PINK1 Rabbit polyclonal antibody (Novus Biologicals, BC100-494, 1:1,000, 2% BSA), Parkin (Prk8) mouse monoclonal antibody (Cell Signaling, 4211, 1:1,000, 2% milk), Rab5 (C8B1) rabbit monoclonal antibody (Cell Signaling, 3547, 1:1,000, 5% BSA), VDAC (D73D12) rabbit monoclonal antibody (Cell Signaling, 4661, 1:1,000, 2% BSA), GAPDH (14C10) rabbit monoclonal antibody (Cell Signaling, 2118, 1:10,000, 2% BSA), LC3 A/B rabbit antibody (Cell Signaling, 4108, 1:1,000, 2% BSA).
7. Immunofluorescence (Chapters 4-6)

7.1 Immunofluorescence – General Method

Cells were fixed with 3.7% PFA (ThermoFisher, 50980487) in 200 mM HEPES (Sigma, H4034) for 15 min at 37°C. Coverslips were washed 3X with PBS and replaced with blocking solution (5% goat serum (Sigma, G9023) and 0.3% Triton-X100 (Acros Organics, 215682500) in PBS) for 60 min. Blocking solution was replaced with indicated primary antibodies in antibody solution (1% BSA and 0.3% Triton-X100 in PBS) at 4°C overnight. After primary incubation, coverslips were washed 3X in PBS and incubated with indicated secondary antibodies in antibody solution for 60 min at room temperature, covered from light. After secondary incubation, coverslips were washed 3X in PBS and then mounted using Fluoroshield with DAPI (Sigma, F6057).

7.2 Immunofluorescence – Antibodies

MAP2 chicken polyclonal antibody (Abcam, ab5392, 1:1,000), Parkin rabbit polyclonal antibody (ThermoFisher, PA5-13399, 1:50), PINK1 Rabbit polyclonal antibody (Novus Biologicals, BC100-494, 1:100), Lamp1 (1D4B) rat antibody (Santa Cruz Biotechnology, sc-19992, 1:100), PINK1 Rabbit polyclonal antibody (Novus Biologicals, BC100-494, 1:100). Secondary antibodies were used donkey anti-mouse or donkey anti-rabbit HRP (Jackson Immuno Research Labs, NC9832458 and NC9736726), Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 633 goat anti-rat (Life Technologies).

8. MitoQC Analysis (Chapters 5-6)

For each coverslip containing mitoQC neurons, 12 random photos were taken utilizing a Zeiss Axio Observer.Z1 inverted microscope with a Colibri.2 module containing 4 LED based filter sets (49 DAPI: 365 nm excitation/445 nm emission, 38 HE GFP: 470 nm excitation/525 nm emission, 43 HE DsRed: 560 nm excitation/605 nm emission, and
81 HE FR: 625 nm excitation/680 nm emission. DAPI channel contained a 160 ms exposure at an intensity of 20%. GFP channel contained 160 ms at an intensity of 100%. mCherry channel contained a 1300 ms exposure at an intensity of 100%. Far red channel contained a 250 ms exposure at an intensity of 30%. Images were taken at a magnification of 63X oil immersion. Each image was deconvolved using the regularized inverse filter method on Zeiss Zen Pro software. Images were further processed in Fiji ImageJ. The following was performed using FIJI’s (ImageJ) batch processing feature in three separate phases: processing, segmentation, and red puncta analysis. The workflow is listed below:

**8.1 Processing:**
- Process > Subtract Background (Rolling Ball Radius = 10.0)
- Process > Filters > Unsharp Mask (Radius = 1.0 pixels, Mask Weight = 0.6)
- Process > Enhance Local Contrast (block size = 127, histogram bins = 256 maximum slope = 3.00, mask = none, checkmark “fast” box)[269]
- Process > Filters > Median (radius = 2.0 pixels)

**8.2 Segmentation:**
Trainable Weka Segmentation was employed to segment red particles. Trainable Weka Segmentation is a plugin in ImageJ that uses a combination of visualization tools and machine learning algorithms to produce a pixel-based segmentation [270]. The program was trained to segment “mitophagy” particles (red-only), “healthy mitochondria” (green/yellow), and “background” (“black”). Once the classifier was trained, photos were segmented using the category “probabilities” as an output.

**8.3 Red Puncta Analysis:**
- Image > Stacks > Stacks to Image
- Close “Background” and “Healthy mitochondria” windows
- Select window titled “Mitophagy
- Image > Adjust > Threshold (0.31, $1 \times 10^{31}$)
- Process > Binary > Convert to Mask
- Analyze > Set Scale (9.7 pixels/micron
- Analyze > Analyze Particles (size = 0.0-Infinity, circularity = 0.0-1.0, show = nothing, check summarize)

Results of red puncta counts were tabulated into an excel sheet for each image and normalized to the number of cells analyzed.

9. Machine Learning Mitochondrial Classification (Chapters 4-6)

Machine learning-based classification of mitochondrial objects was performed in R computing language using the R Caret package [271]. Fiji ImageJ was used for image processing and mitochondrial object measurement collection. R studio was used for all computation.

9.1 Feature Extraction

The green channel of the MitoQC images were used for mitochondrial morphology analysis. Processing was done in the same workflow as above. Trainable WEKA segmentation was employed to segment green mitochondrial signal using “Labels” as an output. Segmented images were then analyzed to obtain measurements of each mitochondrial object. Workflow is listed below:
- Image > Type > 8-bit
- Image > Type > 16-bit
- Image > Type > 8-bit
- Process > Binary > Make Binary
- Analyze > Set Scale (9.7 pixels/micron)
- Analyze > Measure (if mean is \(\leq 0.75\) then invert (Edit > Invert), otherwise continue)
- BioVoxxel_Toolbox (ImageJ plugin) > Extended particle analyzer (Area = 0.3-Infinity, Extent = 0-1, Perimeter = 0-Infinity, Circularity = 0-1, Roundness = 0-1, solidity = 0-1, compactness 0-1, AR = 0-Infinity, Feret AR = 0-Infinity, Ellipsoid angle = 0-180, Max Feret = 0-Infinity, Min Feret = 0-Infinity, Feret angle = 0-180, Coefficient of Variation = 0-1, Show = Nothing, Redirect to = none, Keep borders = none, check “display results”)

Measurements were then expanded using “extended descriptors” macro from the Applied Superconductivity Center [272]. 32 measurements total were recorded for each object: area, perimeter, circularity, feret, FeretAngle, Min Feret, aspect ratio (AR), Round, Solidity, Feret AR, Compactness, Extent, CircToEllipse Tilt, AR_Box, AR_Feret, Round_Feret, Compact_Feret, Elongation, Thinnes Ratio, Angle_0-90, Feret_Angle_0-90, Convexity, Roundness corrected to AR, area equivalent diameter, perimeter equivalent diameter, spherical equivalent diameter, interfacial density, Hexagonal Side, Hexagonal Perimeter, Hexagonal Shape Factor, Hexagonal Shape Factor Ratio, and Hexagonality.

9.2 Random Forest Classifier

Hand classified mitochondrial objects (n=2342) containing the 32 measurements (predictors) listed above were imported into R Studio as the train/test set. These objects were labeled with one of four different classifications: networks (n=236), unbranched(n=851), swollen (n=458), or punctate (n=797). The data was then split based off of their classifications into the training (80%) and the test (20%) sets using the
createDataPartition function from the caret package [271]. A random forest algorithm (rf) from the caret package was trained using the training set through 25 iterations utilizing 32 predictors. The final model consisted of 500 trees with an mtry = 2, which refers to the number of randomly selected predictors considered at each split within the tree.

10. **Statistical Analysis (Chapters 4-6)**

Statistical analysis was performed using GraphPad Prism. PCA was done in R using the built-in function prcomp(). Data is presented as means ± SEM. All experiments were independently repeated in the lab and data were collected using 4-8 biological replicates with 2-3 technical replicates for each experiment. Differences between three or more groups were analyzed using either one-way ANOVA with either Tukey post hoc test (for comparisons between all groups) or Dunnett’s post hoc test (when comparing groups to just the control). Student’s t-test was used to detect differences between two groups. Differences between conditions across time (Lenti-cre vs Lenti-empty) were analyzed using a two-way ANOVA with Dunnett’s post-hoc analysis for differences across time points compared to control. Multiple comparisons to detect differences between conditions were computed using Sidak’s post-hoc test. Differences were considered significant when p < 0.05.
CHAPTER 3: STUDY OVERVIEW

As discussed in Chapter 1, ischemic brain injury caused by cardiac arrest and stroke continue to be the leading causes of death and disability in the U.S. [273]. Mitochondria are key regulators controlling cell survival (via the production of ATP that fuels cellular processes) or, conversely, cell death (via the initiation of programmed cell death) [11, 13]. Therefore, it is imperative to have stringent quality control mechanisms in place to ensure a healthy mitochondrial network. Mitochondrial quality control is carried out through cellular processes that couple mitochondrial fission to the subsequent removal of dysfunctional mitochondria via mitophagy. Establishing a balance between mitochondrial fission/fusion is critical for the disposal of dysfunctional mitochondria that may be potentially harmful to the cell, while maintaining the required amount of mitochondrial content to fuel normal cellular processes. Pathological states, such as I/R injury, disrupt these processes, tipping the scales to excessive fragmentation and possibly upregulated mitophagy. The subsequent loss of mitochondrial content results in insufficient energy production and ultimately cell death. Although these processes have been a major focus of study in the context of I/R injury, quantitative investigation of fission/fusion and mitophagy have been confounded by the dynamic nature of mitochondria. Furthermore, it is poorly understood how i) the pathways of mitochondrial dynamics and mitophagy interact with one another and ii) how disrupting mitochondrial quality control mechanisms contributes to cell death during I/R injury. Given this background, my dissertation will address the following objectives:

Objective 1: Develop a robust method to accurately and precisely measure mitochondrial morphology during OGD/R in primary cortical neurons.

As reviewed in Chapter 1, it is well established that mitochondrial fragmentation
observed during I/R injury is a pathological process associated with cell death. However, most studies are limited in their characterization of mitochondrial morphology. Morphology is typically classified as two different conditions: fused and fragmented. Furthermore, these studies are either purely qualitative or use semi-quantitative scoring system to profile mitochondrial morphology, both of which lack an accurate and precise assessment of different mitochondrial morphologies that are physiologically relevant. In order to overcome this, our goal was to develop a semi-automated machine learning-based classification system for mitochondrial objects. To accomplish this, we employed ImageJ to measure all mitochondrial objects contained in a series of images and imported that data into R studio to classify mitochondrial objects into four different categories (networks, unbranched, swollen, punctate) using the “random forest” machine learning algorithm. We then applied this classification system to mitochondria within primary cortical neurons subjected to OGD/R. We hypothesize that mitochondria undergo extensive remodeling following OGD/R, resulting in a fragmented morphology (see Chapter 4).

Objective 2: Characterize mitophagic flux during OGD/R.

As reviewed in Chapter 1, there is evidence to suggest that mitophagy is upregulated following I/R injury. However, whether upregulation of autophagy is beneficial or detrimental to cell fate remains controversial. Historically, these studies measured mitophagy through protein expression/translocation and colocalization of mitophagy proteins as well as protein expression of mitochondrial content, thus limiting the ability to accurately assess mitochondrial clearance. Furthermore, while mitophagy may be inferred by measurements of mitochondrial content, it fails to provide any insight into the events leading up to the actual clearance of mitochondria. To address these issues, we
utilized the novel transgenic mito-QC reporter mice in an OGD/R model. The reporter allele contains a CAG promoter and mCherry-GFP-mtFIS1 fusion protein inserted into the Gt(ROSA)26Sor locus on chromosome 6. mCherry is stable in acidic pH (pKa 4.5) while GFP (pKa 5.9) is quenched in the acidic lysosomal environment, allowing for identification of mitochondria in autolysosomes. In addition, this model provides precise and temporal resolution of mitophagic flux. We hypothesize that mitophagic flux is increased following OGD/R (see Chapter 5).

Objective 3: Investigate the role mitochondrial dynamics on mitophagic flux during OGD/R.

The final objective was to investigate how the mitochondrial fission protein Drp1, regulates mitophagic flux following OGD/R. It is understood that mitochondrial fission is necessary for mitophagy to occur, but less in known about the complex relationship between mitochondrial dynamics and mitophagy and its role in maintaining cell viability [18, 196]. To elucidate these mechanisms, Drp1 floxed (Drp1\textsuperscript{flox}) mice were crossed with mitoQC reporter mice and used to study Drp1’s effect on i) mitochondrial dynamics, ii) mitophagy, and iii) cell viability following OGD/R. We hypothesize that Drp1 significantly alters mitochondrial quality control and increases cell viability (see Chapter 6).
CHAPTER 4: MITOCHONDRIAL REMODELING FOLLOWING OGD/R: TIPPING THE SCALES TO A FRAGMENTED MORPHOLOGY

1. Rationale

Mitochondria are highly dynamic organelles constantly undergoing fusion and fission. These two events are essential to maintain mitochondrial homeostasis and cell stability by providing an equilibrium of long interconnected networks as well as small fragmented mitochondria [14, 15, 274]. In pathophysiologic states, such as I/R injury, there is substantial evidence that excessive mitochondrial fragmentation is associated with cell death [15, 240, 243, 274-279]. To date, studies focused on mitochondrial fragmentation in the context of I/R injury have been limited by their classification of mitochondrial morphology. Qualitative and semi-quantitative scoring methods lack precision and accuracy of physiologically relevant mitochondrial morphologies and investigator-associated bias in scoring may confound the results.

Accordingly, we developed a non-biased semi-automated machine learning-based classification system to characterize mitochondrial morphology during OGD/R in primary cortical neurons. We hypothesize that (i) utilizing a machine learning-based classification can provide accurate quantification of mitochondrial dynamic states during, and ii) mitochondria will undergo significant remodeling during OGD/R, resulting in extensive fragmentation.

2. Experimental Design

Primary cortical neurons were isolated from mitoQC reporter mice (containing fluorescent proteins fused to fis1 for mitochondrial localization) and plated onto glass coverslips. After 14 days in vitro (DIV14), cells were subjected to 2.5h of OGD followed by up to 6h of reoxygenation (6R). Coverslips were fixed with 3.7% PFA in 200 mM
HEPES (Fig. 4-1) at the end of OGD within the hypoxic chamber (time = 0R), or at 1, 2, 4, and 6 hours after reoxygenation (1R, 2R, 4R, and 6R) Coverslips were imaged and analyzed for mitochondrial morphology using the green (GFP) channel for quantification.

![Experimental schematic](image)

**Fig. 4-1. Experimental schematic.** Primary Cortical Neurons were isolated from mitoQC transgenic mice from P0-P1. Experiments were conducted on DIV14. Cells were subjected to a 2.5h OGD, and a 6h Reoxygenation period. Cells were fixed at 0R, 1R, 2R, 4R, 6R, and compared to control.

### 3. Results

#### 3.1 Identification of Distinct Mitochondrial Morphologies

Analysis of primary cortical neurons from mitoQC transgenic mice with fluorescent microscopy revealed four distinct mitochondrial morphologic groupings: network, unbranched, swollen, and punctate (Fig 4-2A). To validate the independence of these observed morphologies, mitochondrial objects (mitochondrial localized-GFP) were segmented and measured using my newly developed semi-automated image processing workflow detailed in Chapter 2. Comparison of morphologies across size and shape descriptors identified distinct groupings of characteristics in these morphologic categories (Fig 4-2B-C). The shape descriptors circularity ($\frac{4\pi \times \text{area}}{\text{perimeter}^2}$), interfacial density ($\frac{\text{perimeter}}{2 \times \text{area}}$), and area equivalent diameter ($2 \times \sqrt{\frac{\text{area}}{\pi}}$) were significantly different among all groups. Aspect ratio (AR) (the ratio of an object's width to its height, $\frac{\text{width}}{\text{height}}$), was also significantly different between networks vs. swollen, networks vs. punctate, unbranched vs. swollen, and unbranched vs. punctate. AR was not statistically different between networks vs. unbranched as well as swollen vs. punctate categories. Next, a principal component
analysis was applied to visualize group differences between mitochondrial objects (Fig 4-2D). A total of 32 measurements were collected for each object and z-scores were calculated for each variable to compare differences across all morphologies. A summary of the scaled measurements is shown across each morphology in Fig. 4-3. Variables were grouped by similarity. Networks display high z-scores for variables that measure size (such as area and perimeter) and, conversely, have low z-scores for variables that measure spherical shape (such as circularity or roundness). The unbranched morphology displays high z-scores for variables that measure length (such as aspect ratio and elongation), while also containing low values for circularity and roundness. Punctate and swollen mitochondria display high z-scores for variables that measure spherical shape, specifically

**Fig 4-2: Identification of distinct mitochondrial morphologies.** A) Four distinct morphologies were identified in primary cortical neurons from mitoQC transgenic mice: Network, unbranched, swollen, and punctate. B-C) Mitochondrial morphologies differ significantly in circularity, aspect ratio, interfacial density, and area equivalent diameter. D) PCA of mitochondrial objects. Each point represents a single mitochondrial object, each color represents a different morphology: network (blue), unbranched (purple), swollen (green), and punctate (red). Difference across groups were computed using one-way ANOVA with Tukey post-hoc analysis. Multiple comparisons were pairwise. ****p<0.0001. Scale bar = 6 µm.
circularity and roundness, and contain lower z-scores that measure size like area and perimeter. Punctate and swollen mitochondria mainly differ in size, where swollen objects have higher z-scores in area equivalent diameter, ferret, and hexagonal perimeter. Importantly, interfacial density (Fig. 4-3: intfc_D.microns.1.) displays the greatest difference between swollen and punctate morphologies.

3.2. Machine Learning Classification of Morphology: Model Performance

In order to develop the model for the classification of mitochondrial morphology, a total of 2342 mitochondrial objects were segmented from 4 different images (2 from control conditions, and 2 from fragmented conditions), measured, and classified. The mitochondrial objects were split on the basis of their classification into a training set (80%, 1874 objects) and a test set (20%, 468 objects). A machine learning model was developed based upon the training set, and later validated using the test set. The model was built upon Random Forest classification.

![Fig. 4-3. Comparison of scaled measurements across different morphologies. 32 different measurements were collected for each mitochondrial object. Z-scores were calculated for each variable to compare differences across all morphologies. Variables were grouped based on similarity. Red color corresponds to a high z-score, while blue corresponds to a low z-score.](image)
methodology. In our model, a mitochondrial object is sent through a number of parallel decision trees, in which each tree provides an output or vote. Each object is assigned one of the four morphologies at the conclusion of a decision tree based on the morphology with the majority of votes. The final model consisted of 500 trees with an mtry = 2, which refers to the number of randomly selected predictors considered at each split within the tree.

For validation of model accuracy, the test set of mitochondrial objects were run for predicted morphologies. Accuracy was determined using a confusion matrix, which cross tabulates observed and predicted classes to observe error tendencies of the model (Fig4-4A) [271]. The columns represent the referenced morphologies and
rows represent the predicted morphologies. The confusion matrix revealed that out of the
468 objects within the test set, only two objects were mislabeled, a swollen mitochondria
for punctate and an unbranched mitochondria for swollen. The overall accuracy
(computed as the proportion of total correct predictions,
$$\frac{\text{true positive (TP)} + \text{true negative (TN)}}{\text{TP} + \text{TN} + \text{false positive (FP)} + \text{false negative (FN)}}$$) was calculated as 99.57% with a kappa (accuracy
accounting for chance agreement,
$$\frac{\% \text{agreement observed} - \% \text{agreement expected by chance}}{1 - \% \text{agreement expected by chance}}$$) of
99.39% (Fig. 4-4B).
Additionally, the model
presented highly specific
and sensitive predictions for
each morphology.
Sensitivity ($$\frac{\text{TP}}{\text{TP} + \text{FN}}$$) and
specificity ($$\frac{\text{TN}}{\text{FP} + \text{TN}}$$) was
above 99.41% for each
morphology. Precision, the
ratio of correct positive
prediction to the total
predicted positives
($$\frac{\text{TP}}{\text{TP} + \text{FP}}$$),
was over 98.90% for all
morphologies. Finally
balanced accuracy, the
average between sensitivity
and specificity,
was calculated as 99.39%.
and specific for each group \(\frac{\text{Sensitivity} + \text{Specificity}}{2}\) was over 99.32\% for all morphologies. Representative images as well as classified object masks (green = networks, blue = unbranched, yellow = swollen, and red = punctate) are displayed by in Fig. 4-4C-D respectively, as an example of our classification system.

### 3.3 Machine Learning Prediction of Morphology during OGD/R

Visually, primary neurons subjected to OGD/R have changes in mitochondrial morphology (Fig. 4-5A). After applying our machine learning classification system, total mitochondrial objects and area were tabulated for each morphology over control, 0R, 1R, 2R, 4R, and 6R timepoints (Fig. 4-5B). Chi-squared tests demonstrated significance in both the number of mitochondrial objects and the area of mitochondrial objects.

To quantify differences between time points for each morphology, percent objects and area were calculated (Fig. 4-6A). Significant differences were observed in percent objects for each morphology (Fig. 4-6B). Percent networks were significantly decreased at 0R (p<0.05) and 1R (p<0.01) before returning to control levels at 2R, 4R, and 6R. Percent unbranched objects demonstrated a significant decrease at 0R (p<0.0001) and persisting throughout reoxygenation (1R: p<0.0001, 2R: p<0.01, 4R: p<0.01, and 6R: p>0.001) as compared to control. In contrast, percent swollen objects were significantly increased at 0R (p<0.01), 1R (p<0.01), 2R (p<0.05), 4R (p<0.01), and 6R (p<0.01) as compared to control and percent punctate objects were also significantly increased at 0R (p<0.01) and 1R (p<0.01) when compared to control.

Changes in percent area were only observed for swollen and punctate morphology in regard to percent area (Fig. 4-6C). Percent area of swollen mitochondria was significantly increased at 1R vs. control (p<0.05). Additionally, percent area of total punctate objects was significantly increased at 0R (p<0.05) and 1R (p<0.01) as compared
with control. Percent area of networks and unbranched objects remain unchanged.

![Graphs showing mitochondrial objects and area changes](image)

**Fig 4-6: Comparisons of mitochondrial morphologies during OGD/R.** A) Stacked bar graphs showing percent mitochondrial objects and percent area over OGD/R. B) Individual comparisons of percent mitochondrial objects for each morphology over OGD/R. C) Individual comparisons of percent mitochondrial area for each morphology over OGD/R. Differences across time were computed using one-way ANOVA with Dunnett post-hoc analysis. Multiple comparisons were assessed by comparing the means of each time point with the mean of the control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n = 6 biological replicates.

### 4. Summary

In summary, we report that by utilizing a semi-automated machine learning program to classify mitochondrial morphology, the mitochondrial network undergoes
extensive remodeling during OGD/R in primary cortical neurons. Specifically, there is a loss of networks and unbranched mitochondria accompanied by an increase in swollen and punctate mitochondria. While, significant differences in mitochondrial area were only observed in swollen and punctate mitochondria.

Networked mitochondria are significantly decreased 0R and 1R from control and appear to come back to control levels at 2R, while there were no differences in area of mitochondrial networks throughout OGD/R. Networks most likely undergo a transition from smaller networks to unbranched mitochondria at 0R and 1R, while larger networks remain intact. This would account for the loss of network mitochondria, but no observable changes in mitochondrial area. Networks in control conditions constitute roughly 55%-65% of mitochondrial content, which is the majority of mitochondrial area. Unbranched mitochondria decrease significantly over the entirety of OGD/R as compared to control, while unbranched area was unchanged. These data suggest that unbranched mitochondria divide and transition into punctate and swollen mitochondria. When networks divide and mitochondria transition to the unbranched pool, we predict that the smaller unbranched mitochondria are the first to fragment, while longer unbranched mitochondria remain intact. Similar to networks, this could account for the lack of a significant decrease in unbranched mitochondrial area.

Both punctate and swollen mitochondria numbers are significantly increased following OGD and during reperfusion. Interestingly, punctate mitochondria increased at 0R and 1R before returning to control levels, while swollen mitochondria remained increased across the entirety of OGD/R. This suggests a possible transition from punctate mitochondria to swollen mitochondria. Previously, our lab reported that, during OGD, extensive mitochondrial fragmentation occurs in a Drp1-dependent manner due to energy
deprivation [240]. Upon reoxygenation, ROS production will damage mitochondria and induce mitochondrial permeability transition pore (mPTP) opening, resulting in mitochondrial swelling, the release of programmed cell death factors, and ultimately induction and execution of cell death [13, 280]. Therefore, these data suggest that punctate mitochondria returning back to control levels is potentially the transition of these mitochondria from a punctate morphology to a swollen morphology upon induction of cell death processes. Similarly, punctate area was significantly increased at 0R and 1R, while swollen area was only increased at 1R. Both punctate and swollen mitochondria contain relatively small areas, therefore the more objects that are present, the larger area those objects will occupy. However, swollen mitochondria vary widely in size compared to punctate mitochondria. This variability could explain why significant increases were only observed at 1R, where it appeared the most extensive alterations of mitochondrial architecture were taking place.

In conclusion: utilizing a highly accurate, non-biased, semi-automated machine

**Fig. 4-7. Summary of mitochondrial morphologies changes during OGD/R.** Data were calculated as percent change from control for both mitochondrial objects and mitochondrial area. Data was analyzed individually for each morphology using one-way ANOVA Dunnetts post-hoc analysis to detect differences across time points versus control. *p<0.05, punctate; #p<0.05, swollen; $p<0.05, unbranched; †p<0.05, networks. n = 6 biological replicates.
learning-based classification system, we were able to characterize mitochondrial
dynamics with high resolution. These data support that mitochondria undergo extensive
fragmentation throughout OGD/R and provide a highly sensitive and specific tool that may
allow detailed analysis of the transition of morphologic states in multiple disease states.
CHAPTER 5: MITOPHAGIC FLUX DURING ISCHEMIA/REPERFUSION

1. Rationale

Maintaining a healthy mitochondrial network is critical for cell survival. Mitochondria must have active degradation of dysfunctional mitochondria through mitophagy that is counterbalanced with biogenesis of mitochondrial lipids and proteins to maintain a healthy mitochondrial network and meet the energetic needs of a cell. Dysfunctional mitochondria produce ROS that can damage mitochondrial and cellular components, and without recycling of mitochondrial components, the cumulative consequences of excessive mitochondrial dysfunction can lead to release of mitochondrial pro-death signals such as cytochrome C. Therefore, it is essential that dysfunctional mitochondria be degraded through mitophagy. As reviewed in Chapter 1, I/R injury induces the accumulation of dysfunctional mitochondria and there is evidence to suggest that mitophagy is upregulated during this process. Mitophagy is a dynamic process, making assessment complicated. Numerous studies have investigated mitophagy through the conversion of LC3-I to LC3-II, colocalization of mitochondria and lysosomes, expression levels of PINK1 and Parkin, and the loss of mitochondrial content. LC3 conversion reveals changes in autophagosome initiation, and colocalization studies provide poor resolution of protein or organelle interaction. Although LC3, PINK1, and Parkin levels may increase, this cannot be interpreted as conclusive evidence of increased mitophagy, and depending on the goal of the analysis, these data are unable to identify protein molecules that have already been degraded by mitophagy. Furthermore, while mitophagy may be inferred by measurement of mitochondrial content, it fails to provide insight into the events leading to the actual clearance of mitochondria.

To address these issues, we performed OGD/R on primary cortical neurons
isolated from the novel mitoQC reporter mouse (C57BL/6-Gt(ROSA)26Sortm1(CAG-mCherry/GFP)Ganl/J) (Fig. 5-1) [267]. The reporter allele contains a CAG promoter and mCherry-GFP-mtFIS1 fusion protein inserted into the Gt(ROSA)26Sor locus on chromosome 6. During normal physiological conditions, mitochondria are tagged with both mCherry and GFP fluorophores, providing an overlapping signal for both red and green fluorophores on mitochondria. When mitochondria are sequestered to the lysosome, the acidic environment quenches the GFP (pKa 5.9), while mCherry (pKa 4.5) remains stable, resulting in a loss of the GFP signal, while mCherry fluorescence remains intact. This allows resolution of mitochondria inside auto/endo-lysosomes. We hypothesize mitophagic flux will be significantly increased following OGD/R.

2. Experimental Design

Primary cortical neurons were isolated from mitoQC reporter and WT mice between P0-P1. MitoQC neurons were plated on 12 mm glass coverslips at a density of 300K cells, while WT neurons were plated on 60 mm dishes at a density of 5 million cells.
On DIV14 neural cultures were exposed to 2.5 hours of OGD and 6 hours of reoxygenation (Fig. 5-2). Samples were taken at 6 different time points (control, 0R, 1R, 2R, 4R, and 6R) for both PFA fixation or fractionation. Coverslips were fixed with 3.7% PFA in 200 mM HEPES, and 60 mm dishes were scraped down for homogenization and mitochondrial fractionation. For the 0R timepoint, coverslips were fixed inside the hypoxic chamber at the end of OGD, while 60 mm plates were taken out of the hypoxic chamber and immediately processed for fractionation. Coverslips were imaged and analyzed for the presence of red puncta (mitophagy), while mitochondrial fractions were probed for specific protein markers associated with mitophagy. (Chapter 2 - Methods)

3. Results

3.1. Mitophagy is Increased Following OGD/R

To evaluate mitophagic flux during the progression of ischemia/reperfusion, cells exposed to OGD/R were analyzed for the status of mitophagic flux as indicated by accumulation of mCherry fluorescence in the absence of mitochondrial GFP fluorescence (mCherry puncta). Utilizing ImageJ, mCherry puncta were segmented and counted from twelve images for each coverslip (Chapter 2 - Methods). Counts were normalized to cell number to provide average mCherry object counts per cell. mCherry puncta were significantly increased at 4R vs. control (p<0.05) (Fig. 5-3A). To validate mCherry as a marker of mitochondria localized to lysosomes, mCherry puncta were analyzed in cells
co-labeled with immunofluorescence for the lysosome marker LAMP1 and analyzed for co-localization (Manders Correlation Coefficient) of LAMP1 and mCherry puncta (Fig. 5-3B). Colocalization was quantified to identify the extent of mCherry positive puncta containing LAMP1 positive puncta during the progression of ischemia/reperfusion injury in cells exposed to OGD/R (Fig. 5-3B). The results show significant increases across all time points of OGD/R when compared to control (0R: p<0.05, 1R: p<0.01, p<0.001, 4R:
p<0.05, 6R: p<0.01). In addition, total LAMP1 puncta number per cell was significantly increased at 4R vs. control (p<0.05), matching the increases in mCherry puncta. These data support the validity of quantifying mCherry puncta as an index of mitochondria in lysosomes and demonstrate a significant increase in mitophagic flux at 4 hours of reoxygenation.

3.2 Mitophagy Via the PINK1/Parkin Pathway in Ischemia/Reperfusion Injury

As reviewed in Chapter 1, mitophagy occurs through multiple pathways. My studies focus on mitophagic flux following OGD through the PINK1/Parkin pathway. This pathway is the most well characterized mitophagy pathway, and has been implicated as a major therapeutic target for various neurodegenerative diseases such as Alzheimer’s and Parkinson’s Disease [281-285]. In addition, previous studies have suggested its involvement in ischemia/reperfusion injury in both heart and brain [260, 286-288]. To investigate the activation of this pathway, I evaluated the co-localization of PINK1 and Parkin to mitochondria, as mitochondrial localization is considered a reliable marker for mitophagy activation. WT cells were immunolabeled with PINK1 or Parkin and co-immunolabeled with the antibodies against the Beta subunit of ATP synthase (ATPB) as a mitochondria marker (Fig. 5-4A and B). Colocalization of PINK1 or Parkin with ATPB was analyzed with Pearson’s Correlation Coefficient (PCC) in ImageJ. PINK1 colocalization with mitochondria was significantly increased from control at 0R (p<0.05), 1R (p<0.01), and peaking at 2R (p<0.001) (Fig. 5-4C). Parkin colocalization was significantly lower from control at 0R (p<0.001), while being significantly increased from control at 1R (p<0.01), 2R (p<0.01), 4R (p<0.001), and 6R (p<0.0001) (Fig. 5-4C).

A complimentary methodology to evaluate mitochondrial localization of PINK1 and Parkin was also used to verify activation of this mitophagy pathway. Western blot of
mitochondrial fractions during the progression of OGD and reoxygenation revealed PINK1 and Parkin accumulation in mitochondrial fractions (Fig. 5-4D). Pink1 translocation was significantly increased at 2R (p<0.05), while Parkin translocation was detected from 5 min of reoxygenation to 4R (5min: p<0.05, 1R: p<0.05, 2R: p<0.01, 4R: p<0.05) (Fig. 5-4E). These data suggest that PINK1 translocation occurs early following reoxygenation and peaks at 2R, just before our MitoQC reporter data suggests degradation is occurring. It also suggests that Parkin translocates to mitochondria early in reoxygenation and persists.
through late reoxygenation, perhaps until dysfunctional mitochondria are cleared. This is supported by evidence provided by Ordureau et al., that revealed a feedforward mechanism for Parkin translocation and mitochondrial ubiquitination [149]. Accordingly, as an additional indicator of Parkin activity, we analyzed mitochondrial fractions for general protein ubiquitination (Fig. 5-4D). Mitochondrial protein ubiquitination is a direct effect of Parkin activity on the mitochondria, tagging mitochondria to degradative systems for removal [153, 289]. We observed a significant increase of mitochondrial ubiquitination at 2R (p<0.05), corresponding to the timepoint where peak increases in PINK1 and Parkin translocation were detected (Fig. 5-4E). These data suggest that following I/R injury, PINK1/Parkin pathway is activated and mitophagy is induced.

3.3. Mitochondrial Clearance Through the Rab5 Endosomal Pathway

Our data show an increase in mitochondrial ubiquitination, activation of the Pink/Parkin pathway of mitophagy, and accumulation of mitochondrial contents in lysosomes. Mitochondria transport to the lysosome for degradation can occur through two distinct pathways, the autophagosomal and the endosomal pathways. We evaluated the induction of both pathways to gain insight into how mitochondrial clearance may occur following an ischemic insult in neurons. To evaluate the autophagosomal pathway, we probed for LC3 conversion via proteolytic activation from LC3-I to LC3-II (an indicator of autophagy activation). To evaluate the endosomal pathway, we assayed the expression level of Rab5 (endosomal marker), and its presence in mitochondrial fractions as an indicator of mitochondrial sequestration in endosomes (Fig. 5-5A). Whole cell lysates revealed no significant changes in LC3 conversion, but a significant increase of Rab5 expression at 4R (p<0.01) when compared to control (Fig. 5-5B). Similarly, mitochondrial fractions revealed no significant changes in LC3 conversion, but demonstrated significant
increases from control in Rab5 expression at both 2R (p<0.01) and 4R (p<0.05). These data suggest that mitochondria are sequestered in Rab5 endosomes for transport to lysosomes, and not through autophagic pathways involving LC3 conversion.

3.4 Mitochondrial Morphology Classification of mCherry Puncta

In Chapter 4, my mitochondrial morphology classification system revealed significant alterations to mitochondrial architecture during OGD/R. Specifically there was a significant increase in punctate morphology during OGD and early reoxygenation, which reverted to control levels at 2R. Additionally, there were significant increases in swollen mitochondria during OGD that persisted throughout the entirety of reoxygenation. To determine if a specific morphology is most likely to be sequestered for mitophagic degradation, I employed our classification system to identify mCherry puncta morphology. Segmented mCherry puncta masks were generated and analyzed using our classification.
system (Fig. 5-6A). Total mCherry puncta objects were counted for 4 biological replicates and groups compared for statistically significant differences using a Chi-squared test (Fig. 5-6B). Punctate morphology represented the majority of mCherry puncta with a total count of 38,449 objects throughout OGD/R, consisting of 85.9% of red puncta (Fig. 5-6B, C). Interestingly, the number of unbranched objects totaled to 3,727, representing 8.5% of mCherry puncta. Swollen mitochondria contained the third highest number of objects totaling to 2,848, which represented 5.58% of red puncta. Networks contained the lowest number of objects at 12, representing 0.02% of red puncta objects. mCherry puncta were compared to classified mitochondrial objects measured in Chapter 4 to compare sizes (Fig. 5-6D). For all classifications, mCherry puncta demonstrated lower average area when compared to mitochondrial classified objects.

Fig. 5-6. Mitochondria morphology classification of mCherry puncta. A) mCherry puncta were segmented from images of mitoQC neurons and analyzed using our mitochondrial morphology classification system. B) Total mCherry puncta counts over OGD/R. C) Stacked bar graphs representing percent red puncta over OGD/R. D) Violin plots displaying relative distributions of area for mCherry puncta (mCherry only) and mitochondrial (GFP-Fis1). mCherry puncta: n = 4 biological replicates, 44,536 objects. Mitochondria: n = 1 biological replicate, 43,434 objects. Scale bar = 10 μm.
networks had an average area of $8.822 \pm 2.252 \, \mu m^2$ as compared to mitochondrial networks ($25.467 \pm 56.991 \, \mu m^2$). Unbranched mCherry puncta had an average size of $1.440 \pm 0.981 \, \mu m^2$ as compared to unbranched mitochondria ($2.871 \pm 1.812 \, \mu m^2$). Swollen mCherry puncta had an average area of $1.681 \pm 0.649 \, \mu m^2$ as compared to swollen mitochondria ($2.126 \pm 1.066 \, \mu m^2$). mCherry puncta that were classified as punctate had an average size of $0.382 \pm 0.233 \, \mu m^2$ as compared to punctate mitochondria ($0.582 \pm 0.215 \, \mu m^2$). These data suggest that punctate mitochondria are most likely tagged for degradation. In addition, macro-mitophagy may occur on unbranched or network objects, but objects are smaller than $10 \, \mu m^2$.

4. **Summary**

In summary, we report that mitophagic flux is significantly increased following OGD/R, indicated by the increased presence of mCherry puncta within neurons extracted from mitoQC reporter mice. This is accompanied by increases in the number of lysosomes present during reoxygenation and significant increases in colocalization of mCherry puncta with Lamp1-positive lysosomes. Increases in mitophagic flux correspond to the activation of the PINK1/Parkin pathway of mitophagy during ischemia/reperfusion. This was demonstrated by an increase in both PINK1 and Parkin in mitochondrial fractions as well as increased colocalization of PINK1 and Parkin with the mitochondrial marker ATP synthase (ATPB). In further support of mitophagy induction, the primary target of Parkin activity, mitochondrial ubiquitination, was significantly increased following OGD/R. In our model of OGD/R of primary cortical neurons, autophagic sequestration through LC3 containing autophagosomes does not appear to be activated. However, the increase in Rab5 in mitochondrial fractions is indicative of activation of the endosomal pathway. Therefore, mitochondrial degradation is likely carried out through the endosomal
pathway in the setting of ischemia/reperfusion injury. Lastly, our mitochondrial morphology classification system revealed that the predominate state of mitochondria undergoing mitophagy is a punctate morphology.
CHAPTER 6: THE ROLE OF DRP1 IN MITOCHONDRIAL DYNAMICS AND QUALITY CONTROL

1. Rationale

Mitophagy and mitochondrial dynamics have been well studied separately in response to I/R injury, but investigations aimed at elucidating the interplay between these two components of mitochondrial quality control are limited. Overwhelming evidence suggests that excessive mitochondrial fission is directly linked to cell death while, in contrast, mitochondrial fusion is protective [231, 233, 241, 242, 260, 279, 290-294]. Yet, these studies fail to explain: i) why fusion might be protective, ii) why fission is causally linked to cell death, iii) why pharmacologic inhibition of fission is only protective when initiated prior to ischemia [242, 279], but, paradoxically, is detrimental when initiated during reperfusion [259], and finally, iv) whether fission is necessary to dispose of damaged mitochondria through mitophagy following I/R injury. Previous studies suggest a complex relationship between mitochondrial dynamics and mitophagy where fission inhibition during ischemia may provide improved outcomes [243, 260, 279], but this same inhibition of mitochondrial fragmentation may prevent the mitophagy recovery pathway during reperfusion that is needed to remove damaged mitochondria.

Drp1 is a key regulator of mitochondrial fission and has also been implicated as a key component of the mitophagic process allowing damaged portions of the mitochondrial network to be fragmented and sequestered for recycling through mitophagy/biogenesis [196, 198, 199, 236]. Overexpression of Drp1 in HeLa cells resulted in a 70% decrease in mitochondrial mass and endophilin B1, a Drp-1 dependent mediator of fission, was found to colocalize with autophagic markers LC3, Atg5, and Atg9 in response to nutrient starvation [196, 198, 199]. In addition, there is evidence to suggest that overexpression of Parkin in mouse N2a neuroblastoma cells may protect against OGD/R through
ubiquitination and proteasomal degradation of Drp1 [236]. Despite these associations, the role of Drp1 in mitophagy and ultimately maintaining cell viability in response to I/R injury remains unclear.

We hypothesized that inhibition of Drp1 would prevent disruption of the mitochondrial network and lead to reduced mitophagy. To gain further insight into these mechanisms of mitochondrial dysfunction, we crossed mitoQC reporter mice with Drp1fl/fl mice provided from Dr. Hiromi Sesaki. Drp1fl/fl mice contain loxP sites inserted next to exons 3 and 5, located in an essential GTPase domain [268]. Cortical neurons were isolated from mitoQCki+/Drp1fl/fl mice and subjected to OGD/R to determine how Drp1 knockout affects mitochondrial dynamics, mitophagic flux, and cell viability.

2. Experimental Design

Primary cortical neurons were isolated from QCki+/Drp1fl/fl and Drp1fl/fl mice between P0-P1. QCki+/Drp1fl/fl neurons were plated onto 12 mm glass coverslips for PFA fixation, while Drp1fl/fl neurons were plated onto 60 mm dishes and 24-well plates for fractionation and viability assays. Neurons were infected with either LV-EF1a-empty or LV-EF1a-Cre on DIV7 and experiments were conducted on DIV14 (Fig. 6-1). QCki+/Drp1fl/fl neuron cultures were exposed to 2.5 hours of OGD then fixed with 3.7% PFA in 200 mM HEPES at 6 different time points (control, 0R, 1R, 2R, 4R, 6R).
6R) and analyzed for mitochondrial morphology and mitophagic flux. Drp1fl/fl neurons were analyzed by western blot for protein markers associated with mitophagy at three time points (control, 2R, 4R). In addition, Drp1fl/fl neurons were assessed for cell viability at 6R by MTT assay.

3. Results

3.1 Lentiviral Transduction of Primary Cortical Neurons

To optimize target gene knockout with lentiviral-Cre delivery in primary cortical neurons we first conducted preliminary experiments to evaluate (i) cell viability, (ii) infection rate, and (iii) loss of protein target. Viral toxicity was determined with five escalating concentrations of lentivirus (LV-EF1a-empty-VSVG; concentrations 0.5X, 1X, 2X, 4X, 5X) and viability was assessed 7 days post-transduction by MTT assay. All concentrations had significant decreases in viability, resulting in a ~25% reduction of viability at concentrations of 2X and above (Fig. 6-2A). To validate neuron viral transduction efficiency, cortical neurons were transduced with 0.5X and 1X LV-EF1a-GFP-VSVG and

![Fig. 6-2. Lentiviral transduction of primary cortical neurons. A) Lentiviral toxicity was evaluated over 5 different concentrations of LV-EF1a-empty. Data was assessed by MTT, n = 4. B) Representative images of neuron viral transduction efficiency post-transduction day 7. Neuron cultures were treated with 0.5X and 1X (shown) LV-EF1a-GFP and co-labeled with immunofluorescence for the neuron marker MAP2. C) Quantification of infection rate was calculated by the number of MAP2+ cells containing GFP divided by the total number of MAP2+ cells, n = 3. D) Western Blot and quantification of Drp1 knockout after transduction with 0.5X, 1X, and 2X LV-EF1a-cre in Drp1fl/fl primary cortical neurons, n = 3. Scale bar = 20 μm]
co-labeled with immunofluorescence for the neuron marker microtubule-associated protein 2 (MAP2) on post-transduction day 3, 5, and 7 (Fig. 6-2B). Infection rate was calculated as the number of MAP2+ neurons containing GFP fluorescence divided by the total number of MAP2+ neurons. By 7 days of post-transduction the infection rate for both 0.5X and 1X LV-EF1a-GFP-VSVG was above 95% (Fig. 6-2C). It was assumed that all concentrations above 1X, would yield similar results to 1X concentration in transduction efficiency. Knockout of Drp1 (Drp1KO) was evaluated by transducing Drp1fl/fl neuron cultures with 0.5X, 1X, and 2X LV-EF1a-cre-VSVG concentrations and blotting for the presence of Drp1 in whole cell lysates (Fig. 6-2D). Quantification of the blots revealed a 75% knockout of Drp1 protein in Drp1fl/fl neuron cultures with lentiviral-cre delivery (Fig. 6-2D). Based on these data, 2X lentivirus concentration with a 7-day transduction period yielded the optimal knockout of Drp1.

3.2 Drp1KO Stabilizes Mitochondrial Morphology during OGD/R

To evaluate how Drp1KO affects mitochondrial morphology following OGD/R, QCki/+/Drp1fl/fl cells infected with LV-EF1a-cre and exposed to OGD/R were analyzed utilizing my mitochondrial morphology classification system (Chapter 4 – Mitochondrial Remodeling Following OGD/R: Tipping the Scales to a Fragmented Morphology). Microscopy of mitochondrial morphology demonstrated that, in control neurons exposed to OGD/R, there was an apparent transition from large interconnected mitochondrial objects to small mitochondrial objects. Drp1KO prevented this transition to small mitochondrial objects at all timepoints (Fig. 6-3A). To analyze this morphologic disruption, we applied our machine learning classification system to provide detailed analysis of this morphologic disruption by comparing the number of mitochondrial objects and the area of mitochondria in each morphologic state between conditions (LV-EF1a-empty vs. LV-
EF1a-cre) and across OGD/R timepoints using two-way ANOVA followed by post hoc analysis with Dunnett’s test (Fig. 6-3 B-C).

Classification of the number of networked mitochondrial objects revealed a significant reduction in response to OGD/R (p<0.01), with only weak evidence of a difference between control and Drp1KO groups (p = 0.052). In agreement with the total object counts, the area of mitochondrial networks as a percent of total mitochondrial mass was significantly reduced at early reoxygenation timepoints (0R and 2R; p<0.01) and this reduction in mitochondrial network area was inhibited with Drp1KO compared to control (Fig. 6-3B; Control vs. 0R:p<0.0001, 1R:p>0.05, 4R:p<0.001, and 6R:p<0.05). These data demonstrate that Drp1KO attenuates fragmentation of larger mitochondrial networks during OGD/R.

Consistent with induction of mitochondrial fragmentation, control neurons showed a significant decrease in the total number of unbranched mitochondrial objects at all timepoints post-reoxygenation (control vs. 0R:p<0.0001, 1R:p<0.01, 2R:p<0.01, 4R:p<0.01, 6R:p<0.05), however this decrease in number did not reflect a reduction in the total area of mitochondria in the unbranched state (Fig. 6-3B). This suggests that mitochondria in the unbranched state remain a stable percentage of the total mitochondrial pool during OGD/R but increase in length to account for the decreased total object number. The reduction of unbranched mitochondrial objects during ODG/R is inhibited with Drp1 KO (Control vs. Drp1KO), demonstrating that this transition requires Drp1 dependent fragmentation. Interestingly, the total area of mitochondria in the unbranched state is reduced overall with Drp1KO, but there is no effect of Drp1KO on the unbranched mitochondrial object number. This suggests that Drp1 is essential to maintaining unbranched mitochondrial size, and disruption of this pathway results in a
shift toward fewer, longer unbranched mitochondria without disrupting the number of mitochondria in this state under normal conditions (Fig. 6-3B-C).

Swollen mitochondria are characterized by their small size characteristics similar in many ways morphologically to punctate mitochondria, with the primary morphologic difference being the lower interfacial density, as described in Chapter 4. Swelling of mitochondria is a well-documented precursor to induction of cell death during ischemia/reperfusion injury. The total number of mitochondrial objects and the corresponding area of mitochondrial content in a swollen morphology were significantly altered in response to OGD/R (p<0.01) and this effect was ameliorated with Drp1KO (p<0.01). The number of swollen mitochondrial objects was significantly increased in control cells in response to

![Fig. 6-3. Drp1 KO stabilizes mitochondrial morphology during OGD/R. A) Representative images of QCK</sup>+/Drp1<sup>fl/fl</sup> primary cortical neurons (GFP channel), infected with either LV-EF1a-empty or LV-EF1a-cre, and subjected to OGD/R. B) Individual comparisons of percent mitochondrial objects for each morphology over OGD/R. C) Individual comparisons of percent mitochondrial area for each morphology over OGD/R. Two-way ANOVA was computed to detect differences across time and between conditions. Multiple comparisons across time were assessed by comparing the means of each time point with the mean of the control and were computed using Dunnett's post-hoc analysis. Multiple comparisons between conditions were computed using Sidak post-hoc analysis. *p<0.05 across time, #p<0.05 between condition, n = 8 biological replicates per group, scale bar = 10 µm.](image-url)
OGD/R (0R: p<0.05, 1R: p<0.01, 2R: p<0.01, 4R: p<0.05), while Drp1KO blocked this increase in mitochondrial swelling (Fig. 6-3B). Similar effects were observed in percent area of swollen mitochondria following OGD/R (p<0.001) and between groups (p<0.001). Percent area of swollen mitochondria was significantly increased in control cells throughout OGD/R (Fig. 6-3C: 0R: p<0.01, 1R: p<0.05, 2R: p<0.01, 4R: p<0.05), while Drp1KO prevented any changes in mitochondrial swelling as a proportion of total mitochondrial area (Fig. 6-3C: 0R: p<0.01, 1R: p<0.05, 2R: p<0.05, and 4R: p<0.01). These data suggest that Drp1KO inhibits mitochondrial swelling induced by OGD/R.

Punctate mitochondria are characterized by their small size with no evidence of swelling, thus a higher interfacial density. The number of mitochondrial objects in a punctate morphology was not changed in response to OGD/R (p=0.086) or between control and Drp1KO (p=0.355) (Fig. 6-3B). In contrast, the total area of mitochondrial content in a punctate morphology demonstrated significant increases in response to OGD/R (p<0.01) and between groups (p<0.001). Punctate area was significantly increased throughout OGD/R (Fig. 6-3C: 0R: p<0.001, 1R: p<0.05, 2R: p<0.01, 4R: p<0.05) and this increase was blocked with Drp1KO (Fig. 6-3C: 0R: p<0.001, 4R: p<0.001, and 6R: p<0.05). A significant decrease in punctate area in Drp1KO cells was also observed at 4 hours of reoxygenation (Fig. 6-3B: 4R: p<0.05). These results suggest that OGD/R does not cause an increase in the number of mitochondria in a punctate morphology, but the total area of mitochondria in this state is increased, suggesting punctate mitochondria are larger following OGD/R. Importantly, Drp1KO blocked the increase in punctate area, most likely by preventing mitochondria fragmentation. When considered with the increase in swollen mitochondria, these data suggest mitochondrial fragmentation via Drp1 activity during OGD/R results in a rapid transition to a small
swollen phenotype, consistent with induction of cell death.

3.3 Mitophagic Flux is Independent of Drp1

To evaluate the effect of Drp1KO on mitophagic flux in the progression ischemia/reperfusion injury, \( QC^{+/+}/Drp1^{fl/fl} \) cells infected with LV-EF1a-cre or LV-empty and exposed to OGD/R were analyzed for the accumulation of mCherry puncta as an indicator of mitophagic flux (as described in Chapter 5- Mitophagic Flux during Ischemia/Reperfusion). There was a significant increase in mitophagic flux in response to OGD/R \((p<0.0001)\), but no effect of Drp1KO \((p=0.147)\). mCherry puncta accumulation was significantly increased at 2R \((p<0.01)\), 4R \((p<0.05)\), and 6R \((p<0.05)\) (Fig. 6-4B). Drp1 KO cells demonstrated a significant increase of mCherry puncta at 2R \((p<0.05)\), while 4R \((p=0.069)\) and

Fig. 6-4. Mitophagic flux is independent of Drp1. A) Representative images of \( QC^{+/+}/Drp1^{fl/fl} \) primary cortical neurons, infected with either LV-EF1a-empty or LV-EF1a-cre, subjected to OGD/R and analyzed for the presence of mCherry puncta. B) Quantification of mCherry puncta per cell as well as total LAMP1 puncta per cell during OGD/R. Two-way ANOVA was computed to detect differences across time and between conditions. Multiple comparisons across time were assessed by comparing the means of each time point with the mean of the control and were computed using Dunnett’s post-hoc analysis. Multiple comparisons between conditions were computed using Sidak post-hoc analysis. \(*p<0.05\) across time, \#p<0.05 between condition, \(n=8\) biological replicates per group, scale bar = 10 µm.
6R (p=0.064) was not significant (Fig. 6-4B). Similarly, total LAMP1 particle counts detected significant changes in response to OGD/R (p<0.01), but again no effect of Drp1KO. Total LAMP1 particles were significantly increased at 6R (p<0.05) in control cells (Fig. 6-4B). These data suggest that induction of mitophagic flux following OGD/R is independent of Drp1.

3.5 Drp1-Independent Mitophagy is not Cleared Through the Rab5 Endosomal Pathway

Our data show that ODG/R induces increased mitophagy and activation of the PINK1/Parkin pathway. Our data also suggest that mitochondria were not degraded through the LC-3 mediated autophagy pathway, rather through the Rab5 endosomal pathway (Chapter 5). To evaluate how Drp1KO affects mitophagy during OGD/R, western blot of mitochondrial fractions was performed on Drp1fl/fl cells exposed to OGD/R with and without viral delivery of Cre expression. No significant changes were detected in PINK1 and Parkin translocation, or in general mitochondrial protein ubiquitination (Fig. 6-5C). Interestingly, Rab5 expression in mitochondrial fractions revealed significant increase in response to OGD/R (p<0.05) and a significant reduction in response to Drp1KO (p<0.01). Mitochondrial Rab5 expression was significantly increased in control cells at 2R (p<0.01) and was significantly higher than mitochondrial Rab5 expression observed in Drp1 KO at 2R (p<0.01) (Fig. 6-5C). Mitochondrial Rab5 expression was unchanged in Drp1 KO cells during OGD/R. These data suggest that mitochondria in the absence of Drp1 is not sequestered in Rab5 endosomes for transport to lysosomes.

Lastly, to investigate what effect Drp1 has on viability, Drp1fl/fl neurons infected with LV-EF1a-cre were exposed to 2.5 hours of OGD and 6 hours of reoxygenation. Viability was determined through MTT assay and was compared to Drp1fl/fl neurons
infected with LV-EF1a-empty. Drp1 KO resulted in a 20% improvement in viability (Fig. 6-6) suggesting that Drp1 contributes to the progression of cell death during ischemia/reperfusion.

Fig. 6-5. Drp1-Independent mitophagy is not cleared through the Rab5 endosomal pathway. A) Western Blot of Drp1 KO in whole cell lysates during OGD/R. B) Western Blot of mitochondrial fractions probing for PINK1, Parkin, Ubiquitin, and Rab5. C) Quantification of protein levels across OGD/R, normalized to VDAC. Two-way ANOVA was used to detect differences across time and between conditions. Multiple comparisons across time were assessed by comparing the means of each time point with the mean of the control using Dunnett’s post-hoc analysis. Multiple comparisons between conditions were compared using Sidak post-hoc analysis. *p<0.05 across time, #p<0.05 between condition, n = 4 per group.
4. Summary

In summary, we report that Drp1 stabilizes mitochondrial architecture during OGD/R. This was demonstrated by both mitochondrial number and percent area in Drp1KO cells being unaffected by OGD/R. This is in direct contrast to control cells which demonstrated extensive disruption to mitochondrial morphology with reduced mitochondria in long networks or long unbranched morphologies and increased swollen and punctate mitochondria. The increase in mitophagic flux was unaffected by Drp1KO, as indicated by significant increases in mCherry puncta during OGD/R with Drp1KO. My results suggest that mitophagy is likely mediated through the PINK1/Parkin pathway of mitophagy and that mitochondria are sequestered by Rab5 endosomes for transport to lysosomes. Although no significant differences were observed in PINK1 and Parkin translocation to mitochondria, mitochondrial Rab5 expression was significantly repressed in the absence of Drp1, suggesting that Drp1-independent mitophagy may not be through the Rab5 endosomal pathway. These data in total suggest that improvements in cell viability found with Drp1KO is likely mediated by limiting disruption of the mitochondrial network rather than an effect of Drp1 inhibition on mitophagy.
CHAPTER 7: DISCUSSION

1. Summary of Results

The present study tested 4 hypotheses to investigate the role of mitochondrial quality control utilizing an *in vitro* model of ischemia/reperfusion. We made four novel observations:

i) Mitochondria morphology in primary cortical neurons can be classified into 4 distinct groups utilizing a machine-learning based classification system.

ii) Mitochondria undergo significant remodeling during OGD/R, resulting in extensive fragmentation and swelling.

iii) Mitophagic Flux is significantly increased via the PINK1/Parkin Pathway of Mitophagy and are sequestered by Rab5 endosomes for transport to the lysosome. Furthermore, mitochondria inside lysosomes are primarily punctate mitochondria.

iv) Knockout of Drp1 stabilizes mitochondrial architecture, but has no effect on mitophagic flux during OGD/R.

2. Classification of Mitochondrial Morphology: Current Knowledge and New Contributions

Mitochondria are highly dynamic organelles, constantly undergoing cycles of fission and fusion, generating an equilibrium of long interconnected and small punctate mitochondria at homeostasis. As reviewed by Kulek et al., deviations in this equilibrium have been observed in cell cycle progression and cellular differentiation, oxidative stress, metabolic perturbation, and induction of programmed cell death pathways [274], thereby generating considerable interest in this phenomenon. Despite the effort to study mitochondrial dynamics, investigations have been limited by their measures of mitochondrial morphology. Most studies utilized a qualitative or semi-quantitative
approach by developing a scoring system of fission/fusion profiles [240, 275-278, 294, 295], which lack precision and accuracy of physiologically relevant mitochondrial morphologies. To overcome this, recent studies have shifted to utilizing computational image analysis, commonly referred to as Image Cytometry, which limit observer and selection bias in morphological evaluations and demonstrate high throughput [296-301]. Similar to Leonard et al., our method aimed to measure four distinct morphological phenotypes including the quantification of swollen mitochondria [301], but in mouse primary cortical neurons.

2.1 Identifying Distinct Mitochondrial Morphologies

In Chapter 4, I extensively characterized 4 different mitochondrial morphologies (network, unbranched, swollen, and punctate) and constructed a random forest classifier to predict mitochondrial morphology in primary cortical neurons. The independence of these observed morphologies displayed significant differences in 32 size and shape descriptors. PCA analysis further confirmed 4 distinct groupings and tabulated z-scores revealed how morphologies compared with respect to each descriptor. The differences detected provided confidence of the feasibility of using this approach to classify mitochondrial morphology utilizing machine learning algorithm.

2.2 Model Assessment

Model assessment revealed that mitochondrial morphology prediction was highly specific. The model demonstrated an overall accuracy of 99.57% with a Kappa of 99.39%. Model accuracy was calculated as the proportion of total correct predictions and out of 468 objects within the test set, only two objects were mislabeled. Those mislabeled objects corresponded to confusion amongst swollen and punctate, as well as unbranched and swollen. Swollen and punctate generally have similar shape descriptors, with primary
morphological differences in size and interfacial density. Swollen objects are larger in size and possess a lower interfacial density when compared to punctate objects. Our data suggest that punctate mitochondria likely increase in size and transition to a swollen phenotype under pathological conditions, therefore it is not surprising that swollen and punctate mitochondria may be confused with one another, although this confusion is rare. Unbranched mitochondria are distinguished primarily by shape, specifically aspect ratio (AR) (the ratio of an objects width to its height). Long, skinny mitochondrial objects display large values for AR as compared to small circular mitochondria. Because swollen and unbranched mitochondria can have similar area, small unbranched mitochondria can be classified as swollen mitochondria, but again are rare in occurrence.

The high overall accuracy in our model assessment could be influenced by selecting images from two polarizing environments: highly fused and highly fragmented. Additionally, images analyzed in our model are compressed z-stacks, therefore losing 3-D resolution and potentially confounding results. Despite these concerns, in a relatively flat monolayer of neurons along with high technical replication and classification of hundreds of thousands of mitochondrial objects in a single experiment we report that these mitochondrial morphologies can be accurately predicted using machine learning techniques.

3. Mitochondria Fragmentation Following Ischemia/Reperfusion: Current Knowledge and New Contributions

There is a clear consensus that ischemia/reperfusion is associated with mitochondrial fragmentation and subsequent cell death [240, 302-307]. Recent data published in our lab demonstrate the complex temporal profile of mitochondrial fission during OGD and reoxygenation in the HT22 neural cell line revealing a biphasic response, with an initial but reversible phase of fragmentation observed during OGD, followed by a
second and sustained period of fission [240]. These data highlight the complexity of this phenomenon in the progression of ischemia/reperfusion and importantly, the need for precise and biologically meaningful measurement of mitochondrial morphology and its association with cell death. To address this, I utilized my classification system to measure changes in mitochondrial architecture during OGD and reoxygenation in primary neurons.

3.1 Comparing Mitochondrial Morphologies during OGD/R

As expected, significant changes were observed in all mitochondrial morphologies during OGD and reoxygenation (Ch. 4 – Mitochondrial Remodeling Following OGD/R: Tipping the Scales to a Fragmented Phenotype). The number of mitochondria in a network morphology was significantly decreased during OGD and early reoxygenation before being restored to control levels, although no changes were observed in total area occupied by mitochondrial networks. Networks make up over 60% of total mitochondrial content, suggesting that some mitochondrial networks likely fragment, while remaining networks expand in area and become larger and compensate for a loss in networked mitochondrial number. The number of unbranched mitochondria decreased at all time points throughout OGD and reoxygenation, while the total area of mitochondria in this morphology remained constant. These data suggest that unbranched mitochondria may be a transition point between networks and punctate mitochondria, with networks feeding large unbranched objects into the unbranched pool, while smaller unbranched mitochondria fragment into punctate and/or swollen mitochondria.

The decreases observed in networks and unbranched mitochondria were accompanied by increases in punctate and swollen mitochondria. Punctate objects were increased during OGD and early reoxygenation with corresponding increases in punctate area, before restoring back to control. Swollen objects were increased throughout the
duration of OGD/R with increases in swollen area at 1R. Swollen and punctate mitochondria only make up 5\% of mitochondrial content at control conditions and vary in size, therefore a larger study may find significant differences at additional timepoints. The restoration of punctate mitochondria to control levels at 2R could be due to a transition from punctate to swollen mitochondria. Under periods of ischemia, ATP depletion induces dis-equilibration of Ca\(^{2+}\) across the plasma membrane, resulting in high cytosolic Ca\(^{2+}\) and subsequent increases in intramitochondrial Ca\(^{2+}\) causing mitochondrial swelling [308]. Upon reperfusion, excessive ROS production damages mitochondrial lipids and proteins, this damage, in addition to Ca\(^{2+}\) dis-equilibrium, are major forces that induce pathologic activation of the mitochondrial permeability transition pore (mPTP) [280]. Permeability transition of mitochondria causes mitochondrial swelling and, eventually release of programmed cell death factors, and ultimately cell death [13, 280, 309].

Our analysis of mitochondrial morphology was performed over discrete time points, lacking precise temporal resolution. Monitoring mitochondrial morphology in a single cell over the duration of OGD/R would provide valuable information on the events leading up to the different morphology profiles observed in our study. Nonetheless, our highly sensitive and specific classification system has allowed the detailed analysis of the transition of morphologic states in the progression of OGD and reoxygenation.

4. **Mitophagic Flux in Ischemia/Reperfusion: Current Knowledge and New Contributions**

As discussed in Chapter 1 there is evidence to suggest that mitophagy is upregulated in the ischemia/reperfusion injury. ROS production following reperfusion induces extensive mitochondrial damage and dysfunction, threatening cellular health through the release of pro-apoptotic factors. Therefore, it is critical to sequester and degrade dysfunctional mitochondria through mitophagy. To date, studies have been
limited in their measurement of mitophagy, resorting to methods of protein localization, mitochondrial content, and qualitative measurements of images of mitochondria co-localized to lysosomes. Protein localization and expression do not provide conclusive evidence of increased mitophagy and although visualizing mitochondria being engulfed by autophagosomes/lysosomes confirms that mitochondria’s fate for degradation, it lacks quantitative resolution to understand what is occurring in a population of cells in diverse conditions. Therefore, we aimed to quantify mitophagic flux utilizing the mitoQC reporter mouse, which allowed us to visualize and quantify mitochondria inside auto/endo-lysosomes during OGD/R.

4.1 Mitophagy is Increased Following OGD/R

In Chapter 5 we evaluated the status of mitophagic flux in response to OGD/R. We report that mitophagic flux was significantly increased at 4 hours of reoxygenation, indicated by a significant increase in mCherry puncta. Validation that mCherry puncta were mitochondria inside lysosomes was accomplished by co-localization of mCherry puncta with LAMP1 (lysosome marker) immunofluorescence as well as total LAMP1 particle counts, which both displayed significant increases after OGD/R. As discussed previously, our model is based off cells being measured at discrete time points. Real-time monitoring of mitophagic flux in a single cell would allow precise resolution of the exact change in mitophagic flux occurring from baseline conditions and throughout OGD/R. To account for this, I plated coverslips as biological replicates, so each coverslip for a single time course contained a population of neurons from the same mouse. Additionally, high technical replication within each biologic replicate was performed to account for variation among the population of neurons (12 images per coverslip, ≈ 70 cells/coverslip).
4.2 Mitochondria are Cleared Through the PINK1/Parkin and Rab5 Endosomal Pathway

There are multiple pathways of mitophagy induction and clearance. Studies have demonstrated that mitophagy is primarily activated through the PINK1/Parkin pathway in both heart and brain in response to ischemia/reperfusion [260, 286-288]. Utilizing colocalization techniques and western blot, we found that PINK1 and Parkin translocate to the mitochondria during OGD/R. PINK1 accumulation occurred early during OGD and peaked at 2R, just before clearance of mitochondria. Parkin translocation also occurred early, but persisted through the course of OGD/R. This data is supported by evidence provided by Ordureau et al., as discussed in Chapter 5, of a feedforward mechanism for Parkin translocation and mitochondrial ubiquitination [149]. Mitochondrial ubiquitination was also observed at 2 hours of reoxygenation, which corresponded to the timepoint where peak increases in PINK1 and Parkin translocation were detected. These results confirm that mitophagy is activated through the PINK1/Parkin pathway during OGD and reoxygenation in primary cortical neurons.

To date, most studies have concluded that mitochondria tagged for degradation are sequestered and transported to the lysosome by autophagosomes, as measured by LC3 conversion [260, 286, 287]. Recent literature has suggested a pathway for mitochondrial clearance through PINK1/Parkin mediated Rab5 endosomal transport [161]. To determine whether mitochondria were transported to lysosomes through autophagosomes or endosomes, we probed for the LC3 conversion and Rab5 expression in mitochondrial fractions. We found that mitochondria were being sequestered by endosomes for transport to lysosomes in our model of OGD and reoxygenation.

4.3 Punctate Mitochondria are Sequestered for Degradation

Chapter 4 demonstrated significant mitochondrial fragmentation in response to
OGD/R, with increases in both punctate and swollen morphologies. Previous studies visualizing mitochondria inside autophagosomes by confocal microscopy and electron microscopy techniques demonstrate that these mitochondria are generally small (<1 µm in diameter, ≈0.75 µm²) [166, 310-312]. Utilizing my classification system, I wanted to evaluate the predominant morphology for sequestration of mitochondria tagged for degradation. This analysis revealed that punctate mitochondria represented the majority of mCherry puncta objects at 85.9%, followed by unbranched objects (8.5%), swollen objects (5.58%), and networks (0.02%). These data suggest although there may be some level of “macro” mitophagy, punctate mitochondria are preferentially sequestered for degradation. No objects detected were larger than 10 µm², but the average size for all mCherry objects were 0.549 µm², while mCherry objects identified as a punctate morphology had an average size of 0.382 µm². It is not clear why swollen mitochondria were not sequestered by early endosomes, other than the difference in size. Permeability of swollen mitochondria may also release factors that prevent ESCRT identification. It may also be an issue of availability of autophagosomes or endosomes required to transport mitochondria to lysosomes, and the smaller mitochondrial objects are sequestered first for unknown reasons.

Lysosomes possess an acidic environment designed to degrade its contents. Although mitoQC cells are designed to allow identification of mitochondria inside lysosomes by the quenching of GFP with low pH, it is difficult to assess whether mCherry puncta are mitochondria with mCherry-only fluorescence, or contents of degraded mitochondria filling up the lysosome. Despite this, our measurements are comparable with previous studies and since lysosomes are <1.2 µm in diameter, it is likely punctate mitochondria are sequestered for degradation [166, 310-313].
In Chapter 4, our data suggests that punctate mitochondria return to control levels at 2R, while the swollen phenotype is increased throughout late reperfusion and mitophagic flux is increased at 4R. These data suggest that during early reperfusion, punctate mitochondria face one of two fates: 1) transition to a swollen morphology or 2) sequestration for degradation through mitophagy. These data also suggest that mitophagy may be a restorative process necessary for cellular health in response to ischemia/reperfusion and that the rapid transition to a swollen phenotype prevents sequestration for mitophagy and is consistent with cell death.

5. Drp1 and Mitochondrial Quality Control: Current Knowledge and New Contributions

Investigation of the relationship between mitochondrial dynamics and mitophagy has been a large area of focus. Mitophagy proteins such as Parkin have been implicated to have influence on mitochondrial dynamics through the ubiquitination and subsequent degradation of fission and fusion proteins including Fis1, Drp1, and the mitofusins [236, 314]. Similarly, the mitochondrial fission protein Drp1, has been associated as a key component of mitophagic processes through governing the segregation of dysfunction mitochondria tagged for degradation [196, 198, 199, 236]. Inhibition of Drp1 has been shown to be neuroprotective against ischemia/reperfusion through preventing mitochondrial fragmentation associated with cell death [231, 233, 241, 242, 279, 290-294]. Although inhibition of fission during ischemia may improve outcomes, it remains unclear whether the same inhibition prevents the clearance of dysfunctional mitochondria accumulated during reperfusion.

5.1 Drp1KO Stabilizes Mitochondrial Architecture during OGD/R

Drp1KO was achieved in Drp1\textsuperscript{fl/fl} primary cortical neurons with lentiviral-Cre delivery, resulting in a 75% knockout of Drp1 7 days post-transduction. Utilizing our
machine learning classification system, we were able to reliably classify mitochondrial morphology during OGD and reoxygenation in the absence of Drp1. Drp1KO prevented mitochondrial morphologic disruption at all timepoints of OGD/R as opposed to the extensive fragmentation observed in controls. Specifically, Drp1KO prevented both network and unbranched fragmentation during OGD/R. Controls displayed significant decreases in the total number of networks and unbranched objects during OGD/R. Networks also had corresponding decreases in mitochondrial network area displaying significant group effects when compared to Drp1KO, suggesting Drp1 prevents the fragmentation of larger mitochondrial networks during OGD/R. Consistent with the results in Chapter 4, the total area of mitochondria in the unbranched state showed no significant changes, suggesting that fragmentation occurs to smaller unbranched mitochondria, while larger unbranched mitochondria remain intact. Despite the inhibition of unbranched fragmentation with Drp1KO, the total area of mitochondria in the unbranched state is reduced overall compared with controls. This suggests that Drp1 is essential for maintaining the size of unbranched mitochondria. Preventing fission of networks into large unbranched mitochondria results in a shift of smaller unbranched mitochondria without disrupting the number of mitochondria in this state under normal conditions.

Consistent with mitochondrial fragmentation, controls showed significant increases in the total number of swollen mitochondria as well as the total area of mitochondria in a swollen state. Drp1KO blocked the increase in swollen objects as well as prevented changes in mitochondrial swelling as a proportion of total mitochondrial area. These results suggest that Drp1KO blocks the transition of mitochondria to a swollen morphology induced by OGD/R. In contrast, total punctate objects showed no significant differences in either control or Drp1KO groups. With respect to total area of mitochondria
in a punctate state, significant increases were observed in control cells throughout OGD/R. Drp1KO prevented any increases in mitochondrial punctate area and was significantly decreased at 4R. These results suggest that punctate mitochondria are larger following OGD/R, without observing increases in punctate objects. In addition, Drp1KO blocked the increase in punctate area likely by preventing mitochondrial fragmentation. It is unclear if Drp1KO blocks mitochondrial fragmentation to a punctate morphology, thus indirectly reducing the pool of fragmented mitochondria that can undergo swelling, or if Drp1 plays a direct role in inducing mitochondrial swelling. There is some evidence to suggest Drp1 can play a direct role in mediating mitochondrial outer membrane permeabilization (MOMP) through the association and recruitment of pro-apoptotic proteins such as Bax and Bak, which trigger mitochondrial swelling, the release of cytochrome C, and cell death [241, 315-319]. Considering the role Drp1 may play in mitochondrial permeabilization and the increases in swollen mitochondria we observe in our data, increases in punctate area suggest Drp-1 dependent fragmentation results in a rapid transition to a small swollen phenotype, consistent with induction of cell death.

5.2 Mitophagic Flux is Independent of Drp1

We also evaluated the effect of Drp1KO on mitophagic flux during OGD/R. Significant increases in mCherry puncta were observed in response to OGD/R in controls. Drp1 KO had no effect on mitophagic flux, also displaying significant increases in mCherry puncta following OGD/R. Total Lamp1 puncta was also increased in controls and Drp1 revealed no effect, suggesting that mitophagic flux following OGD/R is independent of Drp1.

Although fission is necessary for mitophagy to occur [196], roughly 45% of mitochondrial objects are punctate mitochondria in both control and Drp1KO during
baseline conditions. Large interconnected networks may be more protected from the deleterious effects of ischemia/reperfusion by mitigating excessive ROS production from equilibration of matrix metabolites, intact mtDNA, and even membrane components such as complex I of the electron transport chain [16, 21-24], leaving punctate mitochondria vulnerable to ROS production and damage. With a large pool of vulnerable punctate mitochondria at baseline conditions, it is not surprising that mitophagic flux is still increased despite inhibited fission, especially if punctate mitochondria are the majority of mitochondria being transported to the lysosome. This likely corresponds to the decrease in punctate area observed at 4 hours of reoxygenation with Drp1KO. Additionally, recent evidence suggests that the mitochondrial Rho-GTPases, Miro1 and Miro2, also play a role in mediating fission in both mitochondria and peroxisomes [320, 321], which may provide another avenue for dysfunctional mitochondria to be sequestered for degradation.

5.3 Drp1-Independent Mitophagy is not Cleared Through the Rab5 Endosomal Pathway

We demonstrated that mitophagy was being activated by the PINK1/Parkin pathway and that mitochondria were being sequestered by Rab5 endosomes for transport to the lysosome. No changes were observed in PINK1 and Parkin translocation as well as general ubiquitination of mitochondrial proteins. Interestingly, mitochondrial Rab5 expression was significantly increased in controls and this increase was prevented by Drp1 KO, suggesting that mitochondria undergoing mitophagy in the absence of Drp1 are not being sequestered by the endosome for transport to the lysosome.

Lastly, Drp1KO was shown to be neuroprotective in primary cortical neurons following OGD and reoxygenation, indicated by a 20% improvement in viability. The improvement in viability is likely caused by an inhibition of mitochondrial fragmentation that leads to mitochondrial swelling and subsequent activation of cell death pathways.
6. Technical Limitations and Merits

A primary limitation to this study is lack of an *in vivo* model to validate our *in vitro* findings. To our credit, we did utilize primary cortical neurons isolated from mice, which are more physiologically relevant than the use of cell lines. Additionally, this study provides a solid scientific foundation for the next phase of this project, evaluating this mechanism in mouse models of brain ischemia/reperfusion. A second limitation as discussed previously is our lack of temporal resolution through real-time imaging. Monitoring neurons in real-time as they respond to OGD/R would provide valuable information in the transition of mitochondrial networks to punctate and swollen mitochondria in regard to mitochondrial fragmentation. A major limitation of live imaging is the limited sampling capabilities in real-time. While our study had less temporal resolution than real-time imaging, we have larger sampling capabilities, thus improving
confidence in our findings at the discrete timepoints we evaluated. Additionally, understanding the events that led to mitochondrial clearance would provide valuable insight in the progression of mitochondrial turnover in pathological conditions. While gaining temporal resolution, real-time imaging sacrifices statistical power, which utilizing our methods demonstrated superiority. Our high-throughput machine learning classification system was able to measure and classify hundreds of thousands of objects for a single experiment. Follow-up studies are ongoing to corroborate our findings with intact biological systems.

7. Conclusions and Future Directions

In consideration of our results, we theorize that mitochondrial dynamics and quality control are disrupted during ischemia/reperfusion and this contributes to cell death. Utilizing a highly sensitive and specific machine learning based classification system we have developed, our data have demonstrated that Drp1 induces extensive mitochondrial fragmentation in response to OGD/R, which is accompanied by an increase in swollen mitochondria. Knockout of Drp1 prevented mitochondrial fragmentation and swelling and is associated with improvements in cell viability. We further demonstrated that mitophagic flux is increased following OGD/R likely as a restorative process for turnover of damaged mitochondria. We propose that mitophagy is activated by the PINK1/Parkin pathway and that mitochondria are sequestered by Rab5 endosomes for transport to lysosomes. We demonstrated that knockout of Drp1 had no effect on mitophagic flux during OGD/R suggesting that mitophagy can proceed independent of Drp1-mediated mitochondrial fragmentation.

This study suggests a possible mechanism of cell death where Drp1 activity and excessive fragmentation shift the balance from physiological fission and healthy
mitochondria to pathological mitochondrial swelling, and cell death (Fig. 7-1). Future studies must further interrogate how enhancement or inhibition of mitophagy may contribute to mitochondrial quality control during ischemia/reperfusion injury in both in vitro and in vivo models.
APPENDIX A

IACUC Protocol Approval Letters

ANIMAL WELFARE ASSURANCE # A3310-01

TO: Thomas Sanderson
Emergency Medicine

FROM: Institutional Animal Care and Use Committee

DATE: May 09, 2016

SUBJECT: Approval of Protocol 16-01-030

TITLE: Dysfunctional Mitochondrial Quality Control Mechanisms Following Cardiac Arrest

Protocol Effective Period: May 09, 2016 - May 08, 2019

Your animal research protocol has been approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC).

Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any changes (e.g. procedures, lab personnel, strains, additional numbers of animals) must be submitted as amendments and require prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol application for committee review and approval.

The Guide for the Care and Use of Laboratory Animals (the Guide, NRC 2011) is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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Protocol ID: PRO00007770
Protocol Title: Dysfunctional Mitochondrial Quality Control Mechanisms Following Cardiac Arrest
Approval Period: 6/16/2017 - 6/16/2020

External Funding Sources:

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Internal Funding Sources:

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ANIMAL WELFARE ASSURANCE # D16-00198 (A3310-01)

TO: Anthony Anzell
Physiology

FROM: Institutional Animal Care and Use Committee

DATE: September 05, 2018

SUBJECT: Approval of Protocol IACUC-18-08-0767

TITLE: Mitochondrial Quality Control During Cerebral Ischemia-Reperfusion Injury

Protocol Effective Period: September 05, 2018 - September 04, 2021

Your animal research protocol has been approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC).

Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any changes (e.g. procedures, lab personnel, strains, additional numbers of animals) must be submitted as amendments and require prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol application for committee review and approval.

The Guide for the Care and Use of Laboratory Animals (the Guide, NRC 2011) is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).
APPENDIX B

Copyright License Agreement for Chapter 1

Mitochondrial Quality Control and Disease: Insights into Ischemia-Reperfusion Injury

Anthony R. Anzell, Rita Maizy, Karin Przyklenk, and Thomas H. Sanderson

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ABSTRACT

THE DYNAMICS OF LIFE AND DEATH: MITOCHONDRIAL FRAGMENTATION, MITOPHAGY, AND SWELLING DURING NEURONAL ISCHEMIA/REPERFUSION INJURY

by

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August 2020

Advisors: Thomas Sanderson, Ph.D. and Karin Przyklenk, Ph.D.

Major: Physiology

Degree: Doctor of Philosophy

Ischemic brain injury caused by cardiac arrest or stroke continue to be leading cause of death and disability in the U.S. While restoration of blood flow is necessary to salvage ischemic tissue, reperfusion paradoxically exacerbates injury via the production of reactive oxygen species, which, damage mitochondria and induce cell death. Therefore, it is critical to have stringent quality control mechanisms to ensure a healthy mitochondrial network. Mitochondrial fragmentation has been well characterized in the progression of ischemia/reperfusion and its association with cell death. Conversely, the role of mitophagy has been controversial regarding whether upregulation of mitophagy serves as a restorative process for the turnover of dysfunctional mitochondria, or have deleterious effects through energy imbalance and induction of apoptosis. Furthermore, the interplay between mitochondrial dynamics and mitophagy in ischemia/reperfusion remains unknown.

Utilizing an *in vitro* model of ischemia/reperfusion in primary cortical neurons, we tested four hypotheses:
i) Mitochondrial morphology in primary cortical neurons can be classified into 4 distinct groups utilizing a machine-learning based classification system.

ii) Mitochondria undergo significant remodeling during oxygen-glucose deprivation and reoxygenation, resulting in extensive fragmentation and swelling.

iii) Mitophagic flux is significantly increased via the PINK1/Parkin pathway of mitophagy and are sequestered by Rab5 endosomes for transport to the lysosome. Furthermore, mitochondria inside lysosomes are primarily punctate mitochondria.

iv) Knockout of Drp1 stabilizes mitochondrial architecture, but has no effect on mitophagic flux during OGD/R.

In support of hypothesis I, we demonstrate the development of a highly sensitive and specific machine-learning based classification system of 4 distinct mitochondrial morphologies: network, unbranched, swollen, and punctate. Using the Random Forest algorithm, our high-throughput classification system demonstrated mitochondrial morphologies can be predicted with accuracy. In agreement with hypothesis II, mitochondrial fragmentation was quantitatively measured utilizing our machine-learning classification system during oxygen-glucose deprivation and reoxygenation. This was demonstrated by decreases in networks and unbranched mitochondria with corresponding increases in punctate and swollen mitochondria. In support of hypothesis III, we demonstrated that mitophagic flux was increased during oxygen-glucose deprivation, by the presence of mCherry puncta in primary cortical neurons isolated from mitoQC reporter mice. Additionally, mitophagy was activated by the PINK1/Parkin pathway of mitophagy and that punctate mitochondria were sequestered by Rab5.
endosomes for transport to the lysosome. Lastly, in support of hypothesis IV, we
demonstrate the KO of Drp1 prevents mitochondrial fragmentation and swelling during
oxygen-glucose deprivation in primary cortical neurons, but has no effect on mitophagic
flux. In conclusion, our results illustrate that in the context of ischemia/reperfusion injury,
Drp1 shifts the balance from physiological fission and healthy mitochondrial turnover, to
pathological fragmentation and swelling inducing cell death.
# AUTOBIOGRAPHICAL STATEMENT
## Anthony R. Anzell

## Education

<table>
<thead>
<tr>
<th>Year</th>
<th>Degree</th>
<th>Field</th>
<th>Institution</th>
<th>Location</th>
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<tr>
<td>2009-13</td>
<td>B.S.</td>
<td>Biomedical Sciences</td>
<td>Grand Valley State University, MI, USA</td>
<td></td>
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<tr>
<td>2014-Pres.</td>
<td>Ph.D.</td>
<td>Physiology</td>
<td>Wayne State University School of Medicine, MI, USA</td>
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## Awards:

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<td>2019</td>
<td>HHT Travel Award to present data at the International HHT Scientific conference</td>
<td>HHT Foundation</td>
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<td>2018</td>
<td>Three Minute Thesis Competition</td>
<td>Wayne State University, Department of Physiology</td>
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<tr>
<td>2017</td>
<td>Three Minute Thesis Competition</td>
<td>Wayne State University, Department of Physiology</td>
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<td>2017</td>
<td>Michael Wider Graduate Student Travel Award</td>
<td>Wayne State University, Department of Physiology</td>
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<td>2016</td>
<td>Graduate Student Representative (elected)</td>
<td>Wayne State University, Department of Physiology</td>
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<td>2015</td>
<td>HHT Travel Award to travel to the HHT scientific conference</td>
<td>Private Donor</td>
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<td>2014</td>
<td>Marion I. Barnhart Graduate Student Award in recognition for outstanding achievement in academic and research</td>
<td>Wayne State University, Department of Physiology</td>
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<tr>
<td>2014</td>
<td>Interdisciplinary Biomedical Sciences (IBS) Fellowship (competitive)</td>
<td>Wayne State University, School of Medicine</td>
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## Peer-Reviewed Publications:

