MITOCHONDRIAL RESPONSE TO AXONAL INJURY

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ABSTRACT

The failure of axonal regeneration is due to myriad reasons both cell intrinsic and extrinsic. In this thesis, I sought to investigate an intrinsic reason for regeneration failure in the CNS. Specifically, I investigated the role of axonal mitochondria in the axonal response to injury. A viral vector (AAV) containing a mitochondrially targeted fluorescent protein (mitoDsRed) as well as fluorescently tagged LC3 (GFP-LC3), an autophagosomal marker, was injected into primary motor cortex, to label the corticospinal tract (CST), of adult rats. The axons of the CST were then injured by dorsal column lesion at C4-C5. We found that mitochondria in injured CST axons near the injury site are fragmented and fragmentation of mitochondria persists for two weeks before returning to pre-injury lengths. Fragmented mitochondria have consistently been shown to be dysfunctional and detrimental to cellular health. Interestingly, transection of axons within the sciatic nerve resulted in mitochondrial fission but did not result in the fragmentation of mitochondria. Inhibition of Drp1, the GTPase responsible for mitochondrial fission, using a specific pharmacological inhibitor (mDivi-1) blocked fragmentation. Additionally, it was determined that there is increased mitophagy in CST axons following spinal cord injury based on increased colocalization of mitochondria and LC3. In vitro models revealed that mitochondrial calcium uptake is necessary for injury induced mitochondrial fission, as inhibiting mitochondrial calcium uptake using RU360, a mitochondrial calcium uniporter inhibitor, prevented injury induced fission. This phenomenon was also observed in vivo. These studies indicate that following injury,

both *in vivo* and *in vitro*, axonal mitochondria undergo increased fission, which may result in an ATP deficit that contributes to the lack of regeneration seen in CNS neurons.

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LIST OF ABBREVIATIONS

μl: Microliter

μg: Microgram

μm: Micrometer

μM: Micromolar

AD: Alzheimer's disease

ADP: Adenosine diphosphate

AIS: Axon initial segment

ALC: Acetyl-L-carnitine

ALS: Amyotrophic lateral sclerosis

APP: Amyloid precursor protein

ATG: Autophagy associated gene

ATP: Adenosine triphosphate

Aβ: Amyloid beta

Bax: Bcl-2 associated X protein

BBB: Blood-brain barrier

CAT: Catalase

CCCP: Carbonyl cyanide m-chlorophenyl

hydrazone

CMA: Chaperone-mediated autophagy

CNS: Central nervous system

CNTF: Ciliary neurotrophic factor

CoQ: Coenzyme Q

CSPG: Chondroitin sulfate proteoglycan

CST: Corticospinal tract

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNDrp1: Dominant negative Drp1

Dnm: Dynamin

Drp1: Dynamin-related protein 1

ECM: Extracellular matrix

ER: Endoplasmic reticulum

ETC: Electron transport chain

FCCP: Trifluoromethoxy carbonylcyanide phenylhydrazone

Fis1: Mitochondrial fission protein 1

GAP: Growth-associated protein

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GED: GTPase-effector domain

GFP: Green fluorescent protein

GPX: Glutathione peroxidase

HD: Huntingtin's disease

hrs: Hours

Htt: Huntingtin protein

IMM: Inner mitochondrial membrane

INF2: Inverted formin 2

iPSC: Induced pluripotent stem cell

KLF- Kruppel-like factor

LC3: Protein light chain 3

LIR: LC3-interacting region

MAP: Microtubule associated protein

MCU: Mitochondrial calcium uniporter

mDivi-1: Mitochondrial division inhibitor 1

MDV: Mitochondiral derived vesicle

Mff: Mitochondrial fission factor

Mfn1/2: Mitofusin 1/2

mHtt: Mutant Huntingtin

MiD49/51: Mitochondrial dynamic protein 49/51

Min: minutes

MOMP: Mitochondrial outer membrane

permeabilization

mPTP: Mitochondrial permeability transition pore

NACA: N-acetylcysteineamide

NgR: Nogo receptor

nM: Nanomolar

NO: Nitric Oxide

OMM: Outer mitochondrial membrane

OPA: Optic atrophy protein

OPC: Oligodendrocyte precursor cell

PD: Parkinson's disease

PFA: Paraformaldehyde

PINK1: PTEN induced kinase-1

PNN: Perineural net

PNS: Peripheral nervous system

PS-1: Presenilin-1

RAG: Regeneration-associated gene

RCG: Retinal Ganglion cell

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROI: Region of interest

ROS: Reactive oxygen species

SCI: Spinal cord injury

SNpc: Substantia nigra pars compacta

SOD: Superoxide dismutase

TDP43: TAR DNA-binding protein 43

TFAM: Mitochondrial transcription factor A

TIM: Translocase of inner membrane

TOM: Translocase of outer membrane

YFP: Yellow fluorescent protein

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CHAPTER 1: LITERATURE REVIEW

Part I: Spinal Cord Injury

Epidemiology, Morbidity, mortality, and economic burden

Spinal cord injury (SCI) is a severe medical issue with high mortality and long-term morbidity. It is estimated that there are approximately 291,000 people in the United States living with SCI (Lasfargues, et al. 1995), with an annual incidence of approximately 17,730 new SCI cases, not including those that die during the incident which caused the SCI (Jain, et al. 2015). Males between the ages of 15 and 35 make up the majority of SCI cases (60%), however trends over the past few decades have demonstrated an increase in the median age at which SCI occurs, which is attributed to a greater proportion of injuries related to falls in the elderly population (van Den Hauwe, et al. 2020). The leading cause of SCI is blunt force trauma due to motor vehicle accidents (48%), followed by falls (21%), and sports injuries (14.6%) (Devivo 2012). SCI can be classified based on severity and extent of the lesion, with incomplete tetraplegia (47.6%) being most common followed by incomplete paraplegia (19.9%), complete paraplegia (19.6%), and complete tetraplegia (12.3%) (Center 2019). Advancements in treatment for acute spinal cord injury over the past few decades have resulted in a substantial decrease in mortality rates for SCI patients in the first year after injury and beyond, although overall life expectancy is still below the normal population (Devivo 2012). Most deaths in SCI patients occur due to acute comorbidities such as pneumonia, respiratory failure, septicemia and other infections, and pulmonary embolus (DeVivo, et al. 1993; DeVivo, et al. 1999). Because of the increases in life expectancy and the prevalence of comorbidities requiring medical care and possible hospitalization, the cost of care for SCI is a nontrivial concern. Depending on the severity of injury and the age at which the patient is injured the lifetime cost of care for an SCI patient can range from $1.5 million to $5 million (Center 2019). Therefore, it is of the utmost importance to find an effective treatment to restore function following SCI. For this to happen a thorough understanding of the mechanisms involved in preventing regeneration following injury in the CNS are needed.

Axonal response to injury: PNS vs CNS

Following injury, axons of the adult mammalian central nervous system (CNS) are unable to regenerate unlike those of the peripheral nervous system (PNS), which show a capacity for regrowth. For an injured axon to regenerate and regrow it must first form a new growth cone, a process that most axons in the mammalian CNS fail to complete. Instead, following injury CNS axons exhibit retraction bulbs, also called dystrophic growth cones, at their tips (Hill 2017).

Mammalian PNS axons begin to form new growth cones as early as 3 hours following axotomy (Erturk, et al. 2007; Pan, et al. 2003). The initial event after axotomy is a large influx of calcium into the injured axon, as disruption of the plasma membrane exposes the axoplasm to the extracellular space (Fig. 1.1B). The influx of calcium following injury is necessary for new growth cone formation to occur (Chierzi, et al. 2005; Kamber, et al. 2009; Kulbatski, et al. 2004). The influx of calcium into the cut end of the injured axon triggers a cascade of events beginning with the activation of voltage-gated calcium channels, further increasing the intracellular calcium concentration (Mandolesi, et al. 2004; Ziv and Spira 1995). Such a vast influx of calcium into the intracellular space can then lead to a reversal of Na^{+}-K^{+} exchange pumps leading to release of calcium from intracellular stores and a sustained elevation of intracellular calcium (Mandolesi, et al. 2004). The increased intracellular calcium concentration has multiple effects on the neuronal cytoskeleton, which are necessary for new growth cone formation. Among these cytoskeletal effects is a wave of microtubule depolymerization, which is spatially and temporally correlated with the retrograde calcium wave, along the approximately 100 µm axonal segment immediately proximal to the axotomy site (Erez, et al. 2007; Sahly, et al. 2006; Spira, et al. 2003). At the same time, actin punctae in adhesion complexes depolymerize, freeing the plasma membrane from the physical constraints of the extracellular matrix (ECM) and freeing the membrane to collapse and seal the injury site as well as assemble the new growth cone (Sahly, et al. 2006). Additionally, the increased calcium concentration stimulates calpain activity leading to the cleavage of submembranous spectrin giving additional freedom of reorganization to the plasma membrane (Gitler and Spira 1998) (Fig. 1.1B). Finally, if the axon is to survive, the cut membrane of the injured axon must reseal, a process that is directly dependent on the influx of calcium described above. This process occurs in two stages. First, the plasma membrane of the cut axon collapses once again separating the intracellular and extracellular space (Spira, et al. 1993). Second, vesicles are transported to the cut axon tip and form multivesicular structures which fuse with the disrupted plasma membrane (Fishman and Bittner 2003; McNeil and Kirchhausen 2005; Spira, et al. 1993) (Fig. 1.1C). Proper resealing of the injured plasma membrane allows for restoration of ionic homeostasis within the intracellular space. Following closure of the ruptured plasma membrane, formation of a new growth cone can begin (Fig. 1.1D).

After recovery of normal intracellular calcium levels, microtubules initially repolymerize with their plus ends directed randomly. Microtubule repolymerization allows for the anterograde delivery of Golgi derived vesicles that will subsequently be used as a source of new membrane in the extending axon (Erez, et al. 2007; Sahly, et al. 2006). Additionally, microtubule repolymerization facilitates the retrograde movement of endocytic vesicles. The reestablishment of normal calcium concentrations also allows for the reassembly of the actin cytoskeleton which generates the mechanical force at the leading edge of the lamellipodium necessary for growth cone movement and axon regeneration. With the actin cytoskeleton intact, microtubules then direct their plus ends into filopodia advancing the growth cone (Bradke, et al. 2012).

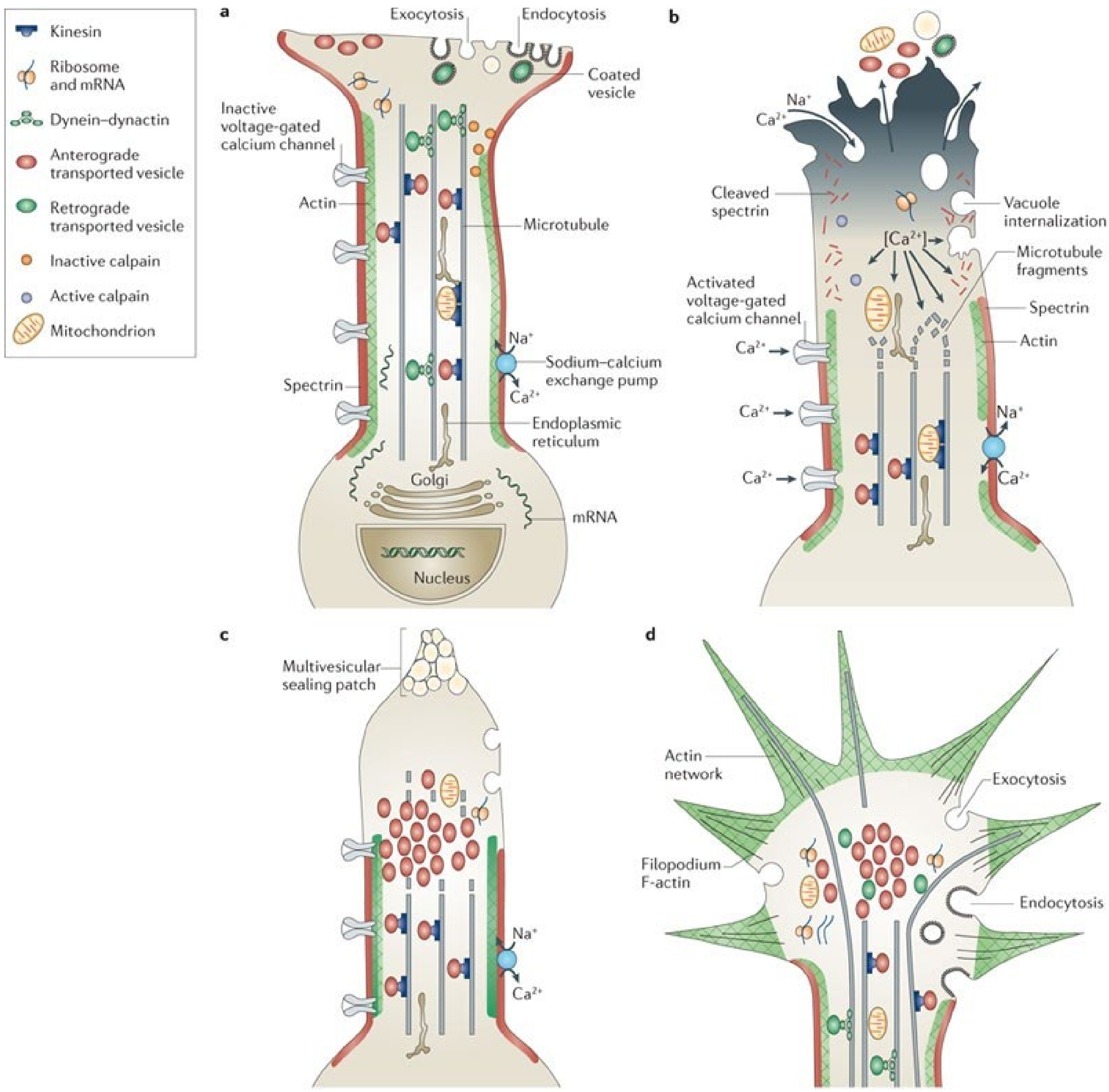

Kinesin
Ribosome and mRNA
Dynein–dynactin
Anterograde transported vesicle
Retrograde transported vesicle
Inactive calpain
Active calpain
Mitochondrion
a
Exocytosis
Endocytosis
Coated vesicle
Inactive voltage-gated calcium channel
Actin
Microtubule
Na+
Sodium–calcium exchange pump
Ca2+
Spectrin
Endoplasmic reticulum
Golgi
mRNA
Nucleus
b
Na+
Ca2+
Cleaved spectrin
Vacuole internalization
[Ca2+]
Microtubule fragments
Activated voltage-gated calcium channel
Spectrin
Actin
c
Multivesicular sealing patch
d
Actin network
Exocytosis
Filopodium F-actin
Endocytosis

Figure 1.1 PNS response to axonal injury (Adapted from (Bradke, et al. 2012)) (A) Various components of the intact neuron take part in the transformation of a cut axonal end into a growth cone. **(B)** Membrane rupture leads to elevation of the free intra-axonal calcium concentration (shown by the grey shading) by diffusion from the cut end, membrane depolarization, activation of voltage-gated calcium channels and release of calcium from intracellular stores such as the endoplasmic reticulum. Among many other processes, the elevated intracellular calcium concentration ($[Ca^{2+}]_i$) leads to the following: microtubule and actin depolymerization, membrane retrieval, activation of calpains (which proteolytically cleave submembrane spectrin), stimulation of vacuole internalization and activation of numerous other enzymes (not shown). These processes are confined to an axonal segment at the tip of the cut axon and lead to a small volume of the axoplasm being lost before the membrane at the cut end collapses. **(C)** In most cases, the membrane of the cut end reseals by membrane collapse and accumulation of vesicles to form a sealing patch. Once a seal is formed, calcium removal mechanisms lower the $[Ca^{2+}]_i$, and this process is followed by microtubule and actin repolymerization. Anterogradely transported vesicles accumulate at the tips of the undamaged microtubule tracks. **(D)** In the final phase of growth cone reconstruction, actin filaments assemble to generate the mechanical force at the leading edge of the lamellipodium. Microtubules polymerize and point their plus ends towards the plasma membrane. Note that proteins are translated both in the cell body (not shown) and at the growth cone.

Failure of any of the events described above to occur will result in the formation of a retraction bulb instead of a new growth cone. Retraction bulbs are defined as a swollen, bulbous axonal end resulting from the continued flow of axoplasm into the injured axon tip. Retraction bulbs are marked by the presence of accumulated organelles, such as smooth endoplasmic reticulum and mitochondria, resulting from continued anterograde transport, as well as disorganized microtubules. The presence of disorganized microtubules is thought to be due to continued microtubule polymerization in the presence of a rigid plasma membrane that does not allow expansion (reviewed in (Hill 2017)). While the precise cause of regenerative failure and retraction bulb formation within the CNS has yet to be determined, numerous obstacles to CNS regeneration have been found encompassing both cell extrinsic and cell intrinsic mechanisms.

Extrinsic mechanisms for the lack of CNS regeneration

Neuronal recovery and regeneration from injury within the CNS does not occur in isolation. It requires complex interactions between the vast network of glial cells and neuronal processes, both on the macro structural level and the molecular levels. Additionally, it involves the communication of both glia and neurons with their extracellular environment. Therefore, it is important to understand the function of glial cells in the response to CNS injury.

The glial cells of the CNS include astrocytes, oligodendrocytes, oligodendrocyte precursor cells (OPCs), and microglia. Under normal physiological conditions oligodendrocytes are responsible for myelination of neuronal processes. OPCs along with some oligodendrocytes are able to remyelinate neurons under certain circumstances. Microglia are phagocytic cells that are the resident immune cells of the CNS. They rapidly react to and provide neuronal protection and participate in wound healing following injury. Astrocytes have a variety of functions in the CNS, including ion homeostasis, neurotransmitter regulation, and blood brain barrier maintenance (reviewed in (Fitch and Silver 2008)). Additionally, following injury astrocytes are the main cell type responsible for walling off damaged areas of CNS tissue to protect it from further damage, forming the “glial scar” (Fitch, et al. 1999; Myer, et al. 2006). Originally, it was thought that the physical barrier of the glial scar was solely responsible for a lack of regeneration found in the CNS, however it has subsequently been shown that the glial scar is far more complex than a simple physical barrier and its role in the prevention of neuronal regeneration following CNS injury involves many cellular and molecular interactions. Moreover, reactive astrocytes within the glial scar upregulate a number of molecules including tenascin, semaphoring-3, ephrin-B2, slit-proteins, and many chondroitin sulfate proteoglycans (CSPGs), which have been shown to contribute to regeneration failure to varying degrees (reviewed in (Fitch and Silver 2008)).

Myelin-associated growth inhibitors

Some of the earliest studied molecules in regard to axon growth inhibition following injury were associated with CNS myelin and myelin debris. These molecules include Nogo (also referred to as Reticulon-4), myelin associated glycoprotein, oligodendrocyte myelin glycoprotein, and Nogo-receptors (NgR) (Schwab 2004; Schwab

2010; Schwab and Strittmatter 2014). In vitro studies demonstrated the ability of these molecules to block neurite outgrowth, however subsequent in vivo studies were inconclusive. Early in vivo studies using antibodies to block myelin associated growth inhibitors in vivo showed long distance axonal regeneration and functional recovery following SCI (Bregman, et al. 1995; Huang, et al. 1999; Schwab 2004). However, follow up studies using genetic deletion of Nogo, myelin associated glycoprotein, oligodendrocyte myelin glycoprotein, and NgR were unable to recapitulate the earlier results, leading to the conclusion that myelin associated growth inhibitors do not play a primary role in regeneration failure following injury (Lee, et al. 2010a; Zheng, et al. 2005; Zheng, et al. 2003). Nevertheless, NgR signaling has been implicated in regulating synapse remodeling following injury indicating that these molecules do have some role in recovery from CNS injury (Schwab and Strittmatter 2014).

Chondroitin Sulfate Proteoglycans

During CNS development CSPGs are important for axon guidance and boundary formation, providing inhibitory axon guidance cues (reviewed in (Carulli, et al. 2005)). In the non-developing CNS, CSPGs are upregulated following injury. These include NG2 proteoglycan, phosphacan, brevican, versican, and neurocan (Jones, et al. 2003a; Levine 1994; McKeon, et al. 1995; Rhodes, et al. 2006). The upregulations of CSPGs has been demonstrated in multiple CNS injury models including brain stab wound (Fitch and Silver 1997), injury to the dorsal root of the spinal cord (Pindzola, et al. 1993), and direct spinal cord injury (Fitch and Silver 1997; Jones, et al. 2003a; Jones, et al. 2003b). CSPG upregulation following injury is rapid and sustained, which in combination with their well-established ability to inhibit neurite outgrowth in vitro (Dou and Levine 1994; Snow, et al. 1990), make CSPGs an ideal candidate to be the primary extrinsic mechanism for the failure of CNS axons to regenerate across CNS lesions. As mentioned, modification or digestion of CSPGs in vitro can reliably counter their growth inhibitory effects and allow for robust neurite outgrowth (Bradbury, et al. 2002; Grimpe and Silver 2004; McKeon, et al. 1995; McKeon, et al. 1991; Steinmetz, et al. 2005). However, in vivo studies are less definitive in the role that CSPGs play in neuronal regeneration failure after injury. Enzymatic treatment to remove sugar epitopes of CSPGs showed long distance regeneration of adult axons through CNS white matter tracts and increased sprouting of injured corticospinal tract (CST) fibers (Barritt, et al. 2006; Steinmetz, et al. 2005). Additionally, a transgenic mouse model in which astrocytes expressed a CSPG degrading enzyme demonstrated local reductions in CSPGs and enhanced CNS regeneration (Cafferty, et al. 2007). However, there is also a growing body of evidence that suggests that CSPGs are not a promising target for the promotion of CNS regeneration. Conflicting studies have shown that CSPG digestion, genetic deletion, or receptor disruption fail to result in consequential axonal regrowth across astrocyte scars (reviewed in (Sofroniew 2018)). Additionally, astrocytes are not the primary producers of CSPGs following CNS injury (Anderson, et al. 2016). Moreover, there are indications that the effects seen in previous studies relating to CSPG degradation may be due to effects on synapse remodeling as opposed to axon regrowth. In the uninjured CNS, CSPGs contribute to the perineuronal net (PNN), where CSPGs regulate developmental and adult synaptic plasticity. Alteration of CSPG levels in the PNN is able to increase axon sprouting and synaptic remodeling following SCI (Dyck and Karimi-Abdolrezaee 2015; Fawcett 2015; Mironova and Giger 2013; Wang and Fawcett 2012). Therefore, CSPGs may not directly inhibit axonal regrowth following injury and may instead have indirect effects. Nonetheless, it is clear that CSPGs also play a role in the lack of regeneration seen after CNS injury. However, by only looking at the extracellular environment one is missing half of the story. It is therefore paramount that any intrinsic barriers to axonal regrowth following injury be examined.

Intrinsic mechanisms for the lack of CNS regeneration

Unlike during adulthood, during development CNS neurons show robust growth potential (Goldberg 2003; Goldberg, et al. 2002a). However, as CNS neurons mature, they lose their intrinsic ability for growth and show an inability to reactivate intrinsic growth programs after injury (Goldberg 2003; Goldberg, et al. 2002b). There are many intrinsic reasons for this loss of regrowth potential including a blunted regeneration-associated gene (RAG) response in mature neurons, changes in the expression patterns of specific genes, changes in molecular transport and trafficking as neurons mature, and energetic problems at the sites of injury.

Regeneration-associated gene (RAG) response

Injury to PNS neurons triggers an intrinsic response in the cell body that results in the activation and repression of hundreds of neuron-intrinsic genes. This pattern of gene activation and repression in response to injury is termed the RAG response. CNS neurons demonstrate a blunted RAG response upon injury. The reasons for a dampened

RAG response in adult CNS neurons are multifaceted. Two possible explanations are discussed below:

First, there are differences in cytoplasmic RNA metabolism between CNS and PNS neurons which alters the RAG response. Following neuronal injury, a histological phenomenon called chromatolysis, described as decoloration of the cytoplasm in sections stained with basophilic dye, occurs. This is caused by scattering of endoplasmic reticulum (ER) and breakdown of ribosomes leading to a reduction in protein synthesis. In PNS neurons chromatolysis eventually abates and protein synthesis once again increases, however most CNS neurons exhibit irreversible chromatolysis (reviewed in (Moon 2018)). One hypothesis is that, the irreversible chromatolysis seen in CNS neurons is caused by the activation of specific ribonucleases which prevent the neuron from synthesizing adequate growth-associated proteins (GAPs) (Zhao, et al. 2013).

Second, CNS and PNS neurons show differences in transcription factor regulation and chromatin accessibility of RAG response genes. It is well established that transcription factors play a major role in the RAG response in injured PNS neurons (Hu, et al. 2016a; Li, et al. 2015c; van Kesteren, et al. 2011; Venkatesh and Blackmore 2017). C-Jun was the first RAG transcription factor to be identified with many others subsequently being found, including ATF3, SOX11, KLF7, and STAT3 (reviewed in (Fawcett and Verhaagen 2018)). As CNS neurons mature there is a developmental decline in chromatin accessibility in the promoter regions of certain RAGs in cortical neurons (Venkatesh, et al. 2016). Therefore, in the adult CNS certain RAGs are unable to be activated by their transcription factor due to inaccessible chromatin preventing the proper pattern of RAG activation needed for axonal regrowth. Specifically, C-Jun and STAT3 were shown to have a high proportion of inaccessible target gene promoters while SOX11 and KLF7 RAG targets were not blocked (Venkatesh, et al. 2018).

Changes in gene expression patterns in adult CNS neurons

As neurons mature there are many changes in the patterns of gene expression. These changes have been shown to play a major role in the lack of regeneration in CNS neurons. Genetic manipulation of genes known to change expression pattern postnatally have had varying levels of success in improving axonal regeneration. Some of the most well studied and promising gene targets include Kruppel-like Factors 4 and 9 (KLF4/9), PTEN, and SOCS3.

Kruppel-like factors are zinc-finger-containing transcription factors that regulate proliferation, differentiation, development, and cell death (McConnell and Yang 2010). In vitro overexpression of both KLF-4 and KLF-9 reduces the capacity of neurons to grow, which correlates with the observation that as neurons mature the expression of KLF-4 and KLF-9 increases (Moore, et al. 2009). Moreover, knockdown or knockout of KLF-4 in adult mice promotes axonal regeneration following optic nerve injury by disinhibiting the Jak-STAT pathway (Moore, et al. 2009; Qin, et al. 2013). Knockdown of KLF-9 showed even more robust regeneration following optic nerve injury (Benowitz, et al. 2017).

PTEN is a protein translation inhibitor that acts by repressing Phosphoinositide-3 (PI3) kinase thereby suppressing downstream signaling via Akt and mTOR. Genetic deletion of *pten* removes the inhibitory signal on Akt and mTOR promoting axonal growth in retinal ganglion cells (RCGs) (Duan, et al. 2015; Park, et al. 2008). Furthermore, when deletion of *pten* is combined with Zymosan, which elevates trophic factor levels and a cAMP analog some injured RCGs are able to regenerate the entire length of the optic nerve (Kurimoto, et al. 2010).

SOCS3 is a repressor of the Jak-STAT signaling pathway and is therefore an inhibitor of neuronal growth. As CNS neurons mature SOCS3 levels increase. Genetic deletion of *socs3* disinhibits the Jak-STAT pathway and allows neurons to respond to trophic signals that promote regeneration such as ciliary neurotrophic factor (CNTF) (Park, et al. 2009; Qin, et al. 2013; Smith, et al. 2009).

Changes in molecular transport and trafficking

For regeneration to occur, the molecules involved in growth must reach the appropriate locations within the neuron. This means that proper transport and trafficking is paramount in the regeneration process. However, in the adult CNS, trafficking of certain molecules in response to injury is reduced. CNS neurons are highly polarized with a somatodendritic and an axonal compartment (reviewed in (Bentley and Banker 2016)). While this polarization is critical for the establishment of distinct structures and functions of axons and dendrites (reviewed in (Maeder, et al. 2014)), it also creates a paradigm in which CNS neurons are unable to regrow after injury. During development growth related molecules are transported to both the somatodendritic and axonal compartments, however as CNS neurons mature many growth-related molecules are excluded from the axonal compartment (reviewed in (Britt, et al. 2016)). In particular, several integrins, TrkA, TrkB, and IGF receptor are all transported into both compartments during development but are excluded from the axonal compartment in the adult CNS reducing growth potential (Andrews, et al. 2016; Franssen, et al. 2015; Hollis, et al. 2009a; Hollis, et al. 2009b). Neurons use a multitude of mechanisms to control molecular distribution, most of which are centered around the axon initial segment (AIS). These include the establishment of a selective filtration barrier by Ankyrin G, without which there is a loss of axonal identity causing axons to resemble dendrites (reviewed in (Yoshimura and Rasband 2014)). The AIS also contains a concentrated area of the GEF Efa6, which activates the GTPase Arf6 affecting the directionality of endosome (Eva, et al. 2017; Franssen, et al. 2015). Proximal to the AIS is the pre-exclusion zone which filters cargo based on the kinesin type that is carrying it (Farías, et al. 2015). Each of these mechanisms and many others together contribute to establish the molecular identity of axons and as a neuron matures this molecular identity changes and develops into one that loses its capacity for growth.

Energetic deficits at the site of injury

Axonal growth is a very energy demanding process and therefore largely dependent on the presence of healthy mitochondria. As CNS neurons mature mitochondrial motility decreases, reducing the ability for mitochondria to respond and shuttle to the site of injury (Lewis, et al. 2016; Mar, et al. 2014; Milde, et al. 2015). This is in contrast to regenerating neurons of the PNS, which show increases in anterograde trafficking of mitochondria following injury that is sustained for up to 21 days (Misgeld, et al. 2007). Additionally, regenerating axons have a higher density of mitochondria within the growth region than non-regenerating axons (Han, et al. 2016). Studies promoting anterograde mitochondrial trafficking, either through genetic deletion of the mitochondrial stop signaling protein syntaphilin in the sciatic nerve (Zhou, et al. 2016) or through overexpression of Armadillo Repeat Containing X-Linked 1 (Armcx1) protein, which aids in the tethering of the GTPase Miro 1 to the mitochondrial membrane, in optic nerve (Cartoni, et al. 2016) demonstrated enhance regeneration following injury . Once present at the site of injury, the primary role of mitochondria is to produce ATP. Increasing mitochondrial respiration after injury increases axonal regeneration (Han, et al. 2016; Zhou, et al. 2016) while inhibiting mitochondrial respiration inhibits axonal regeneration (Zhou, et al. 2016). Therefore, there is a need for increased energy production following injury which is not met in CNS neurons. However, the mechanisms for this energy deficit following injury have yet to be elucidated. For this reason, I chose to focus my studies on the mitochondrial response to axonal injury in the CNS.

Part II: Mitochondrial function and dynamics

Mitochondrial function

The mitochondrion is a double membraned organelle that is vitally important to cell survival. Mitochondria originally evolved from the endosymbiotic relationship of an α-protobacterium and a eukaryotic host. Throughout evolution the mitochondrion retained its bacterial capacity to produce ATP through oxidative phosphorylation while also fully integrating into the metabolism of the host eukaryotic cell (reviewed in (Gray 2012)). As the mitochondria is a double membraned organelle, it also contains two aqueous compartments, the mitochondrial matrix and the inner membrane space. The inner mitochondrial membrane (IMM), which is the location of the oxidative phosphorylation machinery used to make ATP, has many folds and involutions, called cristae, giving it a vastly larger surface area than the outer mitochondrial membrane (OMM). Mitochondria also contain their own genome consisting of 13 genes which encode proteins that form the core components of the oxidative phosphorylation machinery (reviewed in (Pfanner, et al. 2019)). The remainder of mitochondrial proteins are encoded in the nuclear genome and contain mitochondrial localization signals. These proteins are transported to mitochondria following translation and imported into the mitochondria using receptors on the OMM (reviewed in (Kutik, et al. 2009; Neupert and Herrmann 2007). Despite being crucial for energy production within cells, ATP production is far from the only function of mitochondria. Mitochondria also buffer cytosolic calcium, contribute to redox homeostasis within cells, and act as critical regulators of apoptosis. Each of these functions will be briefly described below.

ATP production

ATP is produced through oxidative phosphorylation by the electron transport chain (ETC) located in the IMM. This is done through the stepwise transfer of electrons to each protein component of the ETC, a process that consumes oxygen and establishes an electro-chemical gradient. The ETC consists of four protein complexes, named complex I-IV, which shuttle electrons to their final electron acceptor, oxygen, forming water. As the electrons are shuttled between complexes by mobile electron carriers such as Coenzyme Q (CoQ) and cytochrome *c*, a proton gradient is established. Complex V, also known as ATP synthetase, then uses the energy stored in this gradient to phosphorylate ADP and form ATP (reviewed in (van der Bliek, et al. 2017)).

Cytosolic calcium buffering

The capacity for mitochondria to take up calcium has been known for more than 60 years (Rossi and Lehninger 1963). Moreover, it was originally thought that mitochondria were the main cytosolic calcium stores, since shown to be the case after discovery that the channel responsible for calcium uptake into mitochondria, the mitochondrial calcium uniporter (MCU) shows a low affinity for calcium (Bragadin, et al. 1979; Scarpa and Graziotti 1973). Later it was shown that the ER was in fact the largest calcium store in the cell (Streb, et al. 1983). Nonetheless, mitochondria remain important calcium buffers. Instead of being the main calcium store within a cell, mitochondria act as buffers at high calcium microdomains, which are generally the sites of increased cellular activity (reviewed in (Fonteriz, et al. 2016)). This ability of mitochondria to take up calcium serves dual roles. First, calcium uptake increases the activity of multiple Kreb's cycle enzymes while also increasing ATP production (Jouaville, et al. 1999; McCormack, et al. 1990). Second, fast mitochondrial calcium uptake in high calcium microdomains allows mitochondria to modulate many different cellular processes including general cell stimulation (Park, et al. 1996; Villalobos, et al. 2002; White and Reynolds 1997), neurotransmitter secretion (Montero, et al. 2000), and plasma membrane voltage gated Ca^{2+} channels (Hernandez-Guijo, et al. 2001; Tekmen and Gleason 2010).

Redox Homeostasis

Reactive oxygen species (ROS) are highly unstable oxidizing agents that can cause cellular damage through multiple mechanisms including direct DNA damage and lipid peroxidation. Mitochondria are constantly generating ROS as a normal consequence of oxidative phosphorylation. Most ROS generated by mitochondria come from the activity of complex I and complex III (reviewed in (Murphy 2009)). The most common form of ROS generated by mitochondria is the superoxide anion (O_2^-). Under normal conditions the O_2^- is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) (reviewed in (Auchère and Rusnak 2002)). H_2O_2 itself is an oxidizing agent, albeit much less powerful that O_2^-, so it is also broken down and converted to water by peroxiredoxins, catalase (CAT), and glutathione peroxidases (GPX) (reviewed in (Mailloux, et al. 2013)). If unchecked O_2^- can react with reactive nitrogen species (RNS) such as nitric oxide (NO) to form peroxynitrate ($ONOO^-$). This can then be converted into a number of other damaging reactive species (reviewed in (Valerio and Nisoli 2015)). While under normal physiological conditions, mitochondria only produce 1-5% of intracellular ROS (reviewed in(Wei, et al. 2001)). However, when mitochondria become dysfunctional their production of ROS dramatically increases and surpasses the buffer capacity of the cell. The increase in ROS can then directly damage the ETC lowering the mitochondrial capacity to make ATP as well as damaging mitochondrial DNA. This damage only causes greater production of ROS which can further damage the cell and eventually lead to cell death (reviewed in (Islam 2017)). Therefore, maintenance of healthy mitochondria is of the utmost importance.

Regulation of apoptosis

While mitochondria are responsible for a not insignificant portion of damaging ROS produced within the cell (described above), they are also able to act as damage sensors that can trigger apoptosis if conditions within a cell become too dire. Key to the role of mitochondria in apoptosis is cell morphology. Mitochondria can have many different shapes and conformations due to separate fission and fusion machineries (to be discussed in a later section). When a cell is undergoing apoptosis, its mitochondria become fragmented due to repeated bouts of fission and inhibition of fusion (Lee, et al. 2004). Additionally, the cristae of the IMM remodel. Mitochondrial fission promotes the oligomerization and translocation of the proapoptotic regulator Bcl-2 associated X protein (BAX) to the mitochondria. This translocation facilitates mitochondrial outer membrane permeabilization (MOMP). MOMP allows for the release of various proapoptotic factors into the cytosol, including cytochrome *c*, serine protease OMI/HtrA2, Smac/Diablo, endonuclease G, and apoptosis inducing factor (AIF). The release of these proapoptotic factors then triggers caspase activation and cell death (reviewed in (Suen, et al. 2008).

Mitochondrial Dynamics

The myriad roles that mitochondria play within the cell require mitochondria to be more than simply static energy producers. Instead, mitochondria are highly dynamic organelles that continually undergo transport, fission, and fusion, especially in neurons. The details of mitochondrial dynamics in neurons is discussed below.

Neuronal mitochondrial transport

In neurons, mitochondria undergo bi-directional transport along axons where they are targeted to and maintained at specific sites along the axon, synapses and at the growth cone. Given the unique structure of neurons, neuronal mitochondria face transport challenges not seen in other cell types. Because of the enormous amount of energy needed for neurons to carry out basic physiological processes it is imperative that mitochondria are shuttled to areas of need and that a healthy population of mitochondria is maintained. In neurons, mitochondria must travel lengths measured in centimeters, or in the case of the largest human nerves, meters, as opposed to micrometers in other cell types. Observations made both in vitro and in vivo have shown that at any given time only 30-40% of mitochondria are in motion (Chen, et al. 2016). Mitochondrial motion is far from uniform. Similar to slow transport cargos, motile mitochondria undergo intermittent transport at rates more comparable to fast transport cargo (reviewed in (Black 2016; Smith and Gallo 2018)). Additionally, the distance, duration, and direction of mitochondrial motion is variable, with mitochondria able to switch direction of transport while in motion (Chen, et al. 2016). The remaining 60-70% of mitochondria within neurons are in a stalled state and go long periods of time without movement.

Mechanisms of neuronal mitochondrial transport

Neuronal mitochondria are transported both anterogradely and retrogradely along microtubules powered by ATP generated by mitochondrial respiration (Chen, et al. 2016; Spillane, et al. 2013; Zala, et al. 2013). Transport is accomplished by the motor proteins kinesin KIF5, for anterograde transport, and dynein, for retrograde transport (Lin and Sheng 2015). Binding of the molecular motor proteins to mitochondria is carried out by the adaptor proteins Miro1/2 and Milton/TRAK1/2. Miro is an atypical Rho-like GTPase on the OMM that interacts with TRAK(Glater, et al. 2006). Trak then binds the heavy chain of kinesin or dynein. TRAK1 can bind both the heavy chain of KIF5 and dynein, while TRAK2 preferentially binds dynein (van Spronsen, et al. 2013). Miro contains two calcium sensing EF hand domains. The binding of calcium to the EF hand domains results in Miro binding directly to KIF5 preventing interaction with Trak and preventing movement (Wang and Schwarz 2009). Additionally, myosin 19, an actin associated motor protein can interact with mitochondria and regulate their movement along actin filaments (Quintero, et al. 2009).

As mentioned above, 60-70% of neuronal mitochondria are stationary. Mechanistically the stalled population could be a result of simple inactivity, lack of interaction with a molecular motor, or lack of interaction with the cytoskeleton. However, a number of molecules have been identified that directly tether mitochondria to the cytoskeleton thereby regulating the stalling process. Syntaphilin is an OMM protein that has the ability to bind microtubules (Lin and Sheng 2015). When syntaphilin is deleted in mice the motile pool of mitochondria is vastly increased (Kang, et al. 2008). Syntaphilin mediated mitochondrial stalling occurs through interaction with dynein light chain 8 on microtubules (Chen, et al. 2009). Like Miro, syntaphilin is able respond to calcium, in which case syntaphilin binds to kinesin and sequesters the protein thereby stalling the mitochondria (Chen and Sheng 2013).

Microtubule associated proteins (MAPs) have also been observed to regulate the stalling of mitochondria within neurons. Studies in which Tau was over expressed in vitro have shown increased mitochondrial stalling (Dubey, et al. 2008; Ebneth, et al. 1998; Stoothoff, et al. 2009) attributable to an inability of kinesin to bind microtubules highly decorated with Tau (Trinczek, et al. 1999). Moreover, in mouse hippocampal MAP1B knockout neurons, retrograde transport was increased indicating a role for MAPs in mitochondrial stalling.

While mitochondrial transport is certainly important for meeting the energy requirements of neurons and maintaining a healthy mitochondrial population, it is by no means the only way in which this is achieved. Mitochondria also undergo continual morphological remodeling through fusion and fission.

Mitochondrial Fusion

Mitochondrial fusion involves the joining together of the OMM and IMM of two different mitochondria. This process results in the exchange of mitochondrial content including mitochondrial DNA, which prevents the accumulation of damaged or mutant DNA in any specific mitochondria (Chen, et al. 2005; Yang 2015). Mitochondrial fusion is especially important in times of cellular stress, such as starvation, as it allows for the sharing and mixing of mitochondrial metabolites thereby maximizing the efficiency of mitochondrial metabolism (reviewed in (Silva Ramos, et al. 2016)). Fusion of the OMM and IMM generally occur simultaneously, but given that the fusion machinery for the OMM and IMM is distinct, this can occur sequentially (Malka, et al. 2005; Meeusen, et al. 2004). Fusion of the OMM is carried out by Mitofusin 1 and Mitofusin 2 in humans

(Mfn1 and Mfn 2) (Santel and Fuller 2001) and Fzo1 in yeast (Rojo, et al. 2002). Mfn 1 and Mfn 2 are large GTPases present in the OMM. For mitochondrial fusion to occur mitofusins must be present on apposing mitochondrial membranes. The mitofusins form heterooligomers which promote mitochondrial tethering. Fusion of apposing mitochondrial membranes is then carried out in a GTP dependent manner (Hoppins and Nunnari 2009). Highlighting the importance of mitochondrial fusion in maintaining cellular health is the observation that mutations in Mfn2 that prevent it from participating in fusion results in type 2a Charcot–Marie–Tooth disease, a hereditary peripheral neuropathy (Züchner, et al. 2004).

Fusion of the IMM is carried out by Optic Atrophy 1 (OPA1) in humans and Mgm 1 in yeast. OPA 1 gets its name because when mutated it results in optic atrophy, a condition marked by the death of the retinal ganglion cells that make up the optic nerve (Alexander, et al. 2000; Delettre, et al. 2000). OPA1 is a three-membrane-pass protein that faces the intermembrane space. Unlike mitofusins, OPA1 need not be present on apposing membranes for fusion of IMM to occur, although the exact details of how OPA1 mediates IMM fusion are yet to be worked out (Song, et al. 2009).

Mitochondrial Fission

Mitochondrial fission, the division of one mitochondrion into two daughter mitochondria, is equally important to the health of the cell. Fission is a multistep process centered around the actions of the GTPase Dynamin-related protein 1 (Drp1 in humans and Dnm1 in yeast). Mitochondrial fission is an essential process developmentally as genetic deletion of Drp 1 in mice is embryonically lethal (Ishihara, et al. 2009; Wakabayashi, et al. 2009). In neurons, mitochondrial fission is necessary for the proper distribution of mitochondria to synapses and for branching to occur (Armijo-Weingart, et al. 2019; Wakabayashi, et al. 2009). Mitochondrial fission is also important for mitochondrial quality control and mitophagy, a mitochondria specific form of autophagy (to be discussed in detail in a later section), as decreasing mitochondrial size through fission appears to be required for mitophagy to occur (Buhlman, et al. 2014; Twig, et al. 2008). Additionally, as discussed above, mitochondrial fission participates in apoptosis by promoting the release of cytochrome *c* into the cytosol (Frank, et al. 2001).

Drp1 Structure

Drp1 is the key molecular player in the mitochondrial fission process, forming an oligomeric contractile ring around mitochondria that is responsible for scission (Francy, et al. 2015; Mears, et al. 2011). Drp 1 is an 80 kD cytosolic GTPase that, unlike its dynamin relatives, lacks a lipid-binding pleckstrin-homology domain (Hoppins and Nunnari 2009). Alternative splicing gives rise to eight different isoforms of Drp1 with tissue-specific expression patterns (Howng, et al. 2004; Yoon, et al. 1998). Drp1 is composed of four domains: 1) an N-terminal GTPase domain which harbors the enzymatic activity, 2) a middle domain necessary for oligomerization, 3) an alternative-spliced variable domain that contains most posttranslational modification sites (also called the B-insert), and 4) a C-terminal GTPase effector domain (GED) which interacts with the GTPase domain and is responsible for multimerization (Fröhlich, et al. 2013). In the cytosol, the α-helicies of the middle and GED domains form a stalk, which interacts with the stalk of another Drp1 molecule forming a criss-cross dimer (Fig. 1.2).

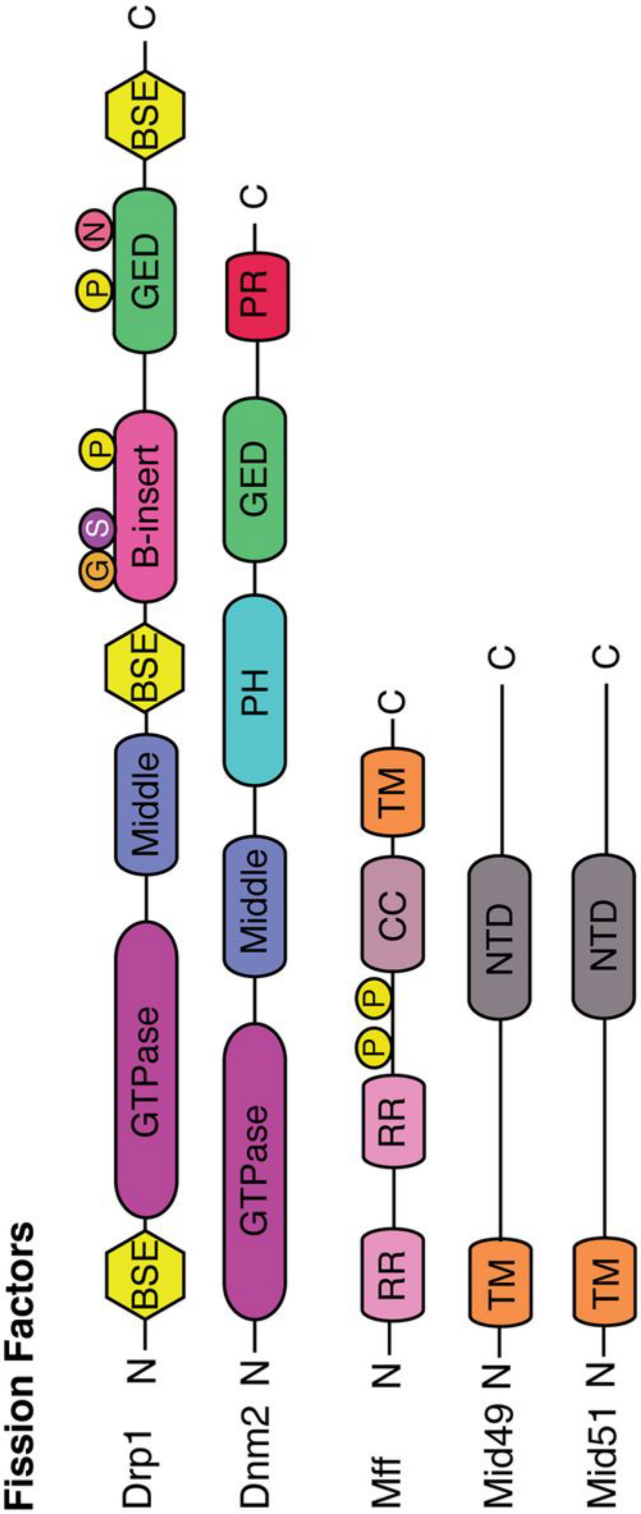

Fission Factors
Drp1
N
BSE
GTPase
Middle
BSE
B-insert
G
S
P
GED
P
N
BSE
C
Dnm2
N
GTPase
Middle
PH
GED
PR
C
Mff
N
RR
RR
P
P
CC
TM
C
Mid49
N
TM
NTD
C
Mid51
N
TM
NTD
C

Figure 1.2 Schematic representation of the structural elements of the fission proteins, and their associated post-translational modifications (Adapted from (Tilokani, et al. 2018)) Illustration of the core machinery proteins involved in mitochondrial. Domains are depicted in different colors. Identified location of post-translational modifications are indicated by P (Phosphorylation), N (S-nitrosylation), S (SUMOylation), G (O-GLcNAcylation), A (Acetylation) or U (Ubiquitination); BSE, bundle signalling elements; CC, coil-coil; GED, GTPase effector domain; HR, heptad repeat; MTS, mitochondrial targeting sequence; NTD, nucleotidyl transferase domains; PH, Pleckstrin homology; PR, Proline rich; RR, repeat regions; TM, transmembrane.

Regulation of Drp1 activity

The activity of Drp1 is regulated by posttranslational modification and protein degradation. Multiple SUMOylation, s-nitrosylation, and phosphorylation sites have been identified, the majority of which are in the variable domain (Santel and Frank 2008).

Phosphorylation is the most extensively studied regulatory mechanism of Drp1 activity. Depending on the site, phosphorylation can be either inhibitory or activating. Phosphorylation at serine 616 (S616) is activating while phosphorylation at serine 637 (S637) is inhibitory. Multiple kinases can phosphorylate Drp1 S616 under different circumstance. Cdk1/cyclin B kinase phosphorylates Drp1 S616 during mitosis (Taguchi, et al. 2007). In the context of cancer, Erk2 has been demonstrated to phosphorylate Drp1 S616 to aid in tumor proliferation (Kashatus, et al. 2015; Serasinghe, et al. 2015). In neurons, Cdk5 kinase phosphorylates Drp1 S616 during NMDA-induced cell death (Jahani-Asl, et al. 2015).

Drp1 S637 is phosphorylated by cAMP-dependent protein kinase A (PKA) during starvation conditions (Gomes, et al. 2011) and exerts an inhibitory effect on GTPase activity by modifying the interaction of the GTPase domain and the GED, leading to elongated mitochondria (Chang, et al. 2010; Cribbs and Strack 2007). Additionally, Drp1 S637 phosphorylation inhibits recruitment of Drp1 to the OMM (Cereghetti, et al. 2008), providing evidence for dual roles for Drp1 S637. The phosphatase calcineurin has been demonstrated to dephosphorylate Drp1 S637 to mediate calcium-induced, Drp1-dependent mitochondrial fission (Cribbs and Strack 2007). Dephosphorylation of Drp1

S637 by calcineurin has little to no effect on Drp1 oligomerization or GTPase activity. Instead, calcineurin mediated Drp1 S637 dephosphorylation promotes recruitment of Drp1 to the OMM (Cereghetti, et al. 2008; Losón, et al. 2013).

While the effects of phosphorylation on Drp1 activity is most well-known other posttranslational modifications have been identified. Small ubiquitin-like modifier (SUMO) is a ubiquitin-related protein, which can be covalently linked to a lysine residue on a target protein. Drp1 SUMOylation occurs at 8 non-consensus sites within the variable domain, four of which are unique to different Drp1 isoforms (Figueroa-Romero, et al. 2009). SUMOylation of Drp1 has been shown to protect against ubiquitination (Harder, et al. 2004). Additionally, SUMOylation contributes to the stabilization of the interaction between Drp1 and the OMM (Wasiak, et al. 2007).

S-nitrosylation is the covalent linking of nitric oxide (NO) to the thiol group of an amino acid residue, most commonly cysteine, which can affect protein function, stability, or subcellular localization (Gould, et al. 2013). Under pathological conditions in which NO is overproduced, Drp1 was found to be S-nitrosylated at C644 resulting in rapid Drp1-dependent mitochondria fragmentation and ultimate cell death (Barsoum, et al. 2006b; Yuan, et al. 2007). These observations remain controversial however, as subsequent studies failed to find an increase in oligomerization or GTPase activity upon S-nitrosylation (Bossy, et al. 2010).

Finally, Drp1 has been shown to be ubiquitinated. Ubiquitination is the covalent attachment of ubiquitin, a 76 amino acid polypeptide, to lysine residues of target proteins, thereby either marking them for proteasomal degradation or altering molecular interactions (Li and Ye 2008). Drp1 has been shown to be ubiquitinated by E3 ubiquitin ligases Parkin (Wang, et al. 2011) and MARCH5 (Yonashiro, et al. 2006). Ubiquitination of Drp1 by Parkin targets Drp1 for proteasomal degradation (Lutz, et al. 2009). The role of MARCH5 is less clear, however. Initial studies concluded that MARCH5 targets Drp1 for degradation resulting in an increase in mitochondrial fusion (Nakamura, et al. 2006; Yonashiro, et al. 2006), while subsequent studies lead to the opposite conclusion (Karbowski, et al. 2007; Park, et al. 2010). The conflicting results of these studies may be explained by observations that other proteins involved in mitochondrial fission and fusion are also substrates for MARCH5, including Mfn1 and Mfn2 (Park, et al. 2010; Park, et al. 2014; Sugiura, et al. 2013). Therefore, more research will be necessary to determine the role of MARCH5 in Drp1-dependent mitochondrial fission.

Molecular mechanism of mitochondrial fission

In short, mitochondrial fission is caused by a contractile ring formed by oligomers of Drp 1, which hydrolyzes GTP to cause contraction and scission of the mitochondrial membrane. However, mechanistically, mitochondrial fission is a multistep process involving the coordination of multiple adaptor and effector proteins. Mitochondrial fission must first begin with a “pre-constriction” step because the diameter of mitochondria is too large for Drp1 to form a contractile ring around them (Fig. 1.3 Step 1). Drp1 oligomers form contractile rings with a diameter of up to 100 nm, while mitochondria have diameters between 0.5-1.0 µm (Ingerman, et al. 2005; Mears, et al. 2011). Pre-constriction begins when juxtaposed segments of ER wrap around the mitochondria. As the ER wraps around the mitochondria two actin nucleators, inverted formin 2 (INF2) (ER-associated) and Spire1C (mitochondria associated), interact to give rise to polymerizing actin fibers that exert pressure on the OMM as they elongate constricting the mitochondria (Korobova, et al. 2013; Li, et al. 2015b; Manor, et al. 2015) (Fig. 1.3 Step 1). Myosin IIa and myosin IIb have also been shown to be involved in pulling the tethered ER and OMM together thus aiding in the pre-constriction (Korobova, et al. 2014). These pre-constriction sites, while necessary for fission, only mark potential fission sites and are independent of Drp1 (Friedman, et al. 2011; Osellame, et al. 2016). As mentioned above, Drp1 lacks a PH domain and cannot directly bind lipids. Therefore, Drp1 interaction with the OMM requires the actions of adaptor proteins (Fig. 1.3 Step 2). Four such adaptors have been identified in metazoans: Fis1, mitochondrial fission factor (Mff), mitochondrial dynamic protein 49 (MiD49), and mitochondrial dynamic protein 51 (MiD51) (reviewed in (Wilson, et al. 2013)). In yeast Fis1 is required for fission, however in metazoans Fis1 has a more ancillary role (Lackner, et al. 2009). Mff is the primary adaptor responsible for Drp1 mediated fission in metazoans (Gandre-Babbe and van der Bliek 2008; Losón, et al. 2013; Otera, et al. 2010). Current models suggest that Drp1 is recruited from the cytosol by MiD49/51, which inhibits the GTPase of Drp1. Then through either posttranslational modification or through interaction with Fis1, MiD49/51 facilitate the interaction of Drp1 with Mff resulting in Drp1 oligomerization and formation of a contractile ring (reviewed in (Wilson, et al. 2013). Following GTP hydrolysis, the contractile ring contracts forming a highly constricted tubule between the two future daughter mitochondria (Fig. 1.3 Step 3). To complete the fission process, dynamin 2 (Dnm2) is then recruited to the highly constricted tubule by an as yet unknown mechanism (Fig. 1.3 Step 4). Dnm2 then hydrolyzes GTP completing the scission event and yielding two daughter mitochondria (Lee, et al. 2016) (Fig. 1.3 Step 5).

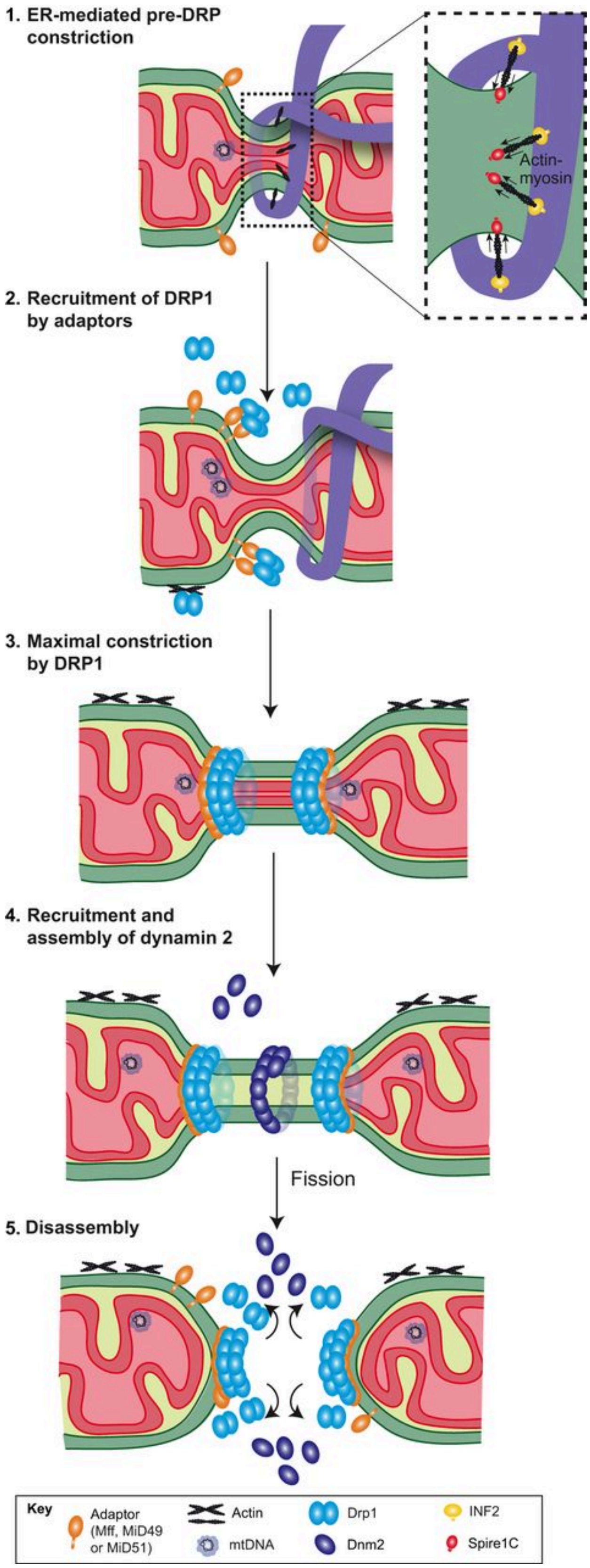

1. ER-mediated pre-DRP constriction
Actin-myosin
2. Recruitment of DRP1 by adaptors
3. Maximal constriction by DRP1
4. Recruitment and assembly of dynamin 2
Fission
5. Disassembly
Key
Adaptor (Mff, MiD49 or MiD51)
Actin
mtDNA
Drp1
Dnm2
INF2
Spire1C

Figure 1.3 Model for mitochondrial fission in animal cells. (Adapted from (Kraus and Ryan 2017) Step 1, the 'pre-Drp1' constriction of mitochondria by the ER constriction machinery takes place, with potential cross-talk between replicating mitochondrial nucleoids. The enlarged view depicts ER-localized INF2 initiating the nucleation of actin filaments, which may associate with mitochondria through Spire1C. Growing actin filaments, together with myosin II, exert pressure on the mitochondrial outer membrane. Step 2, the future fission site is marked for further constriction. This involves binding of the mitochondrial fission adaptor Mff at the neck, which facilitates the recruitment of Drp1. MiD49 and/or MiD51 may also be recruited at this time. Localized changes in lipid composition (e.g. increased cardiolipin) may also facilitate Drp1 assembly. Following adaptor assembly, Drp1 dimers are recruited to the fission site. Different oligomeric forms of Drp1 may be recruited to the mitochondrial surface, including some by actin filaments. Adaptors such as MiD49 and MiD51 may enhance the assembly of the Drp1 contractile ring at the membrane neck. Step 3, GTP hydrolysis fuels the conformational changes, thereby mediating Drp1 constriction, which may enable inner membrane scission to take place. Step 4, following this, Dnm2 is recruited to the constricted mitochondrial neck. Here, additional Dnm2 adaptor proteins might be involved. Step 5, upon GTP hydrolysis, further constriction occurs to complete fission, before the fission machinery is disassembled.

Part III: Overview of Mitophagy

Overview of different forms of autophagy

As noted above, through the process of oxidative phosphorylation mitochondria are the main energy producing structures in cells. However, as a consequence of oxidative phosphorylation, mitochondria produce ROS in the form of superoxide anion, hydrogen peroxide, and hydroxyl radicals, that not only damage other organelles and cellular structures, but can also damage mitochondria (Wallace 2005). If mitochondrial damage becomes too severe, mitochondria can release large amounts of calcium as well as cytochrome *c* into the cytosol and trigger apoptosis (Parsons and Green 2010). Therefore, proper maintenance of mitochondria is vital to cellular health. Mitochondria employ several quality control mechanisms to maintain their health. First, mitochondria have two AAA protease complexes on the IMM that degrade unfolded membrane proteins (reviewed in (Karbowski and Youle 2011; Langer, et al. 2001). This surveillance system also monitors nuclear encoded proteins as mitochondrial genomes only code for 13 of the more than 1000 mitochondrial proteins (Pfanner, et al. 2019). Recently, mitochondrial derived vesicles (MDVs), vesicular buds from mitochondrial tubules carrying damaged mitochondrial proteins have been identified (Soubannier, et al. 2012b). After budding off of mitochondria, MDVs can fuse with lysosomes through the endosomal system, thereby degrading the damaged portion of the mitochondria, while sparing the rest of the organelle (Soubannier, et al. 2012a). Mitochondrial quality control through surveillance of unfolded/misfolded proteins and MDVs accounts for only small percentage of mitochondrial protein degradation. The remaining mitochondrial quality control and protein turnover is carried during mitophagy

Autophagy is the bulk degradation of cytosolic components and organelles through delivery to the lysosome. There are three types of autophagy: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Macroautophagy makes up the majority of autophagic processes going on within a cell. Microautophagy occurs when lysosomes directly engulf portions of the cytoplasm through invaginations of the lysosomal membrane (Mizushima and Komatsu 2011). CMA occurs on proteins that contain a KFERQ amino acid motif. These proteins are recognized by heat shock conjugate 71kDa protein (Hsc70) and other co-chaperones and bound to the lysosomal protein LAMP-2A before being taken up into the lysosome and degraded (reviewed in (Orenstein and Cuervo 2010). Macroautophagy can be non-selective, such as the bulk autophagy induced during starvation that is important for the recycling of essential nutrients, or selective, in which case the cell is able to rid itself of damaged or unwanted organelles. Macroautophagy occurs when a portion of the cytosol or an organelle is enveloped by a double membranous structure called the autophagosome, which then fuses with a lysosome to form an autophagolysosome to facilitate the degradation of its contents (reviewed in (Tanida 2011)).

Mechanistically, macroautophagy consists of five steps: 1) initiation of the isolation membrane, 2) elongation of the isolation membrane, 3) closure of the isolation membrane and autophagosome formation, 4) autophagosome-lysosome fusion, 5) lysosomal degradation (Tanida 2011). The molecular mechanisms of macroautophagy have been carefully worked out using *S. cerevisiae,* in which approximately 30 autophagy associated genes (ATGs) have been identified (Klionsky, et al. 2003). However, a detailed analysis of this work is beyond the scope of this thesis. Instead the focus will be on mitophagy in mammals, which is very similar mechanistically and follows the same five steps described above. Mitophagy is the mitochondrial selective form of the more general process autophagy.

Mechanism of mitophagy

The PINK1/Parkin pathway is most well studied mammalian mitophagy model. PTEN induced kinase-1 (PINK1) is a serine/threonine kinase that contains a mitochondrial localization sequence (Youle and Narendra 2011). In healthy mitochondria, PINK1 is targeted to the OMM where it is constitutively imported to the IMM through the TIM/TOM complex. Once on the IMM, PINK1 is cleaved by multiple proteases, including mitochondrial-processing protease (MPP) and presenilin-associated rhomboid-like protease (PARL) (Deas, et al. 2011; Greene, et al. 2012; Meissner, et al. 2011; Narendra, et al. 2010) (Fig. 1.4). Upon loss of mitochondrial membrane potential, PINK1 import to the IMM is inhibited and PINK1 instead remains on the OMM and interacts with TOM (Lazarou, et al. 2012). The presence of PINK1 on the OMM acts as a damage sensor and recruits the E3 ubiquitin ligase, Parkin from the cytosol to the OMM (Narendra, et al. 2010) initiating mitophagy (Narendra, et al. 2008) (Fig. 1.4). The mechanism by which PINK1 recruits Parkin to the OMM is still not fully understood. PINK1 may directly bind to and phosphorylate Parkin, thereby causing its activation and translocation from the cytosol (Geisler, et al. 2010; Kim, et al. 2008; Narendra, et al.

2008; Narendra, et al. 2010; Shiba, et al. 2009). However, the possibility that PINK1 phosphorylates an unknown substrate that then recruits Parkin to the OMM remains a possibility. Parkin ubiquitin ligase activity increases following translocation to the OMM (Matsuda, et al. 2010), where it can form either lysine (K) 48 polyubiquitin chains, associated with proteasomal degradation (Chan, et al. 2011), or lysine (K) 63 polyubiquitin chains, associated with autophagic degradation (Lim, et al. 2006). It is likely that the formation of these polyubiquitin chains on the OMM is the triggering event for mitophagy (Geisler, et al. 2010; Lee, et al. 2010b). Whether the presence of K48 or K63 polyubiquitin chains initiate mitophagy is still undetermined, as both proteasomal degradation of OMM proteins through K48 polyubiquitination (Chan, et al. 2011; Tanaka, et al. 2010) and K63 polyubiquitination of voltage-dependent anion channel (VDAC) (Geisler, et al. 2010) have been shown to cause prompt mitophagy. Regardless of whether mitophagy is initiated by K48 or K63 polyubiquitination, the presence of polyubiquitin chains on OMM proteins recruits the ubiquitin binding adaptor protein p62/SQSTRM1, which is able to bind to the autophagosome membrane (reviewed in (Youle and Narendra 2011)). In addition to its ability to polyubiquitinate OMM proteins, Parkin along with PINK1 also interact with the Beclin-1-PI3K complex responsible for autophagosome membrane nucleation (Michiorri, et al. 2010; Van Humbeeck, et al. 2011a). Activation of the Beclin-PI3K complex is mediated by the activating molecule of Beclin-1-regulated autophagy (Ambra 1), which is also recruited by Parkin (Van Humbeeck, et al. 2011b). Following nucleation of an isolation membrane and binding of the damaged mitochondria to the isolation membrane through p62/SQSTRM1, elongation of the isolation membrane occurs (Fig. 1.4). The elongation step involves microtubule associated protein light chain 3 (LC3). LC3 is synthesized as pro-LC3 before being cleaved to form LC3-I. LC3-I is conjugated to phosphatidyl ethanolamine (PE) to form LC3-II, which localizes to isolation membranes and is required for elongation and closure of autophagosome membranes (reviewed in (Tanida 2011)). Once autophagosome formation is complete, it can then fuse with a lysosome resulting in the degradation of the damaged mitochondria.

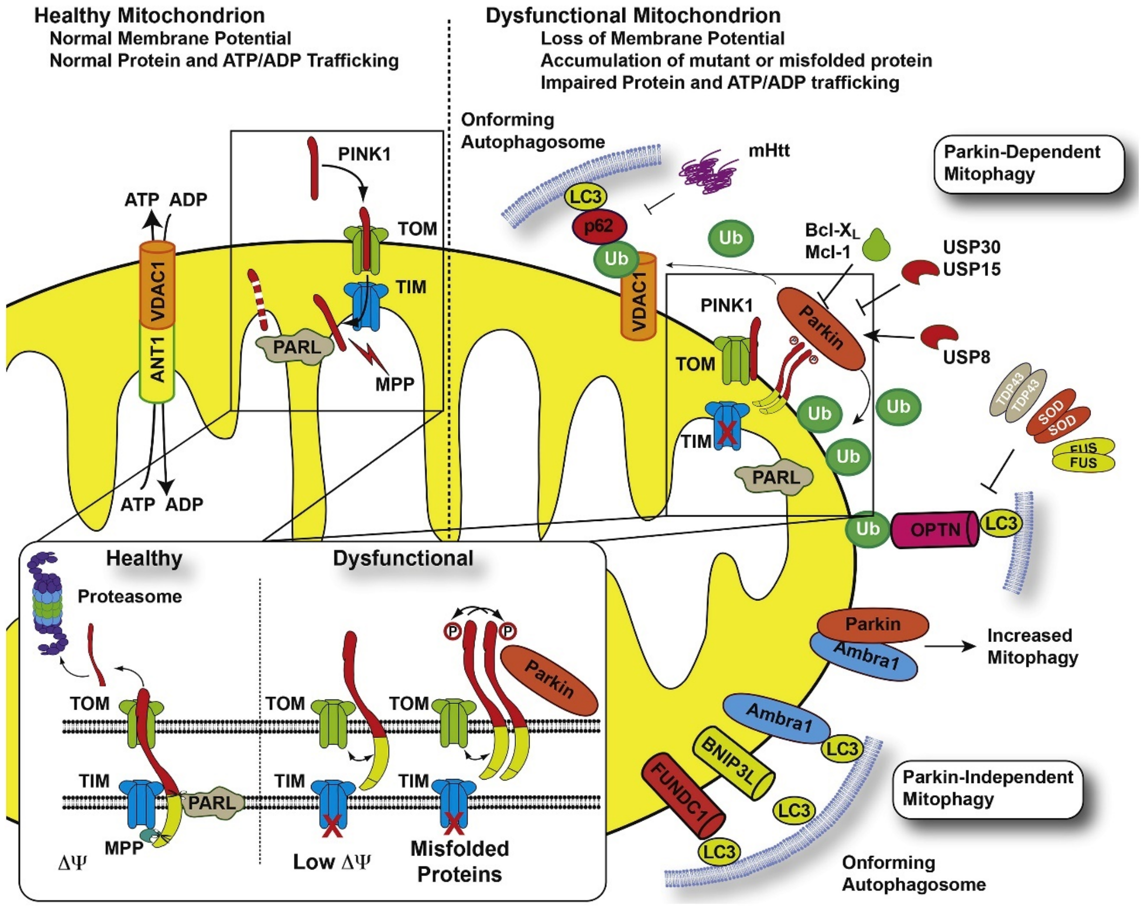

Healthy Mitochondrion
Normal Membrane Potential
Normal Protein and ATP/ADP Trafficking
Dysfunctional Mitochondrion
Loss of Membrane Potential
Accumulation of mutant or misfolded protein
Impaired Protein and ATP/ADP trafficking
Onforming Autophagosome
mHtt
Parkin-Dependent Mitophagy
ATP ADP
PINK1
TOM
TIM
VDAC1
ANT1
PARL
MPP
LC3
p62
Ub
Bcl-X_L
Mcl-1
USP30
USP15
USP8
Parkin
TDP43
SOD
FUS
OPTN
Increased Mitophagy
Ambra1
BNIP3L
FUNDC1
Parkin-Independent Mitophagy
Healthy
Dysfunctional
Proteasome
$\Delta\Psi$
Low $\Delta\Psi$
Misfolded Proteins

Figure 1.4 Events of PINK1/Parkin dependent mitophagy. (Adapted from (Rodolfo, et al. 2018)) The removal of damaged mitochondria through mitophagy is achieved by both PINK1/Parkin-dependent and -independent mechanisms. In healthy mitochondria, sustained mitochondrial membrane potential (ΔΨ) allows for the translocation of PINK1 protein inside the organelle, where it is rapidly degraded by mitochondrial processing protease (MPP) and the Presenilin-associated rhomboid-like protein (PARL). In damaged or dysfunctional mitochondria, loss of MMP and/or accumulation of misfolded/mutated proteins, does not allow for the transfer of PINK1 through TOM/TIM complexes, thus accumulating the protein on the outer mitochondrial membrane (OMM). On the mitochondrial surface, PINK1 auto-phosphorylation signals for the recruitment of the cytosolic E3-ligase Parkin, which is then able to ubiquitinate mitochondrial proteins on the OMM, thus flagging damaged/dysfunctional organelles for recognition by forming autophagosomes. In Parkin-independent mitophagy, other proteins (BNIP3l, FUNDC1, AMBRA1) are able to translocate on the OMM, where they could serve as direct or indirect signals for the engulfment within the forming autophagosome. In neurodegenerative diseases, mutations in the key proteins PINK1 and Parkin could result in either an impairment or an activation of the mitophagic process. In both Parkin-dependent and -independent mitophagy, the presence of mutated/misfolded protein aggregates affects the removal of damaged/dysfunctional mitochondria through mitophagy, possibly by limiting their recognition by the forming autophagosome.

Neuronal Mitophagy

While mitophagy is important in all cell types, the scope of this thesis makes it imperative to highlight mitophagy in neurons specifically. Much of the pathways and mechanisms described above were worked out in non-neuronal cell types, which raises the questions of whether mitophagy in neurons occurs in the same manner. Initial in vitro studies into the role of the PINK1/Parkin pathway in neurons gave mixed results. Induction of mitochondrial depolarization using CCCP/FCCP was unable to induce Parkin translocation to mitochondria in both primary neurons and neurons derived from induced pluripotent stem cells (iPSCs) (Rakovic, et al. 2013; Van Laar, et al. 2011). It was hypothesized that this was due to differences in the bioenergetics of neuronal and non-neuronal cells, as HeLa cells that were forced to rely solely on mitochondrial respiration for energy production as opposed to glycolysis, much like neurons, also showed deficits in Parkin translocation (Van Laar, et al. 2011). However, subsequent in vitro studies demonstrated that Parkin is able to translocate to the mitochondria and initiate mitophagy in response to a variety of stimuli (Amadoro, et al. 2014; Ashrafi, et al. 2014; Cai, et al. 2012; Corsetti, et al. 2015; Joselin, et al. 2012; Koyano, et al. 2013; Van Laar, et al. 2015; Ye, et al. 2015). In vivo studies have yielded equally bewildering results. PINK1 and Parkin knockout mice show at most a mild phenotype with no neuronal loss (Goldberg, et al. 2003; Kitada, et al. 2009; Perez and Palmiter 2005; Von Coelln, et al. 2004). Additionally, decreases in mitochondrial proteins were seen in the midbrain of Parkin KO mice as opposed to the increases one would expect from preventing mitophagy (Palacino, et al. 2004). Clearly PINK1 and Parkin have a role in mitochondrial homeostasis in the mammalian nervous system, however further study is needed to determine the exact details of that role.

As described above, neurons are a highly polarized cell type, with an axonal compartment and a somatodendritic compartment with unique molecular and structure characteristics (Bentley and Banker 2016). Therefore, it is important to understand in which compartment of the neuron mitophagy occurs. Initial studies observed Parkin translocation and mitophagy only in the somatodendritic compartment of neurons (Bingol, et al. 2014; Cai, et al. 2012; Ye, et al. 2015). However, a subsequent study was able to show both Parkin translocation and mitophagy, as seen by mitochondrial colocalization with autophagosomes and lysosomes, occurring in axons by using microfluidic chambers to specifically treat axons with Antimycin A to cause mitochondrial depolarization (Ashrafi, et al. 2014). Taken together these studies allow for the possibility that mitophagy is not region specific within the neuron and can occur anywhere along its length.

While Parkin is certainly a central component of the mitophagy pathway, Parkin independent mechanisms of mitophagy exist and have been observed in neurons. Cardiolipin is a lipid normally present on the IMM that is able to translocate to the OMM when the mitochondria is stressed, where it can bind LC3 and initiate mitophagy (Chu, et al. 2013; Kagan, et al. 2016). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has also been observed to be involved in mitophagy in HD (Hwang, et al. 2015). In these neurons, GAPDH associated with the mitochondria and targeted the mitochondria to lysosomes (Yogalingam, et al. 2013). The mechanism by which this occurred is not yet fully understood. Finally, iron was shown to have a role in neuron mitophagy. Conditional KO of the transferrin 1 receptor resulted in a neuronal iron deficiency which resulted in the accumulation of abnormal mitochondria due to an inhibition of mitophagy (Matak, et al. 2016). Collectively, the studies discussed in this section show the complexity of mitophagy and mitochondrial maintenance in neurons and highlight the need for more research in this area.

Part IV: Mitochondria in neuronal health and disease

Neurons are a unique cell type in that they combine high metabolic demand, high oxidative stress levels , and a highly regulated, compartmentalized cellular structure (Bentley and Banker 2016). To meet metabolic demands, neurons require a high density of mitochondria at a variety of structures along the axon including nodes of Ranvier, growth cones, presynaptic ending, and postsynaptic densities (Berthold, et al. 1993; Morris and Hollenbeck 1993). Aside from producing energy the mitochondria of neurons are also responsible for mitigating the high levels of calcium need for synaptic firing and neurotransmitter release. Additionally, neurons are post-mitotic, non-proliferating cells making mitochondrial homeostasis critically important to maintaining the health of the neuron (Bakthavachalam and Shanmugam 2017). Therefore, it is of no surprise that defects in mitochondrial dynamics, trafficking, and mitophagy have been linked to a host of neurodegenerative disorders including Parkinson’s Disease (PD) (Schapira 2011), Amyotrophic Lateral Sclerosis (ALS) (Cozzolino, et al. 2013), Alzheimer’s Disease (AD), and Huntington’s Disease (HD) (Batlevi and La Spada 2011).

Before discussing the details of how mitochondrial dynamics and mitophagy are involved in neurodegenerative diseases, I will give a brief description of the four diseases for which there is the best evidence of mitochondrial involvement.

Parkinson's Disease (PD)

PD is a progressive nervous system disorder that affects movement. PD is characterized by loss of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) and the formation of intraneuronal aggregates of α-synuclein (Lewy bodies) (Kalia and Lang 2015).

Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive neurodegenerative disorder caused by loss of both upper and lower motor neurons in the brain and spinal cord. ALS is mostly a sporadic disease with only 5-10% of cases showing a pattern of inheritance. Oxidative stress and mitochondrial dysfunction have been shown to cause ALS (Kiernan, et al. 2011). Additionally, several genes have been linked to both genetic and sporadic ALS including superoxide dismutase 1 (SOD1), RNA-binding protein FUS, and TAR DNA-binding protein 43 (TDP43) (Andersen and Al-Chalabi 2011).

Alzheimer's Disease (AD)

AD is a progressive neurodegenerative disorder characterized by brain atrophy and is the most common cause of dementia worldwide. AD is marked by neuronal and synaptic loss as well as accumulation of amyloid β (Aβ) plaques and intracellular neurofibrillary tangles of hyperphosphorylated Tau (Harrington 2012).

Huntington's Disease (HD)

HD is a progressive neurodegenerative disorder marked by increasingly uncontrollable movements, loss of cognition, and psychiatric disturbances (Ross and Tabrizi 2011). It is caused by CAG trinucleotide repeats in the area of large polyglutamine (PolyQ) stretches in the N-terminal region of the huntingtin protein. HD pathogenesis is variable but includes mitochondrial dysfunction (Guedes-Dias, et al. 2016).

Mitochondrial dynamics and neurodegeneration

As described above, neuronal mitochondria are dynamic organelles that experience numerous fission and fusion events in addition to undergoing both anterograde and retrograde transport. The continuous remodeling through fission and fusion acts as a quality control mechanism to isolate damaged and dysfunctional mitochondria and allow for their degradation through mitophagy (Twig, et al. 2008). Defects in the fission/fusion and transport machineries allow damaged and dysfunctional mitochondria to remain in the neuron and are important in the pathogenesis of several neurodegenerative disorders. Under normal conditions, the ubiquitination and degradation of Miro occurs upstream of mitophagy. The degradation of Miro inhibits trafficking of the mitochondria thus making it stationary and able to be placed in an autophagosome (Liu, et al. 2012). However, in PD ubiquitination of Miro is impaired reducing the turnover of Miro. Lack of ubiquitination of Miro inhibits mitophagy of dysfunctional mitochondria contributing to the neuronal loss seen in PD (Birsa, et al. 2014). Additionally, mitochondrial fragmentation through increased or unopposed fission has been shown to be part of PD pathogenesis. Overexpression of pathogenic α-synuclein (A53T or A30P) resulted in OPA1 cleavage thereby preventing fusion and resulting in mitochondrial fragmentation (Guardia-Laguarta, et al. 2014). Furthermore, the G2019S mutation in the Leucine-rich repeat kinase 2 (LRRK2) gene, also known as PARK8, in autosomal dominant PD patients is associated with decreased mitochondrial membrane potential and mitochondrial fragmentation (Rodolfo, et al. 2018). Transgenic mice expressing mutant SOD1 or TDP43 were shown to have increased expression of Drp1 and Fis1 with decreased expression of Mfn1/2 and Opa1 leading to unopposed fission and mitochondrial fragmentation (Wang, et al. 2013). Furthermore, expression of Miro1 is decreased in the spinal cords of human ALS patients and mouse models. This would result in a trafficking deficit that would prevent healthy mitochondria from reaching the most energy dependent regions of neurons and eventually lead to neuronal loss (Zhang, et al. 2015b). Trafficking defects also play a role in AD pathogenesis. An early event in AD is the increase in phosphorylation of Serine 262 of Tau, which can be initiated by the knockdown of either Miro1 or Trak1/2 (reviewed in (Rodolfo, et al. 2018). Additionally, Miro1 protein levels are decreased and there is an impairment in mitochondrial/ER tethering and mitochondrial transport in cells expressing the E280A presenilin-1 (PS-1) mutation, a commonly used model of AD (Sepulveda-Falla, et al. 2014). In HD, mitochondrial fission/fusion dynamics appear to play an important role in disease pathogenesis. Both human patients and mouse models of HD increased mRNA and protein levels of Drp1 with decreased levels of Mfn1/2 leading to a propensity for mitochondrial fission (Costa and Scorrano 2012; Shirendeb, et al. 2012). The increase in fission in HD may be caused by the direct binding of mutant Huntingtin (mHtt) to Drp1 and the subsequent increase in the GTPase activity of Drp1 (Song, et al. 2011).

These observations clearly implicate mitochondrial transport and fission/fusion in the pathogenesis of several neurodegenerative disease. However, deficits in mitophagy may play an even larger role in the development of neurodegenerative diseases.

Mitophagy in neurodegeneration

While other pathways exist, the PINK1/Parkin mediated pathway of mitophagy appears to be the dominant pathway for the removal of damaged and dysfunctional mitochondria (Sterky, et al. 2011). Inhibition or alteration of the mitophagy pathway may set the stage for the development of neurogenerative diseases. Mutations in both PINK1 (PARK6 gene) (Valente, et al. 2001; Valente, et al. 2002) and Parkin (PARK2 gene) (Kitada, et al. 1998; Lücking, et al. 2000; Periquet, et al. 2003) are associated with the autosomal recessive form of PD. Additionally, altered Parkin solubility has been implicated in PD. Parkin solubility decreases with age (Pawlyk, et al. 2003) and with oxidative (Winklhofer, et al. 2003) or nitrosative stress (Wang, et al. 2005), leaving less Parkin available to participate in mitophagy leading to the accumulation of dysfunctional mitochondria.

The evidence for the role of mitophagy in the progression of ALS is still unclear, with contrasting findings that both mitophagy inhibition and induction are present. Decreased Parkin RNA levels have been found in human spinal cord as well as in TDP-43 and RNA binding protein FUS models of ALS, leaving the neurons vulnerable to the accumulation of dysfunctional mitochondria (Lagier-Tourenne, et al. 2012; Polymenidou, et al. 2011). Furthermore, the presence of mutated TDP-43 or SOD1 on mitochondria is thought to interfere with mitophagy (Stribl, et al. 2014). In contrast SOD1 transgenic mice have increased LC3-II (Li, et al. 2008; Morimoto, et al. 2007) and mitochondria contained in autophagosomes and autophagolysosomes in human ALS spinal cord samples (Sasaki 2011). Moreover, G93A-mutant SOD1 transgenic mice have increased levels of p62 in the spinal cord (Gal, et al. 2007). SOD1 binds both p62 and LC3, making it possible that mitochondria that carry mutant SOD1 protein can be preferentially targeted to autophagosome for degradation (Gal, et al. 2009). More research is necessary to determine precise role of mitophagy in the progression of ALS.

In AD, both amyloid precursor protein (APP) and Aβ accumulate on mitochondria and lead to dysfunction. The mechanism by which amyloid accumulation causes mitochondrial dysfunction may be due to interactions with mitochondrial matrix proteins (Du, et al. 2008; Lustbader, et al. 2004), alteration of fission/fusion balance (Manczak, et al. 2006; Manczak, et al. 2011; Manczak and Reddy 2012a), impairment in motility (Calkins, et al. 2011; Reddy, et al. 2012), or disruption of the ETC (Manczak and Reddy 2012b). Regardless of how mitochondrial dysfunction is caused, they must be degraded through mitophagy to maintain neuronal health. However, accumulations of autophagosomes containing mitochondria have been observed in AD brains suggesting an impairment of mitophagy (Nixon, et al. 2005). Recent work has shown that the impairment in mitophagy in AD is in the fusion of autophagosomes with lysosomes

(Bordi, et al. 2016). An additional role for mitophagy in the progression of AD has been shown in the hAPP mouse model and AD patient samples. Observations showed strong induction of Parkin-mediated mitophagy early in AD. As the disease progressed there was a depletion of cytosolic Parkin resulting in an impairment in mitophagy leading to the accumulation of dysfunctional mitochondria (Ye, et al. 2015). Therefore, impairments in autophagosome and lysosome fusion as well as mitophagy induction later in disease may contribute to the pathogenesis of AD.

HD patients and animal models have impairments in the removal of dysfunctional mitochondria indicating a defect in the process of mitophagy. Multiple mechanisms for diminished mitophagy in HD neurons have been proposed, including reduced interaction of dysfunctional mitochondria and p62 caused by mHtt protein (Martinez-Vicente, et al. 2010). In support of this theory, recent work showed that normal Htt protein serves as a scaffold for p62 and the mitophagy initiation machinery (Rui, et al. 2015). The effects of mHtt protein on mitochondrial delivery to autophagosomes can be rescued by over expression of PINK1 in both fly and mouse HD models (Khalil, et al. 2015). A second theory proposes that mHtt protein causes a defect in the transport of autophagosomes to lysosomes. It was observed that mHtt protein disrupted the Kinesin and Dynein motors responsible for axonal transport of autophagosomes preventing fusion of the autophagosome and the lysosome, resulting in accumulation of mHtt and dysfunctional mitochondria (Wong and Holzbaur 2014). In contrast, it has been observed that mHtt has no effect on basal levels of autophagy in the hypothalamus (Baldo, et al. 2013). However, mitophagic activity varies across brain regions (Diedrich, et al. 2011) making it possible that mHtt can impair mitophagy in those regions most affected in HD while having no effect in others.

Mitochondrial based therapeutics in the treatment of neurodegenerative diseases

The need for effective therapies to slow or combat neurodegenerative disorders cannot be overstated. The economic and societal burden of neurodegenerative disorders is increasing (Olesen, et al. 2012), however treatments for neurodegenerative diseases are mainly symptomatic and have had very limited clinical success (reviewed in (Budd Haeberlein and Harris 2015)). Because mitochondrial dysfunction has been linked to may neurodegenerative disorders, mitochondrial directed therapeutics have become a promising new research direction in the field. Studies in various animal models of neurodegenerative diseases targeting defective ATP production, disrupted mitochondrial dynamics, disrupted calcium homeostasis, and inappropriate mitochondrial protein aggregation among others have been very successful (reviewed in (Chaturvedi and Beal 2013; Kumar and Singh 2015; Moreira, et al. 2010). Specifically, treatments to combat oxidative mitochondrial damage using CoQ_{10}, an antioxidant, MitoQ, a mitochondrially targeted antioxidant, and Bendavia (SS31), a mitochondrially targeted inhibitor of ROS production have been successful in animal models (McManus, et al. 2011; Miquel, et al. 2014). Furthermore, enhancement of mitochondrial biogenesis through the increase of transcription factors PGC1α and NRF2 have been beneficial in animal models (reviewed in (Johri and Beal 2012)). Finally, pharmacological inhibition of Drp1 and thus mitochondrial fission was protective in animal models of PD and AD (Bido, et al. 2017; Wang, et al. 2017).

Yet, despite the success of mitochondria targeted therapies in animal models, translation of these therapies to the clinic have been unsuccessful (reviewed in (Onyango, et al. 2016)). In particular, CoQ_{10}, MitoQ, and NRF2 induction were ineffective in AD and PD patients (Chaturvedi, et al. 2009; Moreira, et al. 2010; Snow, et al. 2010). One explanation for the translation failure of the various mitochondria targeted therapies mentioned is that clinical trials generally include symptomatic patients, often in later stages of disease, while animal studies often introduce intervention before the onset of symptoms. Because neurodegenerative diseases are progressive by nature, the interventions that are successful in animal models may be introduced too late in the course of the disease. This issue can be resolved either by performing animal studies in which the intervention is introduced after the onset of the equivalent of clinical symptoms or through improvements in early diagnosis of neurodegenerative disease. While major hurdles still exist, the study of neurodegenerative disease therapeutics directed at mitochondria, in particular those aimed at reducing mitochondrial damage, mitochondrial dysfunction, and improving mitochondrial quality control, is still a promising field that may yet have a major impact on clinical outcomes.

Part V: The role of mitochondria in spinal cord injury

As highlighted above, SCI has a reasonably high prevalence worldwide and can be accompanied by significant comorbidities and financial burden. For this reason, it is imperative that an effective treatment to restore functionality is discovered. Yet, despite major efforts no such treatment exists. The lack of effective treatment can be attributed to the complexity that SCI presents. In addition to the primary injury to the neurons of the spinal cord and the lack of regenerative response inherent in CNS neurons (discussed in Part I), SCI involves a cascade of events in the minutes to hours after injury, deemed the secondary injury. The effects of the secondary injury can be more severe than the original insult and last for weeks to months (reviewed in (Oyinbo 2011). Secondary injury can lead to progressive axon demyelination (Totoiu and Keirstead 2005), neuronal cell death (Anwar, et al. 2016; Beattie, et al. 2002), microglia activation and inflammation (Qiao, et al. 2010; Qiao, et al. 2015), glial scar formation (Shibuya, et al. 2009), and additional mitochondrial dysfunction (Oyinbo 2011). The events of secondary spinal cord injury can be summarized as such: the primary SCI results in disruption of the spinal cord vasculature leading to vasoconstriction, which results in hemorrhage, edema, hypoperfusion and ischemia (reviewed in (Baptiste and Fehlings 2006; Graumann, et al. 2011). The degree of ischemia after SCI has been correlated with the amount of functional loss (reviewed in (Tator and Fehlings 1991)). Additionally, the effects of secondary injury reduces local oxygen delivery thereby reducing the ability of mitochondria to maintain homeostasis (reviewed in (Kundi, et al. 2013)). The loss of mitochondrial homeostasis further perpetuates the secondary SCI and promotes neuronal cell death (Sullivan, et al. 2007), further highlighting the importance of mitochondrial health in the recovery from spinal cord injury.

Loss of mitochondrial function during secondary SCI has far reaching effects. A lack of ATP production impairs the functionality of ATP-dependent pumps required for the restoration of ionic balance and the reuptake of neurotransmitters leading to excitotoxicity, calcium overload, and eventual cell death (reviewed in (Choi and

Rothman 1990; Rowland, et al. 2008)). The effects of secondary SCI on mitochondria become self-propagating as glutamate excitotoxicity leads to increased influx of calcium into neurons, which under normal conditions is buffered by mitochondria (Demediuk, et al. 1990; LoPachin, et al. 1999; Young and Koreh 1986). However, when calcium concentrations exceed the maximum threshold for buffering by mitochondria, the mitochondrial permeability transition pore (mPTP) is opened. Opening of the mPTP causes mitochondrial swelling, due to the influx of water, with eventual rupture resulting in the release of accumulated calcium, ROS, and proapoptotic proteins into the cytosol (McEwen, et al. 2011; Sesso, et al. 2004). Release of ROS into the cytosol can further damage other mitochondria leading to more ROS production (reviewed in (Sullivan, et al. 2004). Increases in ROS production can lead to formation of peroxynitrite resulting in lipid peroxidation which can further damage other mitochondria (reviewed in (Violi, et al. 1999). Additionally, lipid peroxidation can further damage the surrounding vasculature promoting ischemia and propagating the secondary SCI (reviewed in (Hall, et al. 2016). The clear and pervasive role that mitochondria have in the propagation of secondary SCI make them a promising target for therapeutic intervention. Additionally, it has been shown that following SCI markers for oxidative damage are not seen for approximately 8 hours (Jia, et al. 2016; Sullivan, et al. 2007) demonstrating the presence of a therapeutic window in which mitochondrial homeostasis may be restored.

Mitochondrial targeted therapeutics in SCI

Therapeutics aimed at restoring mitochondrial homeostasis following SCI can be broken down into five main categories, each having varying levels of efficacy. These categories include mPTP inhibition, alternative energy sources, antioxidant, mitochondrial dynamics modifiers, and promoters of mitochondrial biogenesis. I will discuss each group of therapeutics separately.

mPTP inhibitors

As noted above, opening of the mPTP contributes to the propagation of secondary SCI and neuronal cell death. Therefore, finding a therapeutic that can prevent this event may have beneficial effects on recovery from SCI. To date, two different compounds that affect mPTP opening have been tested in the context of SCI: cyclosporin A (CsA) and NIM811. CsA is an immunosuppressant that binds to mPTP and inhibits opening. CsA has been shown to be neuroprotective in the context of traumatic brain injury (TBI) and stroke (Matsumoto, et al. 1999; Scheff and Sullivan 1999; Sullivan, et al. 2000; Sullivan, et al. 1999; Uchino, et al. 2002). However, the efficacy of CsA in SCI is inconclusive, with some studies showing a behavioral and functional benefit (Ibarra, et al. 2003; McMahon, et al. 2009) while others showed no benefit of CsA administration (Rabchevsky, et al. 2001). Regardless of the unresolved efficacy of CsA in SCI, its high toxicity makes it a less than desirable therapeutic (Caramelo, et al. 2004; Schenk, et al. 2010; Szalowska, et al. 2015). For this reason, the CsA analog, NIM811 has been tested. NIM811 shows much lower toxicity while demonstrating a similar ability to bind and inhibit mPTP (Waldmeier, et al. 2002). While only few studies have been conducted using NIM811 in SCI, the results have been positive. NIM811 was able to reduce free radical production and increase ATP production capacity in mitochondria isolated from injured spinal cord (McEwen, et al. 2007). Additionally, NIM811 was able to reduce lesion volume and release of cytochrome *c* and fragmented DNA into the cytosol (Ravikumar, et al. 2007). These limited studies indicate a therapeutic potential for NIM811 and warrant further investigation.

Alternative energy sources

Oxidative damage following SCI results in dysfunctional mitochondria. Part of the dysfunction is the inactivation of enzymes critical for energy production, including but not limited to pyruvate dehydrogenase (PDH) (reviewed in (McEwen, et al. 2011)). PDH is crucial in the formation of acetyl-CoA, a necessary substrate for the citric acid cycle and the production of electron donors for the ETC. This raises the possibility that introduction of an alternate source of energy to replace acetyl-CoA could improve the observed mitochondrial dysfunction following SCI. One such candidate molecule is Acetyl-L-carnitine (ALC). ALC is a component of the IMM that can cross the blood-brain barrier (BBB) and provide acetyl groups for the formation of acetyl-CoA (reviewed in (Pettegrew, et al. 2000). ALC has dual functions as it is also able to produce the antioxidant glutathione (GSH) (Karalija, et al. 2012). The use of ALC in the study of SCI has been limited, however it shows promise as a potential therapeutic. Chronically administered ALC was able to reduce neuronal death and increase the incidence of neuronal sprouting following SCI in rats (Karalija, et al. 2012). Daily injections of ALC increased mitochondrial respiration and gray matter sparing (Patel, et al. 2010) while improving behavioral outcomes following SCI in rats (Patel, et al. 2012). Finally, ALC reduced mitochondrial morphological changes usually seen after SCI by increasing expression of Mfn1/2 and Bcl-2 while decreasing expression of Drp1, Fis1, and Bax (Zhang, et al. 2015c).

Antioxidants

The role of ROS and oxidative damage as described above is very apparent. Therefore, it is logical that therapies designed to either prevent or ameliorate oxidative stress following SCI would be beneficial. However, it is important to note that the use of antioxidant therapies is limited by the fact that increases in ROS after SCI are near instantaneous, resulting in a very short therapeutic window (Bao and Liu 2004; Liu, et al. 1998; Liu, et al. 2004; Xiong, et al. 2007). In spite of this, many antioxidants have been studied as treatments for SCI with some positive results.

α-Tocopherol, a form of vitamin E, scavenges lipid peroxyl radicals thereby reducing lipid peroxidation and improving motor score and sensory function following SCI (Al Jadid, et al. 2009; Anderson, et al. 1988; Bozbuğa, et al. 1998; Morsy and Bashir 2013; Morsy, et al. 2010). However, radical scavenging by α-Tocopherol is 1:1, therefore very high doses, associated with increased mortality (Miller, et al. 2005), would be needed to have clinical efficacy in humans (Roberts, et al. 2007).

N-acetylcysteineamide (NACA), is a membrane permeable variant of *N*-acetylcysteine. Following TBI and SCI in rats, NACA was able to increase GSH levels and in doing so improve mitochondrial bioenergetics, tissue sparing, cognition, and hindlimb motor function (Pandya, et al. 2014; Patel, et al. 2014).

Resveratrol is a polyphenolic compound found in grapes and wine that has been associated with improvements in various animal models of neurodegeneration (reviewed in (Pervaiz and Holme 2009). In rodent SCI models, resveratrol administration has been observed to decreased oxidative stress (Kiziltepe, et al. 2004), improve edema, increase Na^+ K^+ ATPase activity (Yang and Piao 2003), and improve functional recovery (Ates, et al. 2006). Resveratrol works by increasing the activity of SOD and reducing expression of inflammatory cytokines and pro-apoptotic proteins (Liu, et al. 2011).

Finally, tempol, a nitroxide-containing antioxidant that is stable and membrane permeable, has had success in promoting recovery following SCI. Tempol acts by metabolizing many forms of ROS and RNS (Wilcox 2010). Tempol has been observed to improve mitochondrial function, reduce cytoskeletal degradation (Xiong, et al. 2009), reduce white matter loss, and improve locomotor function following SCI in rodents (Hillard, et al. 2004). Unlike other antioxidant therapies, the therapeutic window in which tempol can produce improved locomotor activity may reach several days as compared to the 1-hour window for other antioxidants (Hillard, et al. 2004). While promising, much more work is needed to determine if antioxidant therapy is clinically viable in human SCI. More than likely it will be part of a multifaceted treatment regimen.

Mitochondrial dynamics modifiers

As described above, mitochondrial morphology is strongly correlated with the health and functionality of the mitochondria. Mitochondrial morphology is maintained through regulated fission and fusion (Suen, et al. 2008). SCI causes an imbalance between fission and fusion resulting in detrimental changes to mitochondrial function. Observations have suggested that there is a biphasic pattern to morphological changes to mitochondria following SCI with a predominance of fusion and therefore larger mitochondria for up to 6 hours following injury and a predominance of fission and therefore smaller mitochondria 12-24 hours following SCI (Cao, et al. 2013). The smaller mitochondria correlated with an increase in Drp1 protein expression, decreased mitochondrial membrane potential, and cytochrome *c* release (Jia, et al. 2016). Therefore, an intervention to reestablish the balance between fission and fusion following SCI could prove beneficial in recovery. Only a few studies have directly addressed this issue. These studies were done using a pharmacological inhibitor of Drp1, mitochondrial division inhibitor-1 (mDivi-1) (Smith and Gallo 2017). Pretreatment with mDivi-1 increased ATP production and mitochondrial membrane potential while decreasing the expression of caspase-3 and number of apoptotic cells following SCI in rats (Li, et al. 2015a). Additionally, mDivi-1 treatment after injury was able to improve locomotor function (Liu, et al. 2015). While these studies are promising, they use different treatment windows and do not address how and on what cell type mDivi-1 is most exerting its effect. Therefore, much more research into how mitochondrial dynamic impact recovery from SCI is needed.

Mitochondrial Biogenesis

Mitochondrial biogenesis is a transcriptional program that initiates the repair, growth, and/or division of existing mitochondria (reviewed in (Ventura-Clapier, et al. 2008)). Mitochondrial biogenesis is controlled by peroxisomal proliferator *γ* coactivator

(PGC)-1α, which acts as a master regulator for this pathway (Kelly and Scarpulla 2004). PGC-1α interacts and activates multiple other transcription factors resulting in the transcription of nuclear encoded ETC subunits, antioxidant proteins such as SOD2, and other mitochondrial genes like uncoupling protein 2 and mitochondrial transcription factor A (TFAM). TFAM translocates to the mitochondrial matrix and initiates mitochondrial DNA replication and transcription of mitochondrial encoded genes (reviewed in (Ventura-Clapier, et al. 2008). Interestingly, SCI is associated with decreased PGC-1α and TFAM expression (Whitaker, et al. 2016). Therefore, agents that can increase activity of PGC-1α could increase mitochondrial biogenesis and improve recovery from SCI. While many mitochondrially biogenic agents exist (reviewed in (Scholpa and Schnellmann 2017), no studies have used biogenic pharmacologic reagents in the context of spinal cord injury. Instead, they have been used with positive results in neurodegenerative diseases (reviewed in (Cameron, et al. 2016)). However, lentiviral overexpression of PGC-1α following SCI has been observed to reduce neuronal cell death and improve functional recovery (Hu, et al. 2016b), giving proof of concept that increasing mitochondrial biogenesis pharmacologically can help improve outcomes following SCI.

It is obvious that mitochondrial health and dynamics play a major role in both primary SCI (described in Part I) and secondary SCI. However, the complexity and interconnectedness of multiple cell types make it difficult to determine the exact mechanisms by which mitochondrially targeted treatments are exerting their effects. Furthermore, the compartmentalization of neurons adds yet another layer of complexity as the response of mitochondria to axonal injury has yet to be fully worked out. Therefore, in this thesis I set out to define the response of axonal mitochondria to axonal injury in an effort to advance our understanding of axonal regeneration and perhaps provide therapeutic targets for future study.

CHAPTER 2: STATEMENT OF GOALS

Spinal cord injury (SCI) is a severe medical problem with high mortality and long-term morbidity. Unfortunately, there is no effective treatment available for SCI patients. Following injury, severed axons often undergo dieback/retraction from the site of injury and fail to exhibit subsequent regeneration up to and beyond the injury site (Hill 2017; Hill, et al. 2001). Regenerative failure is known to be due to cell intrinsic and extrinsic factors. Manipulation of specific genes regulating transcription and/or translation selectively in neurons revealed the importance of intrinsic factors in promoting axon regeneration (Benowitz, et al. 2017; Park, et al. 2008). Similarly, multiple extracellular signaling molecules either impair regenerative attempts (e.g., CSPGs, myelin inhibitors) (Filbin 2003; Sharma, et al. 2012) or promote growth (e.g., neurotrophins) (Harvey, et al. 2015; Kelamangalath and Smith 2013; Liu, et al. 2016). Ultimately, for functional regeneration and repair of injured circuitry to occur, it will be necessary to manipulate both intrinsic and extrinsic factors. In this thesis, I set out to elucidate intrinsic mechanisms for axon regeneration failure within the CNS, specifically addressing response of axonal mitochondria to spinal cord injury and how injury-induced mitochondrial changes affect the ability of the degenerating axon tips to mount a growth response.

In neurons, mitochondria are highly dynamic (Barnhart 2016; Saxton and Hollenbeck 2012; Schwarz 2013; Sheng 2014). They undergo bi-directional transport along axons where they are targeted to and maintained at specific sites along the axon, synapses and at the growth cone. They undergo regulated fission and fusion to adjust their distribution and removal if dysfunctional. They also determine sites of axonal branching and provide important functions in axonal regeneration (Cartoni, et al. 2016; Courchet, et al. 2013; Han, et al. 2016; Sainath, et al. 2017; Spillane, et al. 2013; Tao, et al. 2014; Winkle, et al. 2016; Zhou, et al. 2016). The mitochondrion has multiple functions in the regulation of cellular physiology by 1) generating ATP, 2) buffering cytosolic calcium and 3) when dysfunctional, generating reactive oxygen species (ROS) (Brennan-Minnella, et al. 2016; Fonteriz, et al. 2016; Islam 2017; Kasahara and Scorrano 2014). It is well established that neuronal mitochondria are adversely affected in a variety of neurodegenerative disorders (Abeti and Abramov 2015; Arun, et al. 2016; Bertholet, et al. 2016; Golpich, et al. 2017; Krols, et al. 2016; Lane, et al. 2015; Zhang, et al. 2015a) and following traumatic brain injury (Balog, et al. 2016; Chaturvedi and Flint Beal 2013; Hiebert, et al. 2015; Yonutas, et al. 2016), and inhibiting mitochondrial fragmentation rescued degenerating neurons from cell death. The primary features of mitochondrial dysfunction are 1) the fragmentation of mitochondria through repeated bouts of fission into small submicron particles and 2) the concurrent loss of the mitochondrial membrane potential resulting in ATP depletion and generation of ROS. The Drp1 GTPase is the primary mediator of mitochondrial fission and fragmentation (Chang and Blackstone 2010; Cho, et al. 2013; Kageyama, et al. 2011; Otera, et al. 2013; Sesaki, et al. 2014). Activation of Drp1 mediates both normally occurring fission in healthy cells, which in conjunction with concerted fusion maintains mitochondrial homeostasis, and excessive fission during adverse conditions resulting in fragmentation. Impairment of mitochondrial respiration happens acutely following spinal cord injury *at* *the* *tissue* level (reviewed in (McEwen, et al. 2011)). However, if and how *axonal* mitochondria are affected by spinal cord injury is not known. Therefore, the major aim of this thesis was to characterize the spatial and temporal response of axonal mitochondria to spinal cord injury. My main hypothesis was that the rapid and prolonged, Drp1-dependent fragmentation of axonal mitochondria represents a detrimental consequence to injury and underlies the ensuing die back of axons and the formation of retraction bulbs and dystrophic growth cones.

CHAPTER 3: MATERIALS AND METHODS

Animal Care

All surgical and animal care protocols were approved by the Temple University School of Medicine's Institutional Animal Care and Use Committee and performed per the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Sprague-Dawley rats (65–75 days, 200–224 g; Harlan Laboratories) were housed two per cage, on a 12-h light-dark cycle with food and water provided *ad libitum*. Animals were allowed 7 days of acclimatization prior to any experimental procedure. At the time of surgery animals ranged in weight from 225 to 250 g. To prevent infection after surgery, animals received an IP injection of 0.5 ml Cefazolin (10 mg/ml). All animals received subcutaneous injection of 2-3 ml of 0.9% NaCl solution to prevent dehydration following surgery. Analgesia was provided by twice daily oral administration of Rimadyl tablets (1mg) for 3 days beginning immediately post-operatively.

Viral Vectors

AAV2-GFP-P2A-mitoDsRed was generated by subcloning the mitoDsRed from the original adenovirus vector(Nasr, et al. 2008) into MluI and XhoI sites downstream of the P2A of pAM-CBA-eGFP-P2A. To generate AAV2-eGFPLC3-P2A-mitoDsRed the GFP coding region was removed from AAV2-GFP-P2A-mitoDsRed plasmid and replaced with eGFPLC3 (Addgene: #22405). Purified AAV plasmid was packaged using the helper-free method as reported previously (Ayuso et al., 2010; Liu et al., 2014). In brief, HEK293T cells at 70 – 80% confluency were transfected with two packaging plasmids, one carrying AAV *rep* and *cap*, the other with AAV helper functions and the transgene using a polyethylenimine method (PEI) (polyethylenimine, linear, MW 25k, Warrington, PA). Three days post transfection, cell supernatant and lysates were harvested. 40% PEG 8000 was added to precipitate crude virus for 2 hours. AAV samples were double-ultracentrifuged in a cesium chloride gradient with isolated viral fractions dialyzed in 0.1 M PBS/0.5% Sorbital overnight (Ayuso et al., 2010; Liu et al., 2014). Purified fractions were added to HEK293T cells to verify function. Quantitative real-time PCR of purified viral fractions was done to determine viral titer. Viral titer of AAV2 was 1.2 x 10^{13} GC/mL.

Surgical Procedures

Cortical Injections

Animals were anesthetized with a ketamine (67 mg/kg)/xylazine (6.7 mg/kg, i.p.) mixture. Their scalps were shaved, and the animals were placed in a stereotaxic head holder. Under aseptic conditions, a small bone flap was cut in the skull to expose the dorsal surface of the primary somatomotor cortex bilaterally. Bone flaps extended from 1 mm anterior to bregma to 1 mm posterior to bregma and from 1mm from midline laterally to approximately 4.5 mm. Injections into the somatomotor cortex were performed using a beveled glass micropipette pulled to a diameter of 30–40 µm connected to a nanoliter injector (Nanoject, Drummond Scientific). All injections were made using a stereotaxic device or micromanipulator (Narishige International) for precise measurements. All coordinates were determined in relationship to bregma. Bilateral injections of either AAV2-GFP-P2A-mitoDsRed or AAV2-GFPLC3-P2A-mitoDsRed (1.5 µl/injection) were made using the following coordinates: AP axis: +0.5 mm ML axis:

+/- 2.5 mm, AP axis: +0.5 mm ML axis: +/- 3.5 mm, AP axis: -0.5 mm ML axis: +/- 3.0 mm, AP axis: -0.5 mm ML axis: +/- 4.0 mm. Coordinates were carefully identified based on the rat brain atlas(Paxinos and Charles 2007). Following virus injection, all animals were maintained for 4 weeks before cervical spinal cord injury was performed.

Corticospinal tract severing

Animals were anesthetized with a ketamine (67 mg/kg)/xylazine (6.7 mg/kg, i.p.) mixture. Under aseptic conditions, a 1 cm incision is made through the dorsal skin above vertebra C3-C6. The skin is retracted to expose the underlying muscle. The exposed spinotrapezius muscle is cut along the midline with scissors, then spread with a small Alm retractor. The muscles are freed from the cervical vertebrae with scissors. The first thoracic vertebrae has a very prominent dorsal spinal process for easy identification. A full laminectomy of vertebra C4 and C5 was performed, so not to damage the spinal cord. Using micro scissors (Vannas Spring Scissors, Fine Science Tools, Inc) the dorsal column was precisely transected bilaterally (1.5 mm width, 1.1 mm depth). The muscles overlying the spinal cord were loosely sutured together with a 5–0 suture and the wound closed. Animals were allowed to recover at 37°C. Animals were maintained for either 2 hrs, 8 hrs, 1 day, 3 days, 1 week, 2 weeks, or 6 weeks following surgery.

For experiments involving mDivi-1 (Cat# 338967-87-6, Sigma-Aldrich) rats had IP injections of either mDivi-1 (50 mg/kg for 250 g rats) or vehicle (10%DMSO/40% PEG300 in saline) 12 hrs prior to CST severing and immediately after for rats being maintained for 1 day. Rats being maintained for 6 weeks received IP injections 12 hrs prior to CST severing, immediately after surgery, and once per day for 7 days following injury.

Sciatic nerve injections

Animals were anesthetized with a ketamine (67 mg/kg)/xylazine (6.7 mg/kg, i.p.) mixture. Both left and right posterior thighs were shaved. Using aseptic conditions, the sciatic nerve was exposed using a standard biceps femoris-splitting approach. The sciatic nerve was then injected with 8 µl of 2% lysolecithin using a beveled glass micropipette pulled to a diameter of 30–40 µm connected to a nanoliter injector (Nanoject, Drummond Scientific). The injections were done bilaterally. Animals were allowed to recover at 37°C. Following injection, animals were maintained for 3 days.

3 days after injection of lysolecithin, the sciatic nerve was again exposed and injected with retroAAV2-GFP-LC3-P2A-mtDsRed. Again, the injections were done bilaterally. Animals were allowed to recover at 37°C. Animals were then maintained for 3 weeks begore sciatic nerve transection.

Sciatic nerve transection

Animals were anesthetized with a ketamine (67 mg/kg)/xylazine (6.7 mg/kg, i.p.) mixture. Both left and right posterior thighs were shaved. Using aseptic conditions, the sciatic nerve was exposed using a standard biceps femoris-splitting approach. The sciatic nerve was then transected using dissection scissors approximately 2 cm from the sciatic notch. Animals were allowed to recover at 37°C. Following sciatic nerve transection, animals were maintained for either 2hrs, 1 day, 3 days, or 7 days.

Tissue Processing and Histology

At the completion of each experiment, all animals were euthanized by injection of Fatal-Plus (Dearborn, MI, United States) and perfused with saline (0.9% NaCl) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.5). The brain and spinal cord were promptly dissected. Spinal cord samples were dissected with dorsal roots intact for identification of each spinal level. Samples were post-fixed in 4% PFA overnight at 4°C. Tissue samples were then transferred to a series of graded sucrose (10%, 20%, and 30%) where they remained for the following 24 h or until the brain section sunk to the bottom of the container. A 3 cm section of spinal cord with the lesion at the center was removed from the whole spinal cord. The spinal cord samples were serially sectioned, sagittally at 30 μm intervals using a Leica CM3050S cryostat. Sections were collected and placed into cryoprotectant solution, then stored at -20°C until processed for histology.

Immunofluorescence

To amplify the GFP and DsRed signals, sections were permeabilized in 0.3% Triton X-100 with 5% normal goat serum to block non-specific binding sites. Samples were then incubated with chicken-anti-GFP primary antibody (1:1000; #GFP-1020; Aves Labs Inc., Tigard, OR, United States) and rabbit-anti-DsRed primary antibody (1:500; #632496; Takara Bio USA, Inc., Mountain View, Ca, United States) overnight at 4°C. The next day, samples were incubated with donkey-anti-chicken-AlexaFluor 488 secondary antibody (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, United States) and goat-anti-rabbit-AlexaFluor 594 secondary antibody (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, United States) then mounted on glass slides.

Tissue Section Imaging

Imaging was performed using AxioVision software (Carl Zeiss Microscopy, Thornwood, NY, United States). A 100x/1.3 N/A objective with a ~300 nm focal plane was used to generate Z-stacks using 250 nm Z-intervals through 30 µm of tissue sections. In order to maximize the power of the morphological analysis, 1x1 camera binning was used to generate highest spatial resolution in the images. Max-intensity 2D projections were then generated using sets of sections from the Z-stack that contained mitochondria.

Culturing of Primary Neurons

Chicken dorsal root ganglia (DRG) were dissected at embryonic day 7 (E7) or day 14 (E14) (SPF eggs obtained from Charles River Laboratories). At this developmental stage, it is not possible to determine sex and both sexes were used at presumably a 50/50 ratio. Whole explants were cultured (3–4 explants/dish), or the DRGs neurons were dissociated and transfected (as explained below) (1.5 explants/dish). The culturing substrata were previously coated with polylysine (Sigma; Catalog number (Cat#) P9011; 100 µg/ml in borate buffer), for 4 hr and following 3x washing with phosphate buffered saline (PBS) with 25 µg/ml laminin (Life Technologies Cat# 23017–015) in PBS overnight, all incubations were performed at 39°C. Explants and dissociated neurons were cultured in defined F12H medium (Gibco; Cat#21700075) supplemented with NGF (20 ng/ml). For live imaging experiments, explants or dissociated neurons were plated in glass-bottom dishes.

For the preparation of dissociated neurons sensory ganglia were incubated in in Ca^{2+}–Mg^{2+}-free PBS (CMF-PBS), for 10 min at 37°C. Ganglia were then spun down for 1 min, and supernatant was removed. Ganglia were then treated with 0.25% trypsin (Fisher Scientific Cat# MT25005CI), for 10 min at 37°C and spun down for 1 min. Ganglia were then pipette triturated 30 times in F12HS10 media (F12H medium supplemented with 10% fetal bovine serum: Fisher; Cat#MT350111CV) and then spun down for 4 min. Supernatant was removed and cells were transfected as described below.

Rat hippocampal neurons were dissociated from dissected E18 rat hippocampi as previously described(Thomas, et al. 2012). Neurons were cultured in Neurobasal media supplemented with B27 (Thermo Fischer Scientific) in glass bottomed dishes coated with polylysine (Sigma; Catalog number (Cat#) P9011; 100 µg/ml in borate buffer) and laminin (Life Technologies Cat# 23017–015; 25 µg/ml) as described above.

Adult rat DRG dissociated cultures were prepared as described previously (Pacheco, et al. 2020).

P0-P1 mice were placed on ice for 10 min. 1.5 DRGs were dissected per coverslip. DRGs were cut into halves and placed in CMF-PBS in 37° water bath for 15 min. The pellet was spun down for 15 sec, supernatant was removed and replaced with culturing media containing F12H, 50 ng/ml NGF, B27 supplement, and FdU. Explants were then placed in glass bottomed dishes coated with polylysine (Sigma; Catalog number (Cat#) P9011; 100 µg/ml in borate buffer) and laminin (Life Technologies Cat# 23017–015; 25 µg/ml) as described above. Explants were cultured for 72 hrs before performing experiment.

Transfection

For transfection of plasmids into neurons, 40 chicken DRGs were dissociated as described above, and after F12HS10 was removed neurons were suspended in 100 µl nucleofector solution (Lonza Cat# VPG-1002) and gently resuspended through trituration. The DRG cell suspension was transferred to a nucleofector cuvette containing 10 µg of Plasmid DNA, and electroporated using an Amaxa Nucleoporator (program G-13). The electroporated solution was then immediately transferred to a tube containing F12H media as described above prior to plating.

Labeling of Mitochondria In Vitro

For labeling mitochondria to determine mitochondrial fission rates, neurons were transfected with pDsRed2-Mito (Clontech Cat# 632421) as explained above. To measure mitochondrial length, mitochondria were labelled with mitochondria targeted dyes, which were prepared according to the manufacturer's directions. Labeling was performed through incubation for 30 min with mitotracker green (50 nM, Molecular Probes, Cat# M7514), MitoTracker Red (0.05 nM, Molecular Probes, Cat# M7512), or Mitoview 633 (20 nM, Biotium®, Heyward, USA; Cat# 70055-T). Following labeling, the dye containing medium was removed and cultures were washed three times with culturing medium not containing any dye. Imaging was performed at least 30 min after the dye was removed to allow cultures to acclimate.

In Vitro axonal severing

For experiments in which DRG explants were used, explants were plated in glass bottom dishes as described above and cultured for 48 hrs (unless otherwise stated).

Dishes were placed on the microscope stage for 10 minutes prior to severing. Axons were severed by dragging a No. 11 scalpel blade across the axon perpendicular to its direction of growth.

For experiments in which dissociated, transfected neurons were used, neurons were cultured in glass bottom dishes as described above for 24 hrs, with the exception of neurons transfected with GCaMP6, which were cultured for 72 hrs (the time at which expression of GCaMP6 was optimal). Dishes were placed on the stage 10 minutes prior to severing. Severing was performed as previously described (Gallo 2004). Briefly, only axons that exhibited a 'healthy' uniform caliber axon were used in single-axon-severing experiments. Severing of axons was performed approximately 200 µm from the axon tip. For severing, borosilicate glass capillaries (AM Systems, Carlsborg WA; catalog # 625500) were pulled to fine tips using a Pul-1 micropipette puller (World Precision Instruments, Sarasota FL). Capillary needles were mounted on a mechanical micromanipulator (Leica Inc., Bannockburn, IL). The tip was then brought into contact with the substratum and axons severed by maintaining the position of the tip steady while moving the stage relative to the tip. The stage was moved manually in a continuous swift motion resulting in uniform severing of axons.

Immunocytochemistry

Following scalpel blade axotomy, P1 mouse DRG explants were cultures were fixed with a final concentration of 4% PFA (EMS #15710) and 5% Sucrose (Fisher Scientific #S5-500) for 15 min. Blocking was performed prior to primary antibody application for 30 min using PBS supplemented with 10% goat serum (Sigma #G9023) and 0.1% triton X-100 (Sigma #9002-93-1) (GST). To visualize dynamin related protein (Drp1), staining was performed at room temperature for 45 min with the polyclonal antibody rabbit-anti-pDrp1 (Ser616) (1:100, Cell Signaling #3455). Subsequently, goat-anti-rabbit-TRITC conjugated secondary antibody (1:200, Sigma Cat# T6778) was applied for an additional 45 min at room temperature. Cultures were washed 3x in PBS after each stain.

Live imaging

Neurons were imaged using a Zeiss 200 M microscope equipped with an Orca-ER camera (Hamamatsu) in series with a PC workstation running Zeiss Axiovision software for image acquisition and analysis. Cultures were placed on a heated microscope stage (Zeiss temperable insert P with objective heater) for 10 min at a constant 39°C before and during imaging. Imaging was performed using a Zeiss Pan-Neofluar 100_objective (1.3 N.A.), 2 × 2 camera binning and minimal light exposure. For the quantification of mitochondria length, mitochondria labeled with mitotracker green (Chicken sensory neurons) or mitoview 633 (rat hippocampal neurons), were imaged at 30 min intervals over a 180 min timeframe. For mitochondrial fission and attempted fission rate quantification, neurons were co-transfected with pDsRed2-Mito and either pEYFP-C1-Drp1 or pEYFP-C1-Drp1K38A (previously generated in our lab (Armijo-Weingart, et al. 2019)). Neurons were then imaged for 250 frames with a 3 s interframe interval. For analysis of changes in cytosolic and mitochondrial calcium, neurons were transfected with either GCaMP6 or mitoGCaMP2. Neurons were then imaged for 16 frames with a 1 min interframe interval.

Determination of mitochondria length

Imaging was performed as described in the tissue imaging and live imaging sections above. The length of mitochondria in the axon was measured using ImageJ software. Mitochondria length was determined by a line, segmented if the mitochondrion was not strictly linear, running from one end to the other of the mitochondrion. Occasionally, mitochondria in axons overlapped as clearly evidenced by the additive signal of their fluorescence in the region of overlap. Overlapping mitochondria were excluded from analysis to ensure only mitochondria with clearly defined ends were used for quantification.

Determination of rates of fission and fusion from live imaging time lapse videos

Instances of fission were determined manually by the user through frame by frame analysis. Using the settings described in the live imaging section each pixel in the resulting images has a radius of 0.12 μm. A completed fission event was defined as when the fluorescence intensity of the mitochondrion at the site of apparent fission had decreased to the background level of the axonal segment not containing mitochondria and the two resultant mitochondria were separated by over 5 pixels representing 0.6 μm. Attempted fission events were defined as mitochondria undergoing constrictions but not continuing into a full fission event, as the constriction sites returned to exhibit the prior width and intensity.

GCaMP6 and mitoGCaMP2 analysis

All analyses were performed using Zeiss Axiovision software. For GCaMP6, regions of interest (ROIs) were drawn around the cut axon tip and 50 µm proximal to the cut. For mitoGCaMP2, ROIs were drawn around axonal mitochondria proximal to the cut site. Image acquisition was performed using excitation filters of 405 nm and 488 nm (Need specifications on Filters). Fluorescence time courses were computed as the mean pixel intensity of all the pixels within the ROI. At each timepoint, fluorescence intensity for both excitation filters (F_{405} and F_{488}) was calculated as mean fluorescence within the ROI, F, relative to the background F_0 ($F-F_0$). Data is reported as a ratio of F_{488}/F_{405}.

Statistical Analysis

All data were analyzed using Instat software (GraphPad software Inc). The software determines the normalcy of data sets using the Kolmogorov and Smirnov test. All data sets were determined to be non-normally distributed, therefore non-parametric analysis was used (Mann-Whitney test). For multiple comparison tests within experimental designs, non-parametric Dunn's post-hoc tests were used. For data sets representing categorical data falling into bins the Fischer's exact test was used on the raw data, although the data is expressed as percentages for ease of appreciation. One or two tailed p values are reported based on whether the hypothesis did or did not dictate the directionality of the expected change in mean or median, respectively. All graphs were generated using Prism (GraphPad software Inc). Sample sizes and qualitative statistical presentation are denoted in figure legends or figures.

CHAPTER 4: RESULTS

PART I: Characterization of the mitochondrial response to axonal injury in vivo

Spinal cord injury decreases the length of axonal mitochondria

To assess axonal mitochondrial morphology following spinal cord injury, the mitochondria of CST neurons of adult Sprague-Dawley rats were labeled with mitochondrially targeted DsRed (mtDsRed) 4 weeks before performing dorsal column lesions at the C4-C5 level. Labeling was achieved by injecting adeno-associated virus co-expressing GFP and mitoDsRed (mitoDsRed/GFP/AAV) in the forelimb and hindlimb somatomotor cortex. The mitochondria in non-injured axons at the C4-C5 level had a median length of 3.15 μm (Fig. 4.1C). Mitochondria in C4-C5 CST axons severed following dorsal column lesion were analyzed in 100 μm bins from the site of injury as a function of time following injury. At 2 hrs following injury, mitochondria closest to the site of injury exhibited decreased length, while mitochondria more proximal along the injured axon maintained longer lengths although still decreased relative to controls (Fig. 4.1C). Very small mitochondria in the vicinity of the injury site persisted for up to 3 days following injury (Fig. 4.1C). Recovery of mitochondrial length began by 1 week after injury with normal lengths almost fully restored by 2 weeks (Fig. 4.1C).

Mitochondria in the <0.5 μm range are considered dysfunctional (Galluzzi, et al. 2016; Hu, et al. 2017; Reddy 2014). In uninjured axons, less than 0.5% of mitochondria were shorter than 0.5 μm and only 4.4% of mitochondria fell within the 0.5-1.0 μm range. Herein, mitochondria with length <0.5 μm are defined as fragments, while mitochondria 0.5-1.0 μm are considered to be in the low normal range for length. By 2 hrs following injury, the percentage of mitochondria in the submicron range dramatically increased resulting in 53% of mitochondria displaying lengths <0.5 µm and 32% with length between 0.5-1.0 µm (Fig. 4.1D). The presence of increased mitochondrial fragments persisted until 3 days following injury before returning to non-injury control levels by 1-week post injury (Fig. 4.1D). Fragmented mitochondria were identified throughout the distal region of severed axons and the axon tips. However, the length of mitochondria within retraction bulbs, defined as the distal end of the axon exhibiting a bulbous rounded morphology, relative to those in non-retraction bulb segments of axons, continued to exhibit sub-µm fragmentation up to at least two weeks (Fig. 4.1E).

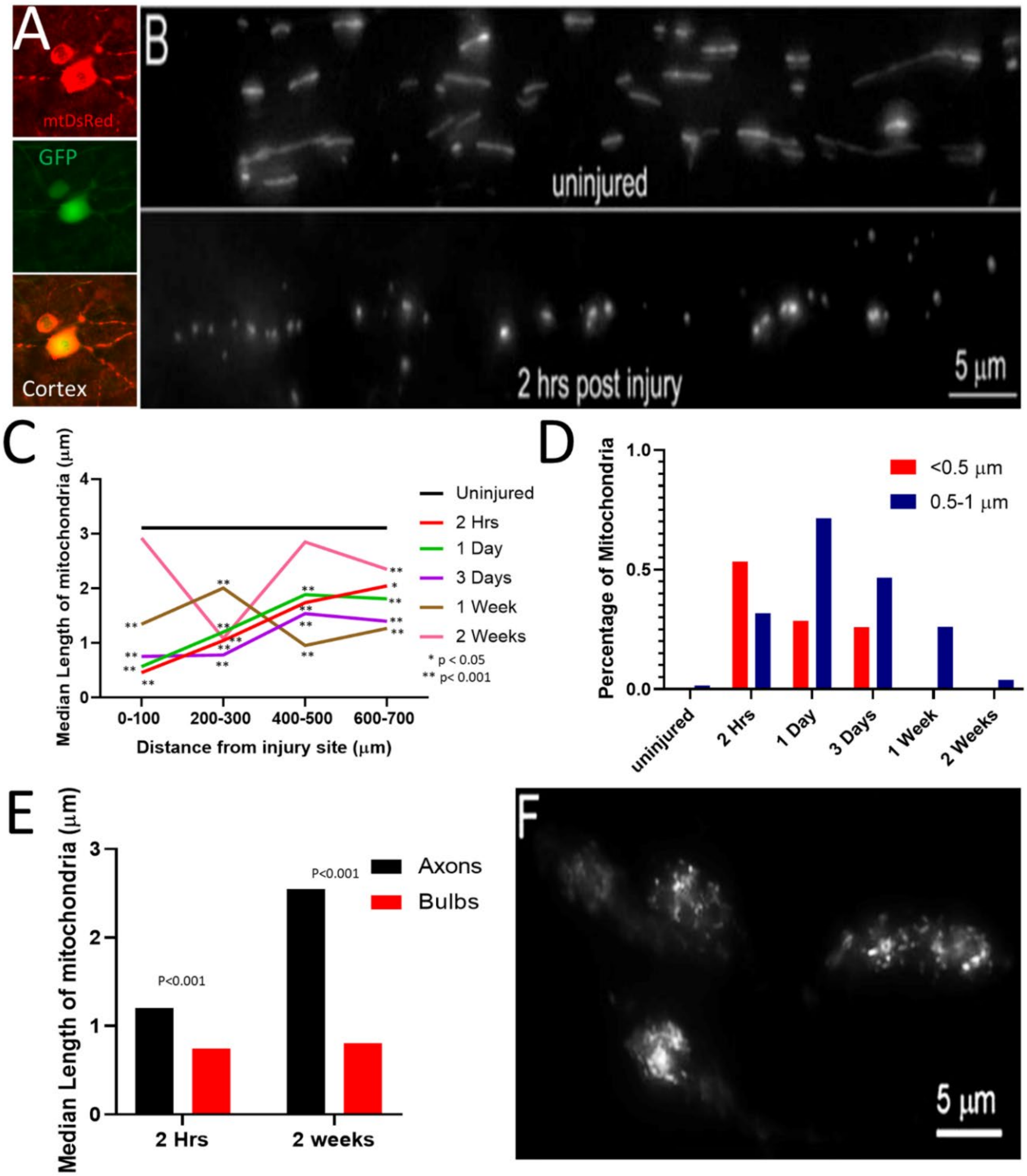
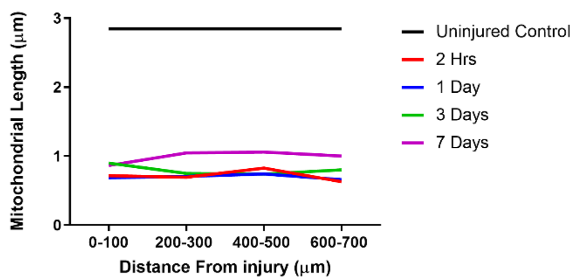

A
mtDsRed
GFP
Cortex
B
uninjured
2 hrs post injury
5 µm
C
Median Length of mitochondria (µm)
Distance from injury site (µm)
0-100
200-300
400-500
600-700
Uninjured
2 Hrs
1 Day
3 Days
1 Week
2 Weeks
* p < 0.05
** p< 0.001
D
Percentage of Mitochondria
<0.5 µm
0.5-1 µm
uninjured
2 Hrs
1 Day
3 Days
1 Week
2 Weeks
E
Median Length of mitochondria (µm)
Axons
Bulbs
P<0.001
P<0.001
2 Hrs
2 weeks
F
5 µm

Figure 4.1 Fragmentation of axonal mitochondria following injury. (**A)** Examples of layer V neurons expressing mtDsRed and GFP. **(B)** Examples of mtDsRed labeled mitochondria in CST axons at C4-5 in the uninjured control and at 2 hrs post injury. **(C)** Graph of the median mitochondrial length as a function of time post injury and distance from injury (Dunn multiple comparison tests). n = 3 rats per group **(D)** Graph of the percentage of mitochondria in the <0.5 and 0.5- 1.0 µm bins as a function of time at 0-100 µm from the injury site **(E)** Graph of the median mitochondrial lengths in retraction bulbs and axons (Mann-Whitney test). **(F)** Example of fragmented mitochondria in bulbs.

Mitochondria in sciatic nerve axons show decreased length but not fragmentation following injury

We next attempted to compare the response of axonal mitochondria in peripheral regenerating neurons. We directly injected the sciatic nerve of adult Sprague-Dawley rats with retro-AAV2 containing cytosolic GFP and mtDsRed 3 weeks before injury. The sciatic nerve was then transected, and the rats were maintained until the appropriate timepoint. Mitochondria in uninjured sciatic nerve axons had a median length of 2.83 µm (Fig. 4.2A). As with experiments in the CST described above, mitochondrial size was measured in 100 µm bins from the site of injury. Mitochondrial length was significantly reduced at all timepoints analyzed (Fig. 4.2A). However, unlike in the CST, the mitochondrial shortening was not dependent on the distance from the injury site showing similar sized mitochondria at all distances from the injury site that were analyzed (Fig. 4.2A). Slight recovery of mitochondrial size was observed by 7 days after injury, but not to the extent seen in the CST. Additionally, very little fragmentation of mitochondria was observed following sciatic nerve injury (Fig. 4.2B). Only 13% of mitochondria closest to the injury site were fragmented at 2 hrs after injury. By 7 days after injury only 5% of mitochondria closest to the site of injury were fragmented (Fig. 4.2B).

A



B

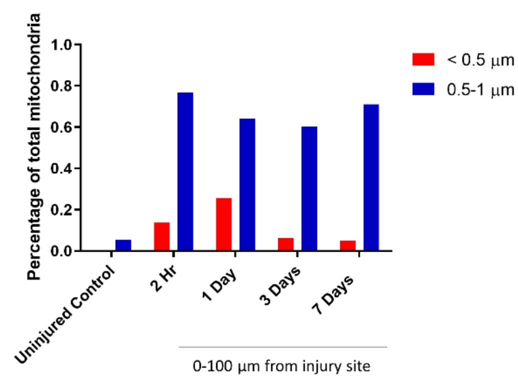


Table 1

Mann-Whitney 2-tailed test
Sciatic Nerve vs CST

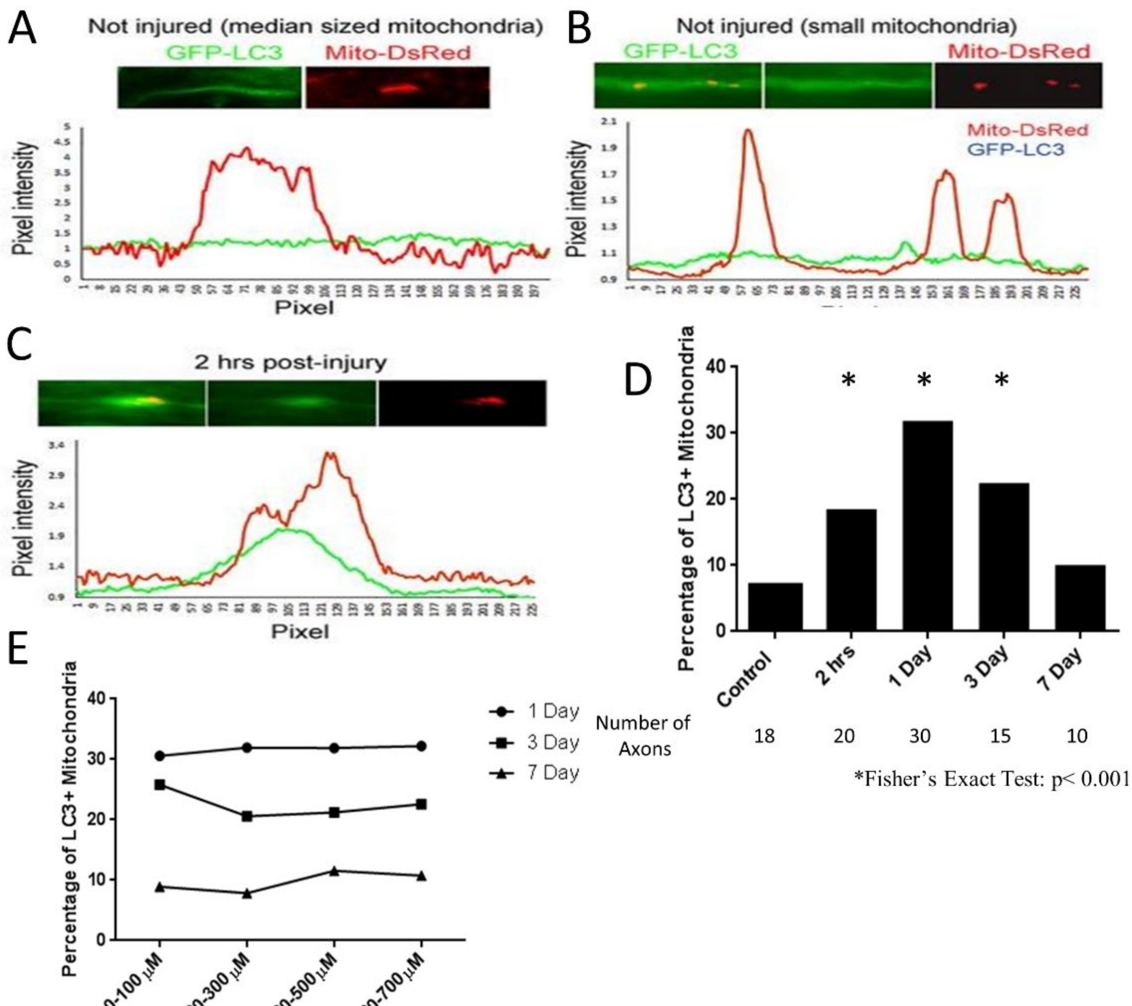
Time Point	2 hr	1 Day	3 Days	7 Days
P-value	P<0.0001	P=0.67	P<0.05	P<0.0001

Figure 4.2 Mitochondrial shortening in sciatic nerve following axotomy (A) Graph of the median mitochondrial length as a function of time post injury and distance from injury (Dunn multiple comparison tests). n = 3 rats per group **(D)** Graph of the percentage of mitochondria in the <0.5 and 0.5- 1.0 µm bins as a function of time at 0-100 µm from the injury site. **Table 1** Comparison of mitochondrial length following axotomy in sciatic nerve and CST (2-tailed Mann Whitney test)

Fragmentation of axonal mitochondria is associated with increased colocalization of mitochondria with mitophagy markers following spinal cord injury

Fragmented dysfunctional mitochondria are removed through mitophagy(Ashrafi and Schwarz 2013). To determine if the mitochondrial fragmentation along CST axons observed after spinal cord injury correlates with increased mitophagy, we examined the association of mitochondria with the autophagocytic marker LC3 (Maday 2016; Maday and Holzbaur 2016). Adult Sprague-Dawley rats were injected with AAV co-expressing GFP-LC3 and mitoDsRed into the somatomotor cortex as described above. Four weeks after injections, the rats received a dorsal column lesion at the C4-C5 level to sever labelled CST axons. Line scans of fluorescent intensities from randomly selected axons were analyzed to measure the degree of LC3 association with mitochondria. Within the C4-C5 level of non-injured rats, GFP-LC3 florescence (Fig. 4.3A, green line) was mostly uniformly distributed with few regions showing increased pixel intensity associated with DsRed labeled mitochondria (Fig. 4.3A, red line). Prior to injury, we observed that only 7.1% of axonal mitochondria were associated with localized increases in LC3. At 2 hours after injury, 18.5% of mitochondria were associated with localized increases in the levels of LC3 (hereafter referred to as LC3+ mitochondria). The increase in LC3+ mitochondria reached its peak 1 day after injury (31.8 % LC3+ mitochondria) but continued until 3 days after injury (22.5% LC3+ mitochondria) before returning to non-injury control levels 7 days after injury (10.1% LC3+ mitochondria) (Fig. 4.3D).

The percentage of LC3+ mitochondria was not dependent on the distance from the injury site, as the percentage of LC3+ mitochondria was the same at all distances from the injury site at each timepoint (Fig. 4.3E). These observations indicate that there is an increase in mitophagy that correlates with mitochondrial fragmentation up to 3 days following spinal cord injury.



A
Not injured (median sized mitochondria)
GFP-LC3
Mito-DsRed
Pixel intensity
Pixel
B
Not injured (small mitochondria)
GFP-LC3
Mito-DsRed
Mito-DsRed
GFP-LC3
Pixel intensity
C
2 hrs post-injury
Pixel intensity
Pixel
D
Percentage of LC3+ Mitochondria
Control
2 hrs
1 Day
3 Day
7 Day
Number of Axons
18
20
30
15
10
*Fisher's Exact Test: p< 0.001
E
Percentage of LC3+ Mitochondria
1 Day
3 Day
7 Day
0-100 μM
200-300 μM
400-500 μM
600-700 μM

Figure 4.3 Injury induced accumulation of LC3 at sites populated by mitochondria. **(A)** Example of LC3 distribution in uninjured axons relative to mitochondria. Line scan shows no increase in LC3 at the mitochondrion. For both traces, values are normalized to the mean intensity in regions not containing mitochondria. **(B)** as in (A) but showing three uninjured small mitochondria not showing LC3 accumulation. **(C)** Two hours after injury, LC3 is observed associated with mitochondria. Positive LC3 accumulation was defined as a maximal increase greater than 15% from the mean baseline in the axon segment defined by the mitochondrion. **(D)** Percentage of LC3+ mitochondria as a function of time post injury. **(E)** Graph of percentage of LC3+ mitochondria as a function of distance from the site of injury. n = 3 rats per group

Inhibition of Drp1-mediated fission prevents injury-induced fragmentation of axonal mitochondria

We next sought to assess the role of Drp1 mediated fission in the injury-induced mitochondrial shortening observed in vivo. mDivi-1 is a cell and blood brain barrier permeable pharmacological inhibitor of Drp1 that blocks mitochondrial fission both in vitro and in vivo (Lackner and Nunnari 2010; Li, et al. 2016; Smith and Gallo 2017). Adult Sprague-Dawley rats were injected with mitoDsRed/GFP/AAV in the somatomotor cortex 4 weeks prior to CST severing at the C4-C5 level. mDivi-1 or vehicle was delivered via IP injections at 12 hrs before injury, and again immediately post-injury for the 1 day survival group, similar to published in vivo protocols (He, et al. 2016). Mitochondria were then analyzed at 2 hrs and 1-day post injury in the distal 0-300 µm of axons. mDivi-1 treatment decreased injury induced mitochondrial fragmentation at 2hrs and 1 day after injury. At 2 hrs, mDivi-1 treatment resulted in mitochondria with intermediate lengths, ranging between the non-injury and injury conditions (Fig. 4.4A). However, the 1-day treatment group exhibited mitochondrial lengths not significantly different than non-injured controls (Fig. 4.4A). Analysis of the percentage of mitochondria in the fragmented range (<0.5 µm) and lower end of the normal range (0.5-1 µm) revealed that mDivi-1 treatment prevented fragmentation at both 2 hrs and 1 day (Fig. 4.4B).

The Drp-1 fission inhibitor mDivi-1 reduces the number of retraction bulbs on CST axons

The fragmentation of axonal mitochondria was rapid and persistent in retraction bulbs even at longer times after injury. In order to determine whether mitochondria fragmentation could be a component of the mechanism that generates or supports retraction bulbs, the effects of mDivi-1 treatment on bulbs at the end of injured axons was examined. Adult Sprague-Dawley rats were injected with mtDsRed/GFP/AAV into the somatomotor cortex 4 weeks prior to the severing of CST axons at the C4-C5 level. The rats received IP injections of either mDivi-1 or vehicle the day before injury and once per day for 7 days after injury. Six weeks following injury, the animals were euthanized, and the spinal cord segments were analyzed. Vehicle injected controls showed cavitation at the injury site, axonal retraction, and numerous retraction bulbs at the distal tips of injured CST axons (Fig. 4.4C). Consistent with a prior report (Li, et al. 2016), animals treated with mDivi-1 showed a decreased lesion volume with lesioned axons adjacent the lesion site. In addition, there was a 73% decrease in the number of retraction bulbs observed at axon tips (Fig. 4.4D).

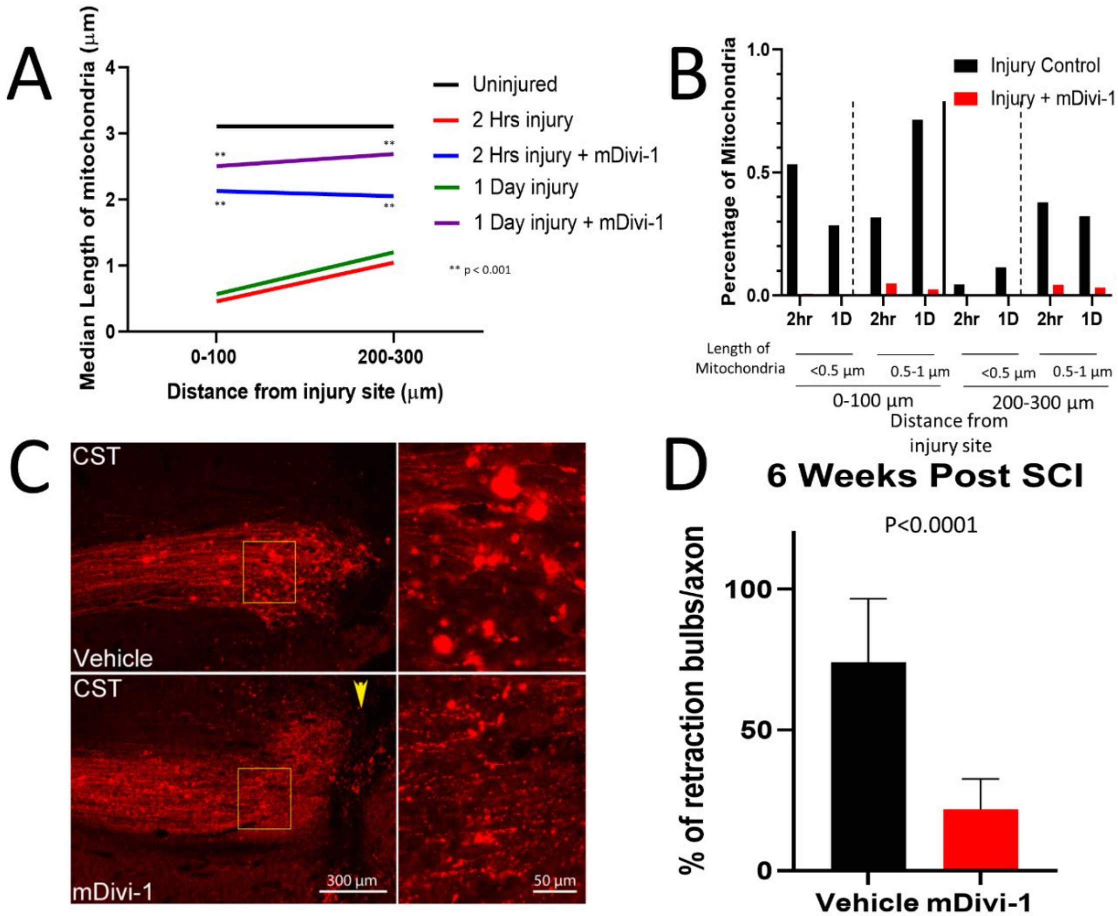

A
Median Length of mitochondria (μm)
4
3
2
1
0
0-100
200-300
Distance from injury site (μm)
Uninjured
2 Hrs injury
2 Hrs injury + mDivi-1
1 Day injury
1 Day injury + mDivi-1
** p < 0.001
B
Percentage of Mitochondria
1.0
0.5
0.0
Injury Control
Injury + mDivi-1
2hr
1D
2hr
1D
2hr
1D
2hr
1D
Length of Mitochondria
<0.5 μm
0.5-1 μm
<0.5 μm
0.5-1 μm
0-100 μm
200-300 μm
Distance from injury site
C
CST
Vehicle
CST
mDivi-1
300 μm
50 μm
D
6 Weeks Post SCI
P<0.0001
% of retraction bulbs/axon
100
50
0
Vehicle
mDivi-1

Figure 4.4 mDivi1 blocks mitochondrial fragmentation and reduces the number of retraction bulbs. (A) Graph of median mitochondrial lengths with and without mDivi-1 treatment (Dunn Multiple Comparison test) n = 3 rats per group Statistical testing done in comparison to time matched vehicle controls. **(B)** Percentage of mitochondria in the <0.5 (fragmented) and 0.5-1 μm ranges with and without mDivi-1 treatment. **(C)** Examples of CST axons adjacent the injury site. In inset: Note that the control exhibits numerous bright spherical retraction bulbs further from the lesion site and adjacent to the cavity. However, with mDivi-1 treatment, no cavities formed, axon did not appear to retract from lesion site, and the number of retraction bulbs greatly decreased. **(D)** Mean % of axons with retraction bulbs. n = 9 rats for each group. The data was analyzed using a Fisher's test on the raw aggregated categorical data within group.

PART II: Mechanistic studies of injury induced axonal mitochondrial fragmentation using in vitro model systems

Development of in vitro model systems to study the fission response of mitochondria to axonal injury

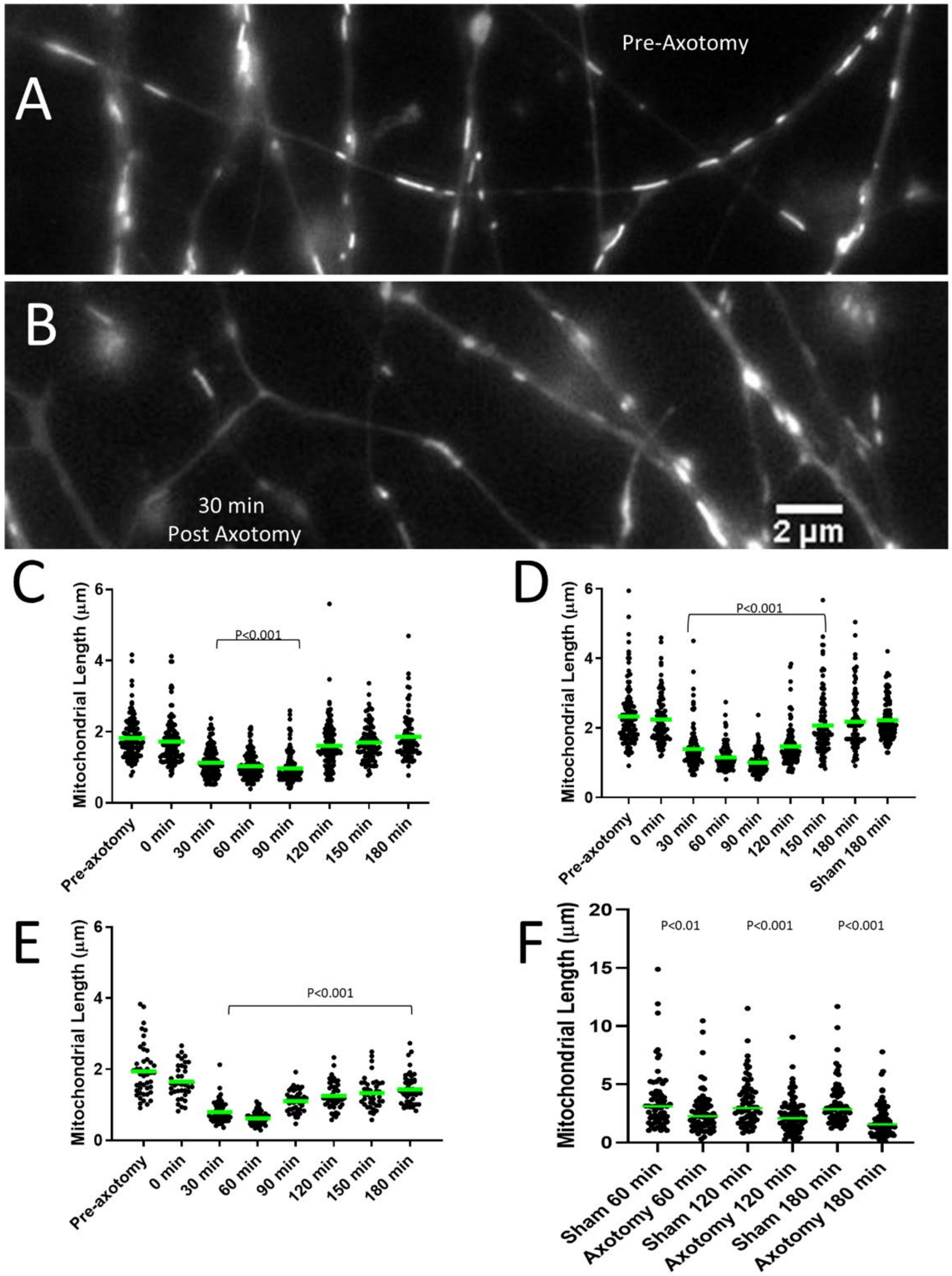
In order to perform mechanistic studies not amenable to in vivo examination and to determine whether the response of axonal mitochondria to axon severing may be cell intrinsic within the cut end of axons, we sought to set up in vitro model systems for examining mitochondrial response to axonal injury. We investigated mitochondrial morphology after in vitro axonal injury using neurons from two species and representing early embryonic, late embryonic and adult stages: (1) Embryonic day (E)7 and E14 chicken DRG explants, reflective of developmental stages when sensory axons are undergoing extension and have reached their proximal targets respectively (Fig. 4.5C, D). (2) E18 rat dissociated hippocampal neurons (Fig. 4.5E) and (3) adult rat DRG dissociated neurons (Fig. 4.5F).

Mitochondria within embryonic chicken sensory axons were labelled with MitoTracker Green dye and axons were severed using a scalpel blade after 48 hrs in culture when axons attained lengths between 1-1.5 mm. Mitochondrial length was monitored using live imaging. Prior to scalpel axotomy, axonal mitochondria showed median lengths of 1.63 µm and 2.41 µm in E7 and E14 neurons respectively (Fig. 4.5C, D), consistent with the prior demonstration of increased length in E14 neurons relative to E7 neurons (Armijo-Weingart, et al. 2019). Following axotomy, both E7 and E14 axons showed a significant reduction in mitochondrial length by 30 min after injury.

Mitochondrial length began to recover between 120 min and 150 min post injury before returning to pre-injury levels 180 min following injury (Fig. 4.5C, D). The recovery from injury was faster in E7 (120 min) than E14 axons (180 min). E18 hippocampal neurons were cultured for 3 days, the time point at which axons attained lengths between 300-400 µm (stage III of in vitro development). Axonal mitochondria were labelled with Mitoview 633. Mitochondrial length was decreased by 30 min after injury and did not recover fully until at least 180 min (Fig. 4.5E). Similar analysis using adult rat DRG neurons with mitochondria labeled using MitoTracker Green showed that lengths were decreased by one hour after injury and the decrease in length persisted until sometime after 180 min (Fig. 4.5F).

We next tested whether injury induced mitochondrial shortening in sensory axons is Drp1 dependent in vitro. E14 chicken DRG explants were incubated with either DMSO (cnt) or 20 µM mDivi-1 for 30 minutes prior to scalpel blade axotomy. Mitochondria within sensory axons were labelled with MitoTracker green. Following axotomy mitochondrial size was monitored for 180 minutes. As with untreated neurons (Fig. 4.5D), DMSO treated neurons demonstrated prominent mitochondrial shortening by 30 min after injury before gradually returning to pre-injury lengths by 180 minutes. Neurons treated with mDivi-1 did not show mitochondrial shortening at any time point following axotomy (Fig. 4.6A). Additionally, we tested whether injury induced mitochondrial shortening in hippocampal neurons is Drp1 dependent in vitro. Axonal mitochondria length was measured in E18 hippocampal neurons (as in Fig. 4.5E) treated with either DMSO or 20 µM mDivi-1 30 minutes before severing using a micro-needle as previously described (Gallo 2004). Mitochondrial length was monitored using live imaging. Prior to axotomy, DMSO treated neurons displayed axonal mitochondria with a median length of 1.86 µm. Median mitochondrial length was significantly reduced at 30 minutes after axotomy. By 180 minutes following injury, median mitochondrial length was still significantly smaller than before axotomy but was trending toward recovery of pre-axotomy length (Fig. 4.6C). Neurons treated with 20 µM mDivi-1 showed no change in median mitochondrial length over the 180-minute monitoring period (Fig. 4.6C). These observations indicate that injury induced mitochondrial shortening in vitro is a Drp1 dependent process.

To further test the hypothesis that mitochondria shortening is due to Drp1 mediated fission, we expressed a dominant-negative Drp1 mutant (Drp1K38A) that lacks GTPase activity and is therefore unable to carry out mitochondrial fission (Barsoum, et al. 2006a; Pitts, et al. 1999). E14 chicken DRGs were transfected with either YFP-Drp1 (control) and mitoDsRed or YFP-Drp1K38A and mitoDsRed. Single axon axotomy was performed using a micro-needle as done previously (Gallo 2004). Because transfection with Drp1K38A allows for unopposed mitochondrial fusion, mitochondria become elongated (20-30 µm or larger) in sensory neurons by 24 hrs of expression (Armijo-Weingart e al., 2019). Thus, comparison of mitochondrial length after axotomy between control and Drp1K38A expressing neurons is not a reasonable analysis. We therefore measured the number of fission events and attempted fission events per 10 microns of mitochondria per minute for each group monitored over a 15-minute period. As previously described (Armijo-Weingart, et al. 2019), a fission event was defined as when the fluorescence intensity of the mitochondrion at the site of apparent fission had decreased to the background level of the axonal segment not containing mitochondria and the two resultant mitochondria were separated by over 5 pixels representing 0.6 µm (Fig. 4.6E). Attempted fission events were defined as mitochondria that underwent apparent constrictions but did not continue into a full fission event, as the constriction sites returned to exhibit the prior width and intensity (Fig. 4.6E). It should also be noted that overexpression of WT Drp1 does not increase mitochondrial fission events at baseline (Armijo-Weingart, et al. 2019). Axotomy resulted in increased fission and attempted fission events in control YFP-Drp1 expressing neurons (Fig. 4.6F, G). The increases in fission and attempted fission events in response to axotomy were abolished by expression of YFP-Drp1K38A (Fig. 4.6F, G).



A
Pre-Axotomy
B
30 min
Post Axotomy
2 µm
C
Mitochondrial Length (µm)
P<0.001
Pre-axotomy
0 min
30 min
60 min
90 min
120 min
150 min
180 min
D
Mitochondrial Length (µm)
P<0.001
Pre-axotomy
0 min
30 min
60 min
90 min
120 min
150 min
180 min
Sham 180 min
E
Mitochondrial Length (µm)
P<0.001
Pre-axotomy
0 min
30 min
60 min
90 min
120 min
150 min
180 min
F
Mitochondrial Length (µm)
P<0.01
P<0.001
P<0.001
Sham 60 min
Axotomy 60 min
Sham 120 min
Axotomy 120 min
Sham 180 min
Axotomy 180 min

Figure 4.5 Axonal injury results in mitochondrial shortening in vitro (A) Example of mitochondria stained with MitoTracker green in E14 chicken DRG axons prior to axotomy. **(B)** Example of mitochondria in E14 chicken DRG axons 30 minutes following axotomy. **(C)** Median mitochondrial length in E7 chicken DRG axons at 30 min intervals following axotomy. Dots represent individual mitochondria. n = 15 axons **(D)** Median mitochondrial length in E14 chicken DRG axons at 30 min intervals following axotomy. n =15 axons **(E)** Median mitochondrial length in E18 rat hippocampal axons at 30 min intervals following axotomy. n = 10 axons **(F)** Median mitochondrial length in adult rat DRG axons at 60 min intervals following axotomy n ≥ 9 axons per group. All statistical analyses done with Dunn's multiple comparison tests.

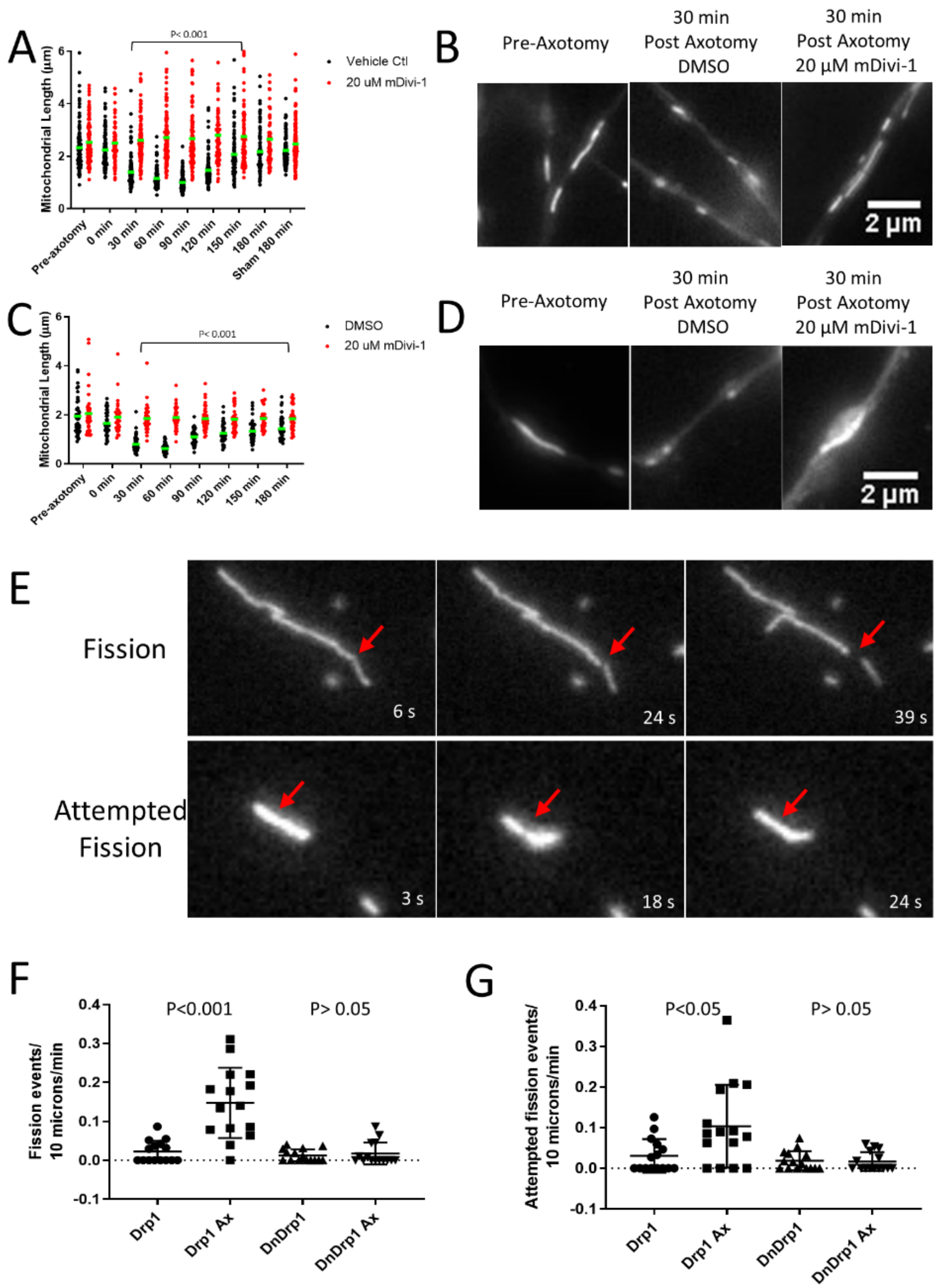

A
P< 0.001
Mitochondrial Length (µm)
Vehicle Ctl
20 uM mDivi-1
Pre-axotomy
0 min
30 min
60 min
90 min
120 min
150 min
180 min
Sham 180 min
B
Pre-Axotomy
30 min Post Axotomy DMSO
30 min Post Axotomy 20 µM mDivi-1
2 µm
C
P< 0.001
DMSO
20 uM mDivi-1
D
E
Fission
Attempted Fission
6 s
24 s
39 s
3 s
18 s
24 s
F
P<0.001
P> 0.05
Fission events/ 10 microns/min
Drp1
Drp1 Ax
DnDrp1
DnDrp1 Ax
G
P<0.05
P> 0.05
Attempted fission events/ 10 microns/min

Figure 4.6 Injury induced mitochondrial fission is Drp1 dependent in vitro (A) Median mitochondrial length in E14 chicken DRG axons with or without mDivi-1. Dots represent individual mitochondria (Dunn's multiple comparison tests). n = 15 axons for each group **(B)** Examples of MitoTracker green stained mitochondria in E14 chicken DRG axons. **(C)** Median mitochondrial length in E18 rat hippocampal axons with or without mDivi-1. Dots represent individual mitochondria (Dunn's multiple comparison tests). n = 10 axons for each group **(D)** Examples of Mitoview633 stained mitochondria in E18 rat hippocampal axons. **(E)** Example of mitochondria labelled with mitoDsRed undergoing fission or attempted fission following axonal injury. At 18 s a constriction is apparent at the site denoted by the arrow but by 24 s the constriction is no longer detectable and the mitochondrion has returned to its prior morphology. **(F)** Graph of fission events per 10 µm of mitochondrial length per minute in E14 chicken DRG axons transfected with either YFP-Drp1 or YFP-Drp1K38A (DNDrp1) following injury (Dunn's multiple comparison tests). n = 15 axons **(G)** Graph of attempted fission events per 10 µm of mitochondrial length per minute in E14 chicken DRG axons transfected with either YFP-Drp1 or YFP-Drp1K38A (DNDrp1) following injury (Dunn's multiple comparison tests). n = 15 axons

Investigations into the functional consequences of axotomy induced mitochondrial fission in vitro

Having established that axotomy results in Drp1-dependent fission both in vivo and in vitro, we next sought to determine the functional consequences if any of this process. We first assessed changes in mitochondrial membrane potential following axotomy. To do this, E7 chicken DRGs were first transfected with mitoGFP. The next day the DRGs were stained with the mitochondrial membrane potential sensitive dye, MitoTracker red (0.05nM) for 30 min prior to single axon axotomy using a pulled glass. Fluorescence was measured at 1-minute intervals for a total of 15 minutes. Mitochondrial membrane potential, as measured by MitoTracker Red fluorescence, began to decrease immediately following axotomy, showing a 10% decrease by 30 seconds after axotomy ($p<0.01$) and a 17% decrease by 2 minutes after axotomy ($p<0.001$) (Fig. 4.7A). Mitochondrial membrane potential remained reduced in comparison to uninjured axonal mitochondria for the entire 15-minute imaging period. We next wanted to determine if axotomy and the observed reduction in mitochondrial membrane potential correlated with any changes in axonal ATP. E7 chicken DRGs were transfected with a fluorescent biosensor of ATP:ADP ratio, Perceval HR (Berg, et al. 2009). Again, DRGs were injured via single axon axotomy using pulled glass and imaged at 1-minute intervals for a total of 15 minutes. We did not observe any changes in the ATP:ADP ratio following axotomy during the imaging period (Fig. 4.7B). It should be noted that positive control experiments in which a mitochondrial poison (e.g. Antimycin A) was used to block ATP production resulted in a decrease in ATP:ADP ratio as measured by Perceval HR fluorescence, demonstrating proper functioning of the construct (data not shown). When ATP fluorescence (488nm) and ADP fluorescence (405 nm) are analyzed separately, one sees that axotomy results in a decrease in ATP (Fig. 4.7C) that is partially matched by a concomitant decrease in ADP (Fig. 4.7D). This may explain why only a small, non-significant reduction in ATP:ADP ratio is seen following axotomy.

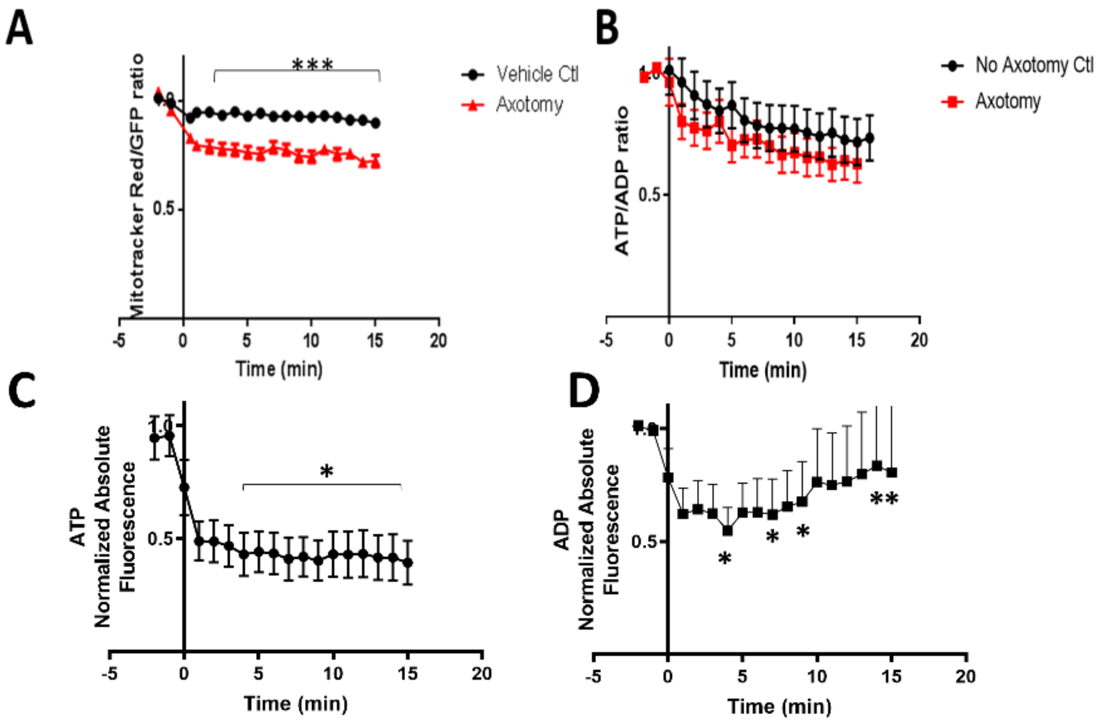

A
Mitotracker Red/GFP ratio
***
Vehicle Ctl
Axotomy
Time (min)
B
ATP/ADP ratio
No Axotomy Ctl
Axotomy
Time (min)
C
ATP
Normalized Absolute
Fluorescence
*
Time (min)
D
ADP
Normalized Absolute
Fluorescence
*
*
*
**
Time (min)

Figure 4.7 Effect of axotomy on axonal mitochondrial membrane potential and axonal ATP in vitro. (A) Graph of fluorescence ratio for Mitotracker Red and mitoGFP in E7 chicken DRGs following axotomy (Dunn Multiple Comparison test). n= 15 axons per group. **(B)** Graph of cytosolic PercevalHR fluorescence ratio (488nm/405 nm) in E7 chicken DRG axons following axotomy (Dunn Multiple Comparison test). n= 12 axons per group. **(C)** Graph of ATP absolute fluorescence from PercevalHR construct (488nm) normalized to pre-axotomy value in E7 chicken DRG axons following axotomy (Dunn Multiple Comparison test). n= 12 axons per group. **(D)** Graph of ADP absolute fluorescence from PercevalHR construct (405nm) normalized to pre-axotomy value in E7 chicken DRG axons following axotomy (Dunn Multiple Comparison test). n= 12 axons per group.

Drp1-dependent injury induced mitochondrial fission is not driven by phosphorylation of Drp1 S616

Next, we investigated the possible activation mechanism for Drp1-dependent injury induced mitochondrial fission. To do this P1 mouse DRG explants were cultured for 72 hrs prior to scalpel blade axotomy. 72 hrs culture was necessary to allow the postnatal DRG axons sufficient time to grow to a length amenable to the experiment. Following axotomy, the P1 DRG explants were fixed at 5 min, 10 min, and 15 min. The fixed samples were then stained with a pDrp1 S616 antibody (Fig 4.8A). Total axonal fluorescence was measured. We observed small increases in pDrp1 S616 fluorescence at each timepoint analyzed with the 10-minute timepoint being the largest. However, none of the observed increases in pDrp1 S616 fluorescence were significant 10-minute (Fig 4.8B). Previous experiments using activators of the ERK1/2 pathway have shown increases in pDrp1 S616 fluorescence (data not shown).

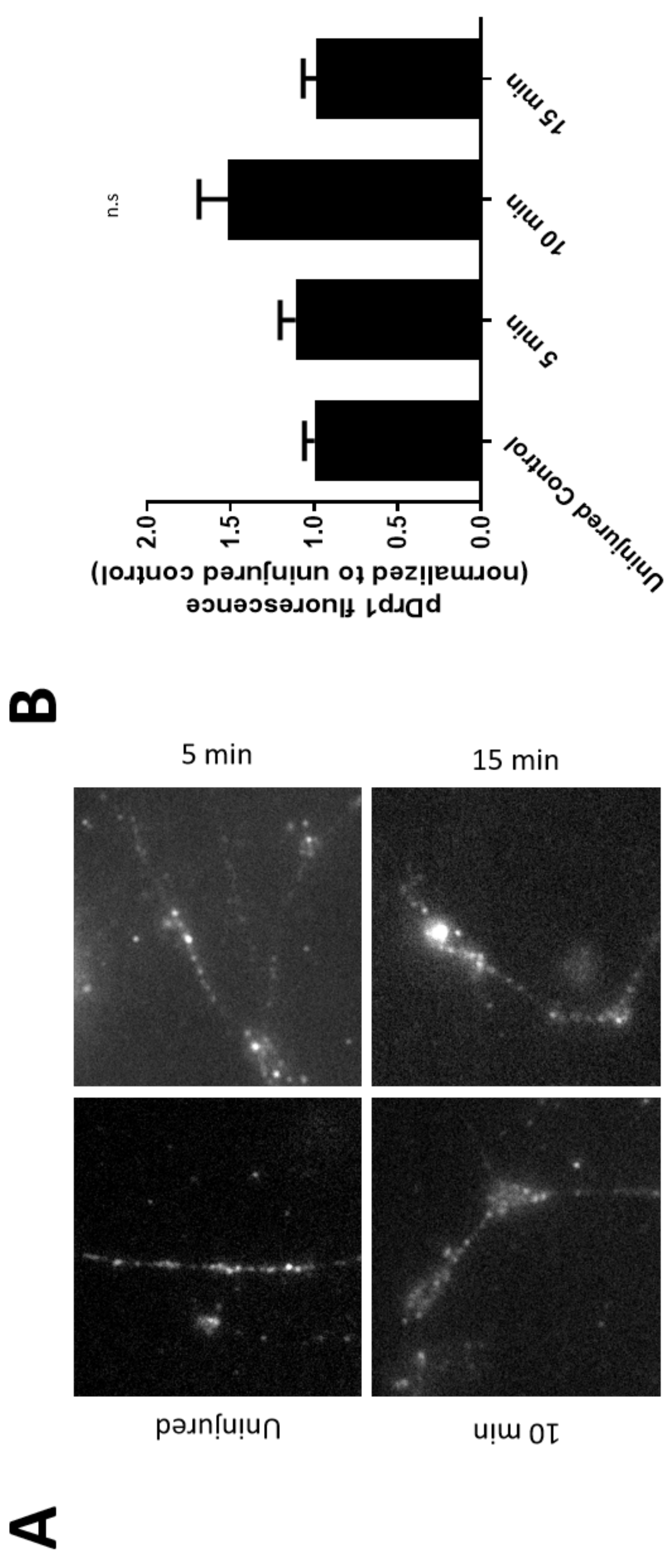

A
B
Uninjured
5 min
10 min
15 min
pDrp1 fluorescence
(normalized to uninjured control)
2.0
1.5
1.0
0.5
0.0
n.s
Uninjured Control
5 min
10 min
15 min

Figure 4.8 pDrp1 S616 activation following axotomy in vitro (A) Representative images of P1 culture mouse DRGs prior to and 5 min, 10 min, and 15 min following scalpel blade axotomy. Fixed cells were stained with pDrp1 S616 antibody (Cell Signaling) **(B)** Graph of total pDrp1 axonal fluorescence following axotomy (Dunn Multiple Comparison Test). n ≥ 10 axons.

Injury induced mitochondrial fission requires mitochondrial calcium uptake

Following axotomy there is influx of calcium into the axon, which is important in the formation of either a retraction bulb or a new growth cone (Bradke, et al. 2012). We first transfected E7 chicken DRG neurons with GCaMP6, a cytosolic, ratiometric calcium sensing protein (Chen, et al. 2013). Individual axons were severed using a micro-needle, as above, and calcium changes were monitored over a 15-minute period with live imaging. As expected axotomy caused a sustained influx of calcium resulting in a 2-fold increase in GCaMP6 fluorescence (Fig. 4.9A, B). To determine whether intra-mitochondrial calcium levels are increased following axotomy, E7 chicken DRG neurons were transfected with a mitochondrially tagged calcium-sensing protein, mitoGCaMP2. We monitored mitochondrial calcium levels for 15 minutes following axotomy, observing a steady increase in mitochondrial calcium culminating in a 3-fold increase in mitoGCaMP2 fluorescence at 15 minutes post axotomy (Fig. 4.9C, D). To determine if this increase in mitochondrial calcium was dependent on injury induced fission, we pretreated neurons with either DMSO or 20 μM mDivi-1 as above. Blocking fission following axotomy had no effect on mitochondrial calcium demonstrating that this was not a fission dependent process (Fig. 4.9C). We next addressed if injury induced mitochondrial fission is dependent on an increase in mitochondrial calcium. Calcium is taken up into mitochondria through the mitochondrial calcium uniporter (MCU) present on the mitochondrial inner membrane (Gunter and Gunter 1994). RU360 is an established inhibitor of calcium fluxes through MCU (Matlib, et al. 1998; Vanderluit, et al. 2003; Zhou, et al. 1998). E7 chicken DRG explants were treated with either 10 μM

RU360 or vehicle and mitochondria were labelled with MitoTracker Green 30 minutes prior to scalpel axotomy. Following axotomy mitochondrial size was monitored for 30 minutes using live imaging. Application of RU360 had no effect on mitochondrial size in the absence of axotomy during the 30-minute imaging period (Fig. 4.9E). At 30 minutes after axotomy, vehicle treated neurons displayed a significantly reduced median mitochondrial length when compared to mitochondrial length prior to axotomy, while neurons treated with 10 μM RU360 displayed no change in median mitochondrial length (Fig. 4.9E). These observations indicate that calcium fluxes through MCU are a required component of the mechanism that drives injury induced mitochondrial fission in vitro. In order to determine whether mitochondrial calcium fluxes through MCU could also have a role in the in vivo response of axonal mitochondrial to spinal cord injury, adult Sprague-Dawley rats were injected with mtDsRed/GFP/AAV into the somatomotor cortex 4 weeks prior to the severing of CST axons at the C4-C5 level. Immediately following injury, gel foam soaked in either vehicle or 10 mM RU360 was placed at the site of injury. The animals were euthanized either 2 hrs or 24 hrs following injury and mitochondrial size as a function of time after injury and distance from the site of injury was analyzed as previously described. Animals treated with RU360 did not show fragmentation in the vicinity of the injury site at either 2 hrs or 24 hrs post injury (Fig. 4.9F), indicating that mitochondrial calcium fluxes through MCU are also a required component of the mechanism driving injury induced mitochondrial fission in vivo.

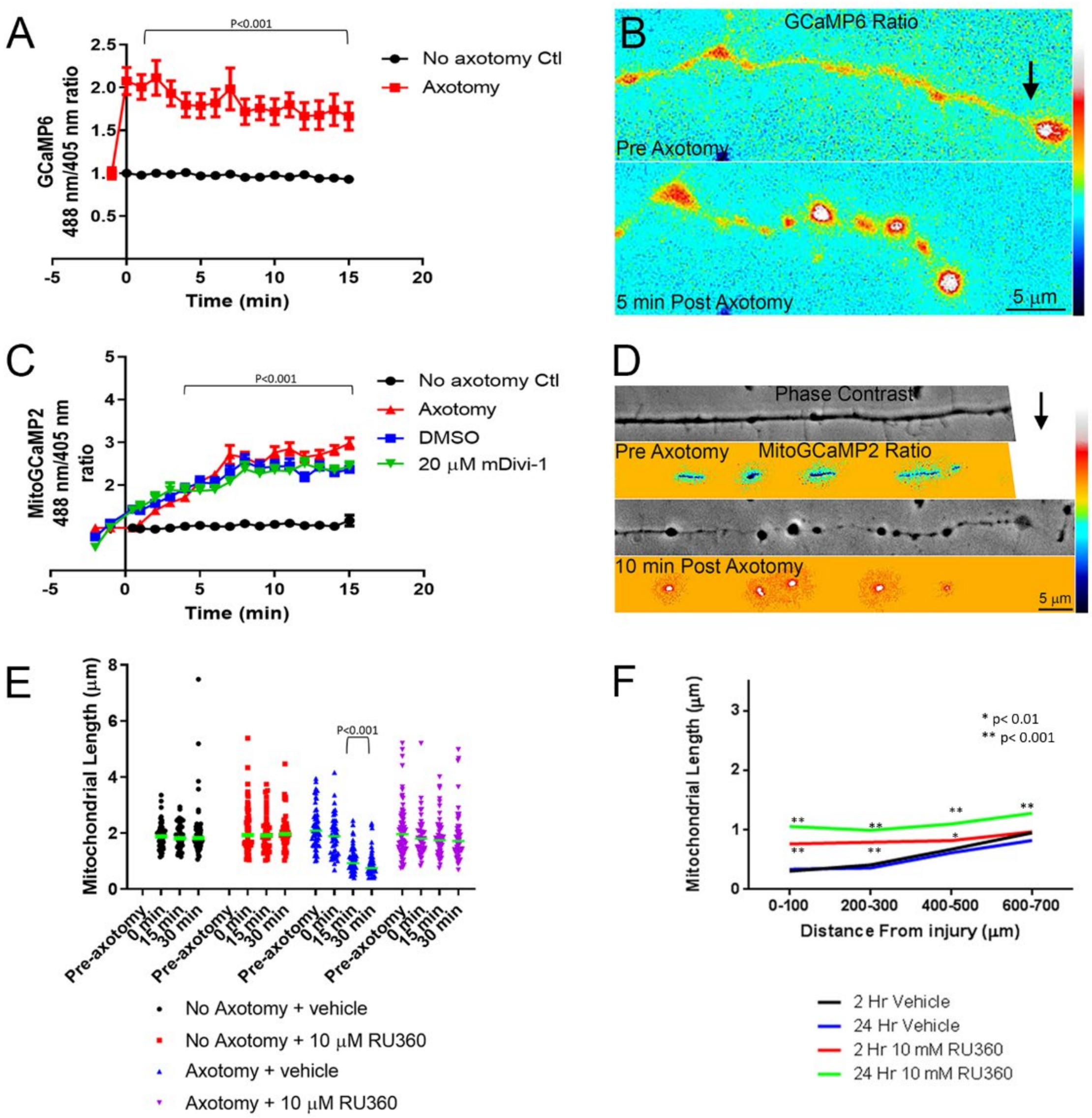

A
P<0.001
GCaMP6 488 nm/405 nm ratio
Time (min)
No axotomy Ctl
Axotomy
B
GCaMP6 Ratio
Pre Axotomy
5 min Post Axotomy
5 μm
C
P<0.001
MitoGCaMP2 488 nm/405 nm ratio
Time (min)
No axotomy Ctl
Axotomy
DMSO
20 μM mDivi-1
D
Phase Contrast
Pre Axotomy
MitoGCaMP2 Ratio
10 min Post Axotomy
5 μm
E
Mitochondrial Length (μm)
P<0.001
Pre-axotomy
0 min
15 min
30 min
No Axotomy + vehicle
No Axotomy + 10 μM RU360
Axotomy + vehicle
Axotomy + 10 μM RU360
F
Mitochondrial Length (μm)
* p< 0.01
** p< 0.001
0-100
200-300
400-500
600-700
Distance From injury (μm)
2 Hr Vehicle
24 Hr Vehicle
2 Hr 10 mM RU360
24 Hr 10 mM RU360

Figure 4.9 Injury induced mitochondrial fission requires mitochondrial calcium uptake both in vitro and in vivo (A) Graph of cytosolic GCaMP6 fluorescence ratio (488nm/405 nm) in E7 chicken DRG axons following axotomy (Dunn Multiple Comparison test). n= 10 axons per group. **(B)** Ratiometric false colored images of the GCaMP6 signal in a representative axon pre and 5 min post axotomy. The black arrow denotes the location of the axotomy. As reflected by the ratiometric measurements by 5 min the GCaMP5 signal is elevated, particularly in axonal swellings that form after axotomy. **(C)** Graph of mitoGCaMP2 fluorescence ratio (488nm/405 nm) in E7 chicken DRG axons following axotomy with or without mDivi-1 (Dunn Multiple Comparison test). n = 10 axons per group. **(D)** Ratiometric false colored images of the MitoGCaMP2 signal in a representative axon pre and 5 min post axotomy. As in panel B the arrow denotes the location of axotomy. In this example the field of view containing the axon was shifted leftward while the axotomy was occurring and is not visible in the pre images. As shown quantitatively in panel C, the ratio in mitochondria is increased by 10 min post injury. **(E)** Graph of median mitochondrial length in E7 chicken DRG axons with or without axotomy and with or without RU360 (Dunn Multiple Comparison test). n = 10 axons per group. **(F)** Graph of median mitochondrial length in adult rat CST axons following injury at the C4-C5 level with or without RU360 (Dunn Multiple Comparison test). n = 3 rats per group. Statistical testing done in comparison to time matched vehicle controls.

CHAPTER 5: SUMMARY AND DISCUSSION

Dysfunctional mitochondria have consistently been associated with neuronal disease and injury (Cherubini and Gines 2017; Galloway, et al. 2012; Luo, et al. 2015; Reddy 2014; Reddy, et al. 2011; Wu, et al. 2017; Wu, et al. 2016). In this study, we used both in vitro and in vivo models to investigate the response of axonal mitochondria to axonal injury. Mitochondria within CST axons undergo rapid fragmentation following severing. Mitochondrial fragmentation was persistent within retraction bulbs even after mitochondria in more proximal regions of the axon had recovered to normal length. In contrast, mitochondria within sciatic nerve axons do undergo injury induced fission but do not become fragmented. Injury induced mitochondrial shortening was also found in three different in vitro models of axonal injury. Drp1-mediated fission of mitochondria into fragments smaller than 0.5 µm is associated with neuronal malfunction and cell death in a variety of neurotoxic and disease states (Balog, et al. 2016; Bertholet, et al. 2016; Galluzzi, et al. 2016; Hu, et al. 2017; Karbowski 2010; Kasahara and Scorrano 2014; Otera, et al. 2013; Pradeep, et al. 2014; Reddy 2014). The observed fragmentation of axonal mitochondria following injury could severely reduce mitochondrial bioenergetics and impair growth from the cut axon tip. We observed that, in vitro, mitochondrial membrane potential was decreased following axotomy (Fig. 4.7A). However, this did not result in a change in the ATP:ADP ratio in the axons (Fig. 4.7B). Further analysis revealed that axotomy did result in a reduction in axonal ATP (Fig. 4.7C) with a partially matched reduction in axonal ADP (Fig. 4.7D). However multiple studies have shown that, at the tissue level, mitochondrial respiration following spinal cord injury is impaired (Jin, et al. 2004; McEwen, et al. 2007; Patel, et al. 2010; Patel, et al. 2009; Sullivan, et al. 2007), and there is a rise in mitochondrial oxidative stress (Jin, et al. 2004; McEwen, et al. 2007; Patel, et al. 2009; Sullivan, et al. 2007). The discrepancy in our results and previous studies may lie in the experimental model. The neurons used in this study are of embryonic and PNS origin and therefore have a higher growth potential than postnatal or adult CNS neurons. Therefore, it is possible that these neurons employ homeostatic mechanisms that allow for the maintenance of ATP:ADP ratio in the face of reduced mitochondrial potential. The neurons may utilize substrate level phosphorylation through the glycolytic pathway to maintain energy balance. Such mechanisms have been seen in other cell types, such as skeletal muscle (Harris, et al. 1986). It is also possible that the 15-minute period during which ATP:ADP ratio was monitored was not long enough to see the energetic effects of an injury induced decrease in mitochondrial membrane potential. Any possible homeostatic mechanisms may have been sufficient to maintain energy balance during the experimental window, however over a longer time scale disfunctions in energy balance may have become more apparent.

A recent study found that following sciatic nerve injury, mitochondrial fission is “adaptive” and prevents motor neuron death and axonal degeneration (Kiryu-Seo, et al. 2016). The authors observed that following injury, approximately 30% of mitochondria were fragmented in the sciatic nerve. It should be noted that this is much higher than we have observed in sciatic nerve. In contrast, if Drp1 was conditionally knocked out in motor neurons less than 7% of mitochondria became fragmented following injury, yet more motor neurons died when the Drp1 gene was knocked out. However, neurons express many different splice variants of Drp1, including those that regulate microtubule stability independent of mitochondrial fission (Strack, et al. 2013). Therefore, this study failed to separate the functions of Drp1 toward mitochondria and microtubules, and destabilization of microtubules is expected to promote axonal degeneration. This may explain inconsistencies in the interpretation of the effects of Drp1 knockout as revealing a role for fission being "adaptive" following injury, with the rest of literature consistently assigning a detrimental role to excessive fission resulting in fragmentation.

These findings raise the question of the physiological purpose for injury induced mitochondrial fission. Speed and distance of mitochondrial movement is inversely related to mitochondrial length (Narayanareddy, et al. 2014; Steketee, et al. 2012). Additionally, mitochondrial fission is necessary for axonal branching (Armijo-Weingart, et al. 2019; Spillane, et al. 2013). Axons of the sciatic nerve have the ability to regenerate following injury. We observed that following transection axonal mitochondria in the sciatic nerve undergo fission and attain lengths between 0.5 µm and 1.0 µm. This raises the possibility that fission of mitochondria into smaller, but not dysfunctional, fragments could increase their transport rate within axons to aid redistribution of healthy mitochondria within axons, increasing the growth potential, much like our observations in sciatic nerve. However, it is unclear why many axonal mitochondria within the CNS become fragmented following injury, rendering them incapable of aiding in neuronal growth and recovery.

Retraction bulbs are formed as a consequence of new growth cone formation failure and the continued flow of axoplasm to the injured tip resulting in a swollen, bulbous axonal end. Retraction bulbs are marked by the presence of accumulated organelles, such as smooth endoplasmic reticulum and mitochondria, as well as disorganized microtubules (Hill 2017). The fragmentation of mitochondria following injury may contribute to the formation of retraction bulbs, a main contributor to the lack of regenerative potential in the CNS. We observed that fragmented mitochondria persist within retraction bulbs up to 2 weeks after injury (Fig. 4.1E), a time at which axonal mitochondria have re-attained lengths comparable to axonal mitochondria in non-injured controls. For axonal regrowth to occur after injury, the injured axon must first form a growth cone, an energy demanding multistep process that relies on ATP produced by healthy mitochondria (Campbell and Holt 2001; Hill 2017; Verma, et al. 2005). Multiple recent studies show that increasing delivery of healthy mitochondria into injured axon tips positively contributes to axonal regeneration (Cartoni, et al. 2016; Han, et al. 2016; Zhou, et al. 2016). Therefore, we posit that the injury induced mitochondrial fragmentation in the CNS results in energy deficiency at the cut axon tip, leading to the formation of a retraction bulb and the failure of growth cone formation. Our observation that inhibiting injury induced mitochondrial fragmentation using mDivi-1 reduced the number of retraction bulbs reinforces this hypothesis. Additionally, inhibiting injury induced mitochondrial fragmentation reduced the lesion volume. It should be noted that the reduction in lesion volume and the reduction in the number of retraction bulbs may be caused by independent effects of mDivi-1 in different cell types.

A previous study demonstrated that mDivi-1 treatment following spinal cord injury prevented astrocyte activation and astroglial scar formation, thereby reducing lesion volume (Li, et al. 2016). Nonetheless, our findings demonstrate that inhibition of injury induced mitochondrial fragmentation prevents retraction bulb formation and may ultimately contribute to axonal regeneration following injury.

Dysfunctional mitochondria are detrimental due to their incapability to produce enough ATP to meet cellular demand, and increased generation of reactive oxygen species (ROS). Dysfunctional mitochondria are removed through mitophagy. Following spinal cord injury, a sharp increase in mitophagy occurs, as measured by LC3-mitochondria colocalization, beginning at 2 hours after injury and peaking at 3 days following injury, before returning to non-injury control levels 7 days after injury. This timeline coincides with the observation that mitochondria in the vicinity of the injury site are fragmented up to 3 days following injury before showing partial recovery of normal mitochondrial length. These observations are consistent with the notion that the injury induced mitochondrial fragments are dysfunctional and must therefore be removed from the axon in the interest of maintaining neuronal health (Ribas and Lingor 2015; Ribas, et al. 2015). Activation of autophagy using a cell permeable Tat-Beclin1 peptide, a key component of autophagosome formation (Molino, et al. 2017), reduces retraction bulb formation acutely, as well as axonal dieback 6 weeks after SCI (He, et al. 2016). In contrast, inhibiting VPS34, a phosphoinositide-3-kinase (PI3K) involved in autophagosome initiation, using 3MA prevents acute axonal degeneration and neuronal degeneration after either optic nerve injury or SCI (Bisicchia, et al. 2017; Knoferle, et al.

2010). Although the underlying models are different and could differentially modulate the rate at which autophagosomes are cleared, another explanation is that 3MA is a general PI3K inhibitor, and not specific to VPS34 (Knight and Shokat 2007; Kong and Yamori 2008). Our observations suggest that the specific promotion of mitophagy following spinal cord injury possibly proves beneficial in axonal regeneration and recovery.

Understanding the mechanism of Drp1 activation following injury may allow for more precise therapeutic targeting of injury induced mitochondrial fragmentation. Therefore, we attempted to determine the pathway of Drp1 activation following injury. Drp1 activation generally occurs through phosphorylation of S616 (Santel and Frank 2008). Phosphorylation of Drp1 S616 can be accomplished by multiple upstream kinases (described in Chapter 2). However, we did not observe any significant increases in pDrp1 S616 following axotomy. While this may be due to technical limitations in our study, this finding also may indicate that following injury Drp1 is instead activated through a different mechanism. Dephosphorylation of Drp1 S637 also can activate Drp1 in different contexts (Santel and Frank 2008). Due to a lack of a reliable pDrp1 S637 antibody we were unable to pursue this line of investigation.

Calcium dynamics following injury are important in determining whether a new growth cone or a retraction bulb will form (Bradke, et al. 2012), thus we investigated whether calcium acts in Drp1-dependent injury induced mitochondrial fragmentation. Following injury, there was an increase of both cytosolic and mitochondrial calcium content. The increase in mitochondrial calcium content was not blocked by application of mDivi-1, indicating that the observed mitochondrial uptake is independent of injury induced mitochondrial fission. Inhibiting mitochondrial calcium uptake using RU360, both in vitro and in vivo, blocked injury induced mitochondrial fission, showing that mitochondrial calcium uptake is a component of the mechanism underlying injury induced mitochondrial fragmentation. Mitochondria are composed of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), both of which must be fully constricted for fission to occur (Kuroiwa, et al. 2008). Constriction of the IMM was shown to be dependent on mitochondrial calcium uptake, and could be blocked by administration of RU360 (Cho, et al. 2017). Thus, the influx of mitochondrial calcium following injury may be required for fission to come to completion due to its role in IMM constriction. Additionally, Drp1 alone is unable to initiate mitochondrial constriction because Drp1 oligomers can only form contractile rings with a diameter of up to 100 nm, while mitochondria have diameters between 0.5-1.0 μm (Ingerman, et al. 2005; Mears, et al. 2011). Therefore, mitochondrial fission requires a pre-constriction step, in which actin filaments promote direct contact of mitochondria with endoplasmic reticulum (ER) (Friedman, et al. 2011; Korobova, et al. 2013). A rise in cytosolic calcium promotes actin dependent pre-constriction, bringing mitochondria into closer contact with ER, allowing for efficient uptake of calcium into mitochondria through the MCU (Chakrabarti, et al. 2018). These results suggest that following injury, a large influx of calcium into the cytosol results in actin polymerization, which drives mitochondrial pre-constriction and mitochondrial-ER contact. This in turn leads to an uptake of calcium into the mitochondria through the MCU and promotes constriction of the IMM priming mitochondria for Drp1-dependent fission. These mechanistic insights may provide yet another target for inhibiting injury induced mitochondrial fragmentation and perhaps promoting axonal regeneration in the future.

CHAPTER 6: CONCLUSION

This thesis details the novel findings that following injury, axonal mitochondria of CST neurons undergo rapid fragmentation in a Drp1-dependent process. Mitochondrial fragments persist in retraction bulbs and may play a role in their initial formation or maintenance. Sciatic nerve axons, on the other hand, demonstrate injury induced mitochondrial fission but not fragmentation. Axonal injury in the CST also results in an increase in mitophagy, indicating that the injury induced mitochondrial fragments are dysfunctional. In vitro models show that axonal injury leads to a reduction in mitochondrial membrane potential but did not show a decrease in ATP:ADP ratio. Mechanistically, phosphorylation of Drp1 S616 does not appear to be involved in the activation of Drp1 following injury. However, we show that mitochondrial calcium uptake is a mechanistic contributor to injury induced mitochondrial fragmentation. These observations pave the way for new strategies aimed at axonal mitochondrial dynamics in the effort to prevent axonal dieback, and to promote axonal regeneration following injury.

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