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Mitochondrial Fission After Traumatic Brain Injury

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MITOCHONDRIAL FISSION AFTER TRAUMATIC BRAIN INJURY

by

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MITOCHONDRIAL FISSION AFTER TRAUMATIC BRAIN INJURY

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Acknowledgements

As I look back the time I spent during my PhD, it is bitter sweet. The PhD process is an incredibly challenging experience that pushes one's limits intellectually and emotionally, but yields a level of growth that can only occur outside of one's comfort zone. Along with most graduate experiences, mine has been riddled with moments of agony, excitement, confusion, curiosity, anticipation, indifference, and enlightenment. Every single experience, bad and good, has been invaluable and absolutely essential to the person and scientist I am today, and for that I do not look back on any moment with resentment, but with gratitude. My experiences during my PhD have taught me resiliency, persistence, and independence, and have resulted in an unwavering enthusiasm (alright, not necessarily "unwavering", but endless!) for science that I am absolutely excited for the work I have yet to realize. I am extremely grateful for every person that has given me the platform to achieve all of my lofty, and often over-ambitious goals and dreams. This work is as much theirs, as it is mine, and I could not have achieved it without the unending support and guidance from my mentors, friends, and family. I have truly accomplished this work standing on the shoulders of giants, and have been so fortunate to have found extraordinary mentors in many professors, lab mates, and peers.

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Well this is three pages and has gotten a little bit unwieldy, so enough with all the ooey-gooey sentiment. *En finalement*, in the words of the wonderful Freddie Mercury, Don't. Stop. Me. Now. Let's do some science!

MITOCHONDRIAL FISSION AFTER TRAUMATIC BRAIN INJURY

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Mitochondrial dysfunction is a central feature in the pathophysiology of Traumatic Brain Injury (TBI). Loss of mitochondrial function disrupts normal cellular processes in the brain, as well as impedes the ability for repair and recovery, creating a vicious cycle that perpetuates damage after injury. To maintain metabolic homeostasis and cellular health, mitochondria constantly undergo regulated processes of fusion and fission and functionally adapt to changes in the cellular environment. An imbalance of these processes can disrupt the ability for mitochondria to functionally meet the metabolic needs of the cell, therefore resulting in mitochondrial damage and eventual cell death. Excessive fission, in particular, has been identified as a key pathological event in neuronal damage and death in many neurodegenerative disease models. Specifically, dysregulation of the primary protein regulator of mitochondrial fission, Dynamin-related Protein 1 (Drp1), has been implicated as an underlying mechanism associated with excessive fission and neurodegeneration; however, whether dysregulation of Drp1 and excessive fission occur after TBI and contribute to neuropathological outcome is not well known. The studies described in this dissertation investigate the following hypothesis: *TBI causes dysregulation of Drp1 and increases mitochondrial fission in the hippocampus, and inhibiting Drp1 will reduce mitochondrial dysfunction, reduce neuronal damage, and improve cognitive function after injury.* Results from these studies revealed four key findings: 1) Experimental TBI increases Drp1 association with mitochondria, and 2) causes acute changes in Drp1-mediated mitochondrial morphology that persists post-injury, indicating increased mitochondrial fission acutely after injury. Additionally, 3) post-injury treatment with a pharmacological inhibitor of Drp1, Mdivi-1, improved survival of newly born neurons in the injured hippocampus, and 4) improved hippocampal-dependent cognitive function after experimental TBI. Taken together, results from these studies reveal that TBI causes excessive Drp1-mediated mitochondrial fission and that this pathological fission state may play a key role in hippocampal neuronal death and cognitive deficits after TBI. Furthermore, these findings indicate inhibition of Drp1 and mitochondrial fission as a potential therapeutic strategy to improve neuronal recovery and cognitive function after injury.

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Chapter 1. Introduction

Mitochondria are beautifully structured organelles and functionally sophisticated metabolic machines that form a highly interconnected network throughout the cell. As a central source of cellular adenosine triphosphate (ATP) and site for many metabolic processes, mitochondrial function is essential for cellular health and survival. Mitochondrial dysfunction is a central feature in the pathophysiology of Traumatic Brain Injury (TBI). Loss of mitochondrial function disrupts normal cellular function in the brain, as well as impedes the ability for repair and recovery, and therefore creates a vicious cycle that perpetuates damage after injury. To maintain homeostasis and cellular health, mitochondrial morphology is regulated by delicately balanced processes of fusion and fission that allow for efficient functional adaptation of the mitochondrial network to meet the metabolic demands of the cell. Imbalance of these processes impedes the ability for mitochondria to accomplish essential metabolic functions, and results in substantial mitochondrial dysfunction, metabolic dysregulation, and cell death. For cells that have high metabolic demands, such as neurons, this balance is particularly critical to maintain an adaptive mitochondrial compartment for neuronal function and survival. Disruption in the balance of mitochondrial fission and fusion has thus been an area of intense investigation in the pathogenesis and progression of neurodegenerative diseases. Whether disruption in the balance of mitochondrial fission and fusion occurs after TBI, and contributes to mitochondrial dysfunction and neuropathology is not well known.

This chapter is a thorough review of the current knowledge for 1) mitochondrial damage and dysfunction in TBI pathophysiology, and 2) mechanisms and regulation of mitochondrial fission and fusion, and will provide the reader with the necessary background for the hypotheses investigated in this dissertation. Additionally, a section is dedicated to mitochondrial structure and function to provide the reader a brief overview of basic mitochondrial biology, with a focus on topics relevant to the design of the described experiments.

1.1 A “Brief” Overview of Mitochondrial Structure and Function

Mitochondria are an ancient double membrane-bound organellar system fundamental to eukaryotic metabolism. Groundbreaking electron microscopy studies in the early 1950s revealed the “fine structure of mitochondria” and introduced the canonical bean shaped organelle we still see today in biology textbooks.(Palade, 1952) Mitochondria are a specified compartment for many fundamental metabolic processes, including ATP synthesis, β-fatty acid oxidation, and amino acid biosynthesis. Each process requires key metabolic machineries that are organized within the intricate architecture of mitochondria and precise interaction and coordination of these metabolic machineries is required to efficiently carry out fundamental biochemical processes, such as oxidative phosphorylation. The structure of mitochondria is therefore strategically built and organized for optimal metabolic function within the mitochondrial compartment. For example, the matrix contains enzymes of the citric acid cycle and its biochemical components that regulate metabolite levels critical for oxidative phosphorylation. Oxidative phosphorylation is executed by the cooperation of the respiratory complexes that form supramolecular assemblies in the inner membrane for efficient electron transport and ATP generation. ATP is then transported out of the inner membrane through one of many specialized transporter proteins, to finally exit into the cytosol through large outer membrane pores to fuel many cellular processes. Therefore, the elaborate organization and structure of mitochondria and the coordination between its molecular components is critical for the function of the organelle. This section will outline the basic structural features of mitochondria as well as the key pathways and mechanisms fundamental for oxidative phosphorylation.

Mitochondrial structure. The intricate architecture of mitochondria creates structurally and functionally distinct subcompartments and membranes that are dynamically regulated by cellular metabolism. Key metabolic machineries for several biochemical processes are organized within these subcompartments and are resident to specific membrane regions (**Figure 1.1**). The outer membrane (MOM) contains large porins (VDACs), and translocases (TOMs) that allow the

passage of ions and proteins less than 5 kDa into the inter membrane space.(Walther and Rapaport, 2009) Several proteins and complexes also reside in the outer membrane that are important for various mitochondrial and cellular processes, including membrane fusion (Mfn1/2), protein sorting and assembly (SAMs), and apoptotic signaling (Bak).(Pernas and Scorrano, 2015) More recently, proteins have been discovered on the MOM that interact with outer membrane proteins of other organelles and form complexes, such as the endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES).(Rowland and Voeltz, 2012) These complexes have led to the regional and functional specialization of mitochondrial-associated membranes (MAM) that have been found to be involved in interorganellar communication and content exchange. The MOM also undergoes fission and fusion, and therefore it's shape is highly dynamic.(van der Bliek et al., 2013) Between the outer and inner membranes is the intermembrane space (IMS), an aqueous subcompartment that contains a milieu of metabolites, ions, and small molecules, and has a pH slightly more acidic than the cytosol.(Herrmann and Riemer, 2010) The IMS is further compartmentalized by the organization of the mitochondrial inner membrane (MIM) and the formation of cristae compartments. The mitochondrial inner membrane (MIM) is a complex and plastic structure that also has the ability to fuse and divide.(van der Bliek et al., 2013) The composition of the MIM is primarily cardiolipin, along with protein complexes, enzymes, and metabolite transporters.(Mannella, 2006; Paradies et al., 2014) Cardiolipin binds several MIM proteins and is a fundamental component of the architecture and function of the MIM.(Mannella, 2006) The MIM forms transient subcompartments, termed cristae, to sequester key molecules essential for oxidative phosphorylation, such as Cytochrome *c*. Protein complexes that compose the electron transport chain (I-IV) are resident to the MIM are found on the sides of the folded membrane portions of cristae.(Mannella et al., 2001) Respiratory complexes can further assemble into supercomplexes that are more efficient for electron transport and oxidative phosphorylation.(Acín-Pérez et al., 2008) Cardiolipin is also thought to play a role in this supramolecular assembly of respiratory supercomplexes.(Bazán et al., 2013) The assembly of respiratory chain complexes in the MIM and the sequestration of essential

metabolites and proteins, thus creates a specialized aqueous environment for efficient oxidative phosphorylation.(Cogliati et al., 2013) Mechanisms underlying the formation of cristae are still relatively unknown. This is currently and area of exciting investigation, and recent advances have provided considerable insights to the remarkable flexibility and adaptability of cristae formation and the control of oxidative phosphorylation. ATP synthase (Complex V of the electron transport chain) dimers have been found to be organized in rows along the cristae membrane and specifically located at the apex.(Davies et al., 2012) At the contact site of the MIM, termed the cristae junction, a few ring-like protein complexes, such as MICOS, Prohibitin, and the dynamin-related protein, Opa1, have also been identified as candidates for formation and stabilization of the structure.(Cogliati et al., 2016) The mechanistic role of these complexes, as well as the functional implications, in the regulation of cristae formation and reorganization for oxidative phosphorylation, remains to be elucidated. Regions of the MIM excluded from the cristae are termed the inner boundary membrane (IBM) and are the site for several transporter complexes that regulate import into the matrix.(Cogliati et al., 2016) In contrast to the MOM, transport into the matrix is highly regulated to maintain balanced concentrations of critical metabolites and ions for oxidative phosphorylation, as well as protect the mtDNA and ribosomes from cytosolic insult.(Wohlrab, 2009) Several essential transporters are located in the MIM, including the adenine nucleotide transporters (ANT) and translocases (TIM) for exchange of ADP/ATP and regulated protein import into the matrix, respectively. The calcium uniporter and Na+/Ca+ exchanger is also located in the IBM for import and regulated extrusion of calcium from the matrix.(Palty and Sekler, 2012) Transport of pyruvate, the end-product of glycolysis, is also highly regulated and has a specialized transporter, the Mitochondrial Pyruvate Carrier (MPC), however given its recent discovery, whether it is specifically localized in the IBM or the cristae membrane has yet to be determined.(Herzig et al., 2012) The matrix is the inner most compartment within mitochondria and houses mitochondrial DNA (mtDNA) and ribosomes, TCA cycle enzymes, metabolites, nucleotides, and ions. The difference in concentrations of ions and protons between the matrix and the inner membrane space creates an electrochemical gradient across the MIM

that drives electron transport and oxidative phosphorylation.(Mitchell, 1961) The functional state of the mitochondrial compartment is highly dependent on the composition in the matrix, therefore several quality control mechanisms have evolved to allow for mixing of matrix contents to maintain homeostasis and preserve function. In summary, the remarkable structural integrity and functional specialization of the mitochondrial membranes and compartments is fundamental to mitochondrial health and function, and therefore underscores the concept of “form follows function”.

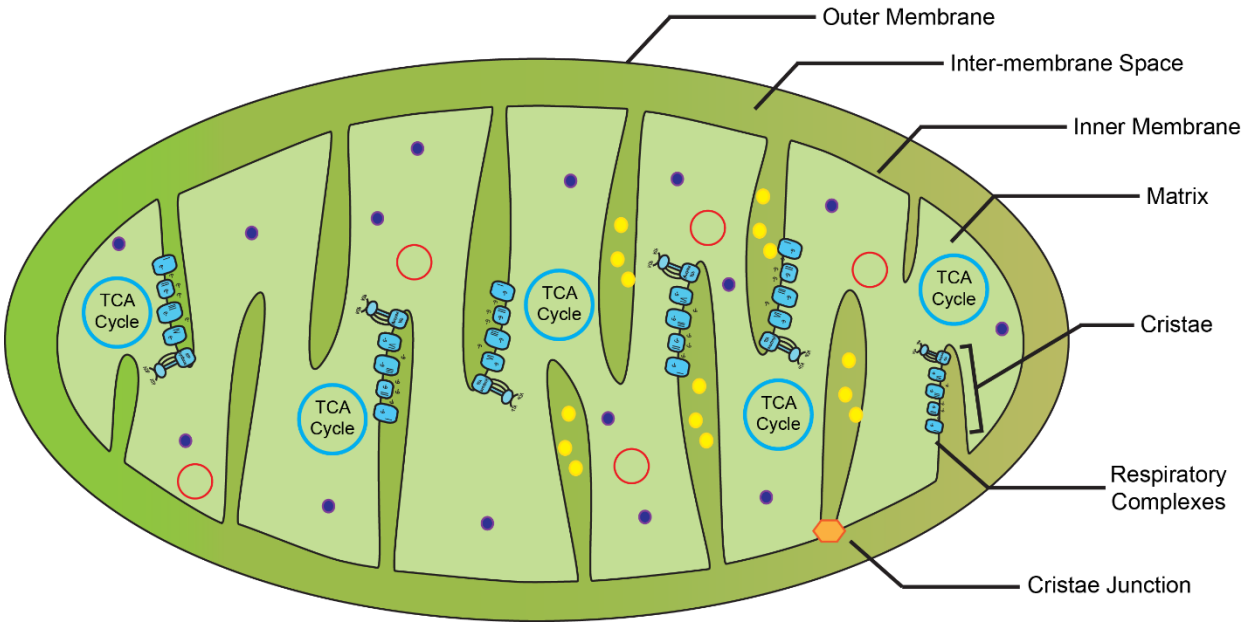

Figure 1.1 Mitochondrial structure.

Oxidative phosphorylation.(Berg et al., 2002) In 1961, Peter Mitchell first proposed the "Chemiosmotic Hypothesis" with the theory that ATP synthesis is coupled to oxidative reactions that create a proton-motive force and form an electrochemical gradient across the inner mitochondrial membrane.(Mitchell, 1961) The gradient formed provides the free energy necessary to execute the phosphorylation of ADP to ATP, thus facilitating *Oxidative Phosphorylation* (**Fig. 1.2**). Mitochondrial ATP synthesis is mechanistically complex and dependent on the coordination and interaction of many chemical processes both external and internal to mitochondria. First, glucose is broken down through a series of enzyme catalyzed reactions in glycolysis (the Embden-Meyerhof-Parnas Pathway). The final product of glycolysis, Pyruvate, then diffuses through porins on the MOM, and is subsequently transported into the mitochondrial matrix via the Mitochondrial Pyruvate Carrier (MPC). Pyruvate decarboxylation by Pyruvate Dehydrogenase (PDH) in the mitochondrial matrix produces Acetyl-CoA, which then enters the The Citric Acid (TCA) Cycle and initiates its feed-forward catabolism through several enzymatic reactions. The reactions of the TCA cycle produce NADH and $FADH_2$, reducing agents required for electron entry into the electron transport chain (ETC). The oxidation of NADH and $FADH_2$ begin a sophisticated process of sequential transfer of electrons to strategically organized complexes of higher reducing potential to provide the energy necessary for the final phosphorylation of ADP.

Complex I and II in the inner mitochondrial membrane (MIM) are the single entry points for electrons into the ETC. Electron transfer from Complex I and II requires redox of Coenzyme Q (CoQ), a soluble quinone in the MIM, and will further be referred to either in its oxidized form (Ubiquinone) or its reduced form (Ubiquinol). Complex I (NADH-Q Oxidoreductase) oxidizes NADH and reduces Ubiquinone to donate two electrons into the electron transport chain. The reduction of Ubiquinone is accompanied by the pumping of four protons into the intermembrane space. Complex II (FADH-Q Oxidoreductase) oxidizes $FADH_2$ via FeS centers within the complex, in which electrons are subsequently transferred to Ubiquinone for entry into the ETC.

No protons are pumped through Complex II with these electron transfers, as it does not span the entire inner membrane. Additionally, Complex II is associated with the TCA cycle as the only membrane bound member that catalyzes the conversion of succinate to fumarate, known as Succinate Dehydrogenase. Therefore, succinate is a direct substrate to drive electron transport via Complex II. Electrons flow from Complexes I and II via Ubiquinol through the ETC to the next electron acceptor, Complex III (Q-Cytochrome *c* Oxidoreductase). Complex III is responsible for transferring electrons from Ubiquinol to Cytochrome *c* and pumping out four protons into the intermembrane space. Transfer of electrons via Complex III is more sophisticated, as Ubiquinol is a two-electron carrier and Cytochrome *c* is a one electron carrier, therefore a process known as the Q cycle is required to manage the sequential transfer of electrons to Cytochrome *c* molecules. Cytochrome *c* resides in the intermembrane space associated with the MIM. The final electron transfer reaction is the coupled oxidation of Cytochrome c with the reduction of molecular oxygen to water via Complex IV (Cytochrome *c* Oxidase). This final electron transfer involves another cycle of redox reactions between cupric ions and iron centers in heme groups within Complex IV. Molecular oxygen binds to one of the iron centers in Complex IV and is then poised for final reduction into water. The reactions at Complex IV "pump" a total of 8 protons from the matrix, in which four protons are required for the reduction of molecular oxygen, and four protons are pumped into the inter membrane space. Therefore, the reduction of molecular oxygen provides the greatest energy transfer in the ETC reactions, and is essential for forming the electrochemical gradient across the MIM.

The final reaction for the synthesis of ATP is the phosphorylation of ADP by Complex V (ATP Synthase). ATP Synthase is a beautiful molecular machine. The structure of ATP synthase was first visualized in pioneering electron microscopy studies and described as a "ball and stick" located in the mitochondrial inner membrane. The "ball and stick" are now identified as two rotary motors, the hydrophobic F_o unit, that forms a channel in the inner membrane, and the soluble F_1 unit, an 85Å diameter ball facing the mitochondrial matrix that mediates the catalytic activity of

ATP Synthase. The two motors are connected by a stalk subunit that provides rotatory control of the F_1 unit in the matrix by the F_o unit in the membrane. The process of ATP synthesis by the rotatory motions of ATP synthase is structurally complex and coordinated mechanism. For the purpose of this brief review, I will only provide a basic explanation of this mechanism, but acknowledge that it is a much more sophisticated process than the provided explanation. Firstly, the energy released from the proton-motive force during electron transport drives rotations of the F_o unit, in which protons from the intermembrane space enter and move through the channel with each rotation. At the F_1 subunit, ATP synthesis occurs in three sequential steps, 1) binding of ADP and free phosphate, 2) ATP synthesis, and 3) ATP release. These steps are mediated by a "binding change" mechanism via conformational changes of individual subunits between "open" and "closed" states. The conformational change that synthesizes ATP also binds ATP with high affinity. Thus, the rotary motion and proton movement through the F_o unit and the stalk controls the rotation of the F_1 unit, and forces a conformational change of the subunit that provides the energy necessary for the release of ATP. ATP is then transported through the Adenine Nucleotide Translocator (ANT) in the MIM and diffuses out of porins in the outer membrane to finally reach the cytosol and fuel fundamental cellular processes.

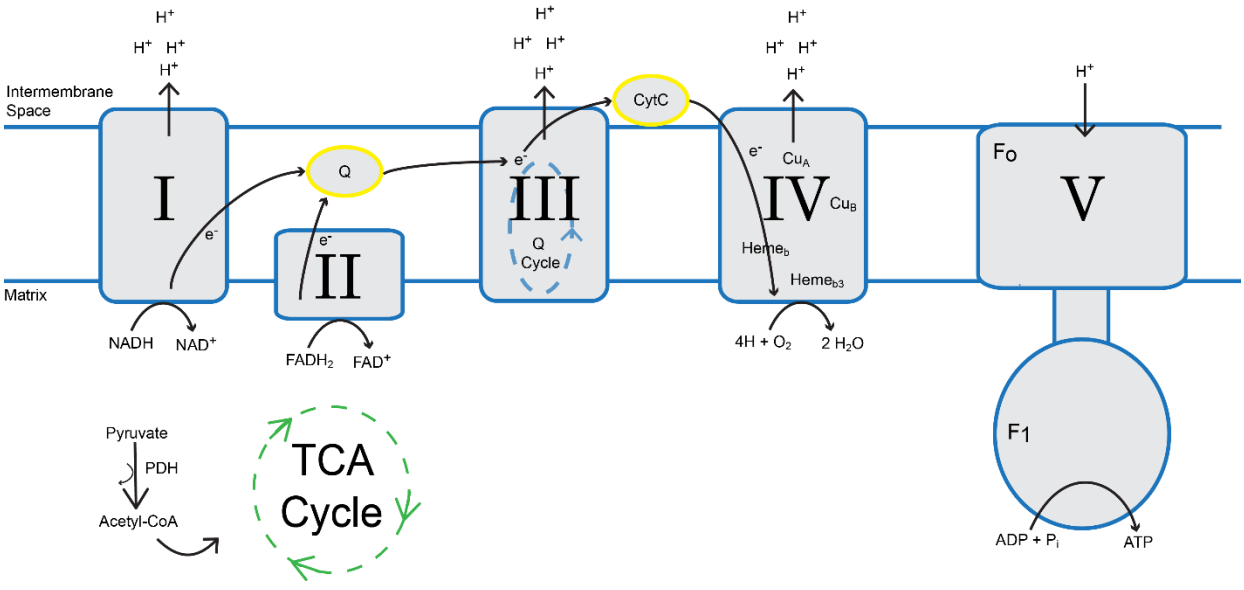

Figure 1.2 Mechanisms of oxidative phosphorylation.

Conclusions. In summary, the intricate architecture of mitochondria creates structurally and functionally distinct subcompartments and membranes that are dynamically regulated for cellular metabolism. Key metabolic machineries, such as the respiratory complexes and ATP synthases, are strategically organized within the mitochondrial membranes and essential metabolites are sequestered in subcompartments to create an optimal environment for metabolism. These metabolic machineries require precise interaction and coordination to efficiently carry out fundamental biochemical processes, such as oxidative phosphorylation and ATP synthesis. The structure of mitochondria is thus strategically built and organized for optimal metabolic function within the mitochondrial compartment.

1.2 Mitochondrial Damage and Dysfunction in the Pathophysiology of Traumatic Brain Injury

Traumatic brain injury (TBI) is a major cause of death and disability in the United States, with an estimated 1.7 million people suffering a TBI admitted to the emergency room each year.(Faul, M., Xu, L., Wald, M.M., and Coronado, 2010) About 53,000 of severe TBIs result in death, however 5.3 million survivors are currently living with serious disabilities. TBI is caused by an external, inertial force to the head, defined as an impact, penetration, or rapid movement of the brain in the skull that causes in disruption of normal brain function. The severity of brain injury can range from mild (concussion), moderate, or severe, and can also be influenced based on a number of criteria, such as the comorbidity of other injuries including skull fracture, hemorrhage, and ischaemia.(Chesnut et al., 1993) Thus, outcome after brain injury is complex and multifactorial, and patients exhibit different neuropathological outcomes depending on many factors, including age, severity, and location of damage. TBI patients typically endure disabling cognitive deficits as a result of injury, particularly in executive functions and learning and memory.(Engberg and Teasdale, 2004; Tsaousides and Gordon, 2009) Brain areas important for learning and memory, such as the hippocampus, are particularly vulnerable to damage causing significant cognitive and functional for months to years after injury.(Christidi et al., 2011; Venkatesan et al., 2014; Walker and Tesco, 2013) Despite significant efforts, no therapies currently available improve neuropathological and cognitive outcomes after TBI. Therefore, it is essential to understand the fundamental pathophysiological mechanisms underlying TBI to develop novel, targeted therapeutic approaches and improve the quality of life in patients who suffer from brain injury.

Pathophysiology of secondary injury: In brief. At the cellular level, TBI involves a complicated and heterogeneous insult to the brain, causing chronic activation of biochemical cascades that result in cell death, regional damage, and lasting dysfunction. The progressive pathophysiology of TBI can be considered to occur in two stages: an initial, primary insult and a

delayed period of secondary damage that precipitates cell death and dysfunction for days to weeks post-injury.(Prins et al., 2013; Werner and Engelhard, 2007) The initial insult is caused by direct mechanical damage to tissue, and consists of irreparable tearing and stretching of membranes, axon shearing, and the consequential release of cellular contents into the extracellular space. Elevations in intracranial pressure and swelling can also further distort tissue and cause reduced cerebral blood flow and nutrient deprivation in the brain. As a consequence of the initial injury, several biochemical cascades are activated that propagate pathological signaling and cause widespread biochemical, physiological, metabolic, and cellular changes that can persist post-injury (**Fig. 1.3**).(Ray et al., 2002) Experimental studies have elucidated several mechanisms underlying secondary pathophysiology after TBI; however, as TBI is a sudden insult, the temporal relationship of each event has not been fully resolved, therefore a hypothetical model currently stands to represent the pathophysiology of secondary injury after TBI.(Prins et al., 2013; Werner and Engelhard, 2007) Two notable events that characterize secondary injury are a “metabolic crisis” and persistent calcium dysregulation after injury. The cause of each event is thought to be intimately linked, in which both separately, and in combination, facilitate the propagation and exacerbation of secondary damage after TBI.(Giza and Hovda, 2001; Weber, 2012) Due to plasma membrane stretching and shearing, normal cellular ionic balances are disrupted, membrane potential is abolished, and a substantial amount of neurotransmitter is subsequently released at presynaptic terminals causing indiscriminate neurotransmission. Thus, a significant energy demand occurs both presynaptically and postsynaptically, to restore ionic imbalances and membrane potential, and to manage persistent synaptic signaling. In steady state, cerebral blood flow is tightly coupled to neuronal activity and glucose uptake. However, reductions in cerebral blood flow acutely after TBI limit the availability of nutrients to the brain, resulting in an uncoupling of energy supply and demand. This creates a “metabolic crisis” after injury as cellular ATP supplies are depleted, and ATPase pumps fail, further exacerbating disruptions in recovery and normal synaptic neurotransmission.(Giza and Hovda, 2001; Werner and Engelhard, 2007) Aberrant excitatory signaling, such as glutamate,

during this time saturates receptors (NMDA, AMPA), and maintains depolarization allowing for continuous entry calcium and sodium entry through voltage gated channels into the postsynaptic cell. Maintenance of intracellular calcium is critical for normal signaling in the cellular environment and cell survival, thus a number of calcium-mediated enzymes and signaling pathways are inappropriately activated or disrupted after injury.(Atkins, 2011) Consequently, massive dysregulation and dysfunction in the intracellular environment ensues, triggering reactive oxygen species production, interleukin signaling and inflammation, organelle damage, and eventual cell death. Aberrant neurotransmission further propagates pathological signaling, spreading the damage, and causing dysfunction in other areas of the brain. Thus, the pathophysiology of TBI is a complex, heterogeneous insult that initiates cellular processes that spread the damage from the initial injury to cause massive, widespread damage and dysfunction across the brain.

Mitochondrial damage and dysfunction is a key feature of secondary injury, and is a critical piece in the puzzle of TBI neuropathophysiology. Mitochondria are essential regulators of intracellular calcium signaling, reactive oxygen species production, cellular metabolism, and cell death. Therefore, each pathophysiological event in secondary damage is intimately linked to mitochondrial damage and dysfunction. Furthermore, as a crucial source of ATP, loss of mitochondrial function creates a vicious cycle of cellular insult that further contributes to the metabolic crisis and cell death after injury. Thus, mitochondrial damage and dysfunction has far reaching implications in the pathophysiology of secondary damage after TBI.

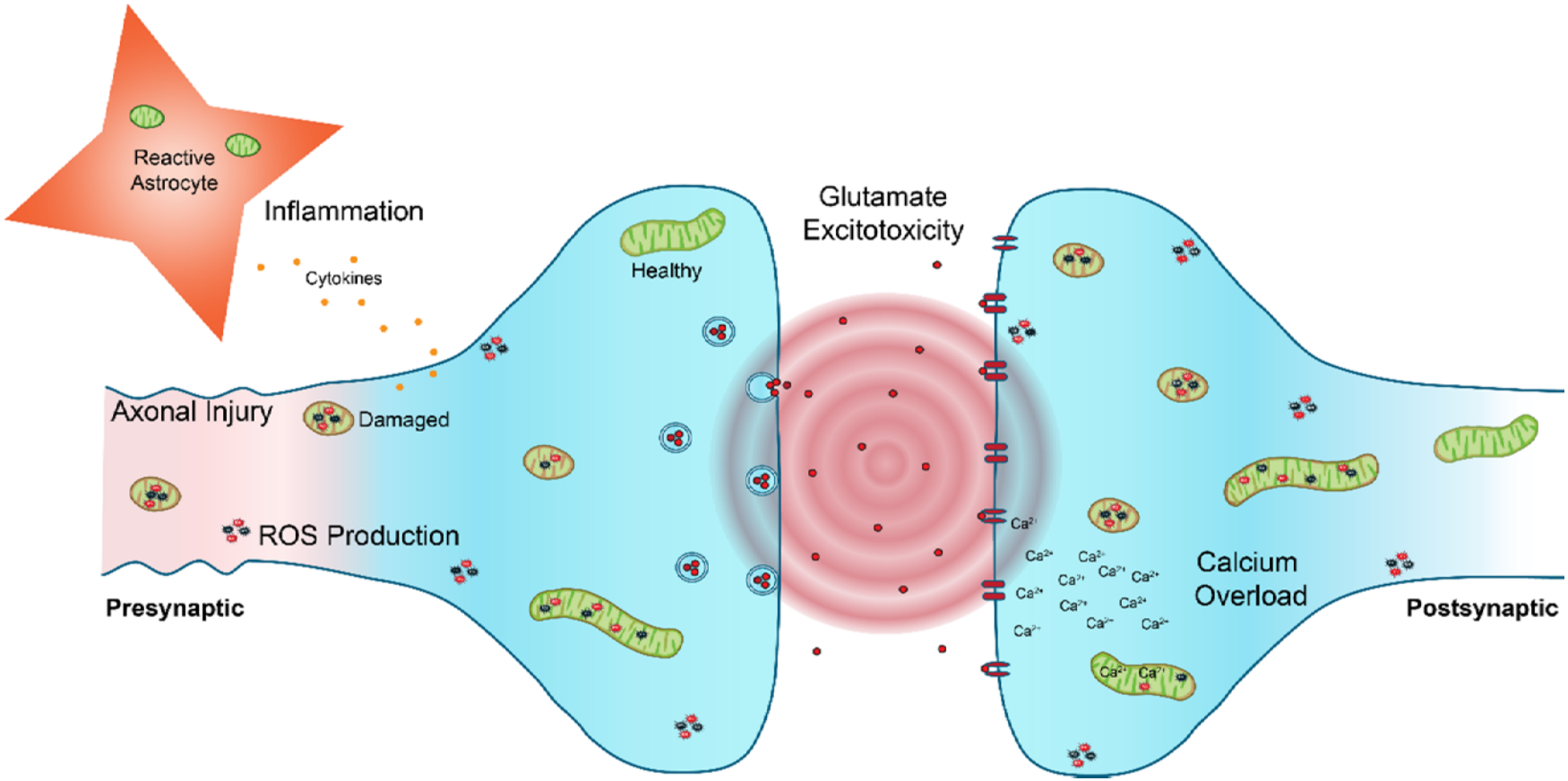

Figure 1.3 Pathophysiology of secondary damage after TBI.

Mitochondrial pathology after TBI. Mitochondrial damage and dysfunction can be associated with many of the physiological and metabolic consequences of secondary brain injury, and is therefore at the center of pathological outcome (**Fig. 1.4**). Both clinical and experimental evidence has demonstrated that acute structural and functional damage of mitochondria in affected brain regions is an early event after TBI, and is thought precede cell death and cognitive deficits.(Cheng et al., 2012; Lifshitz et al., 2004) Injury-induced mitochondrial dysfunction is characterized by decreases in respiratory capacity and ATP synthesis, calcium dysregulation, and oxidative damage.(Hill et al., 2016; Singh et al., 2006; Sullivan et al., 1999; Vink et al., 1990; Watson et al., 2014; Xiong et al., 1997) Several insults converge on mitochondria that contribute to overt dysfunction, including structural damage, reactive oxygen species, and calcium overload, ultimately leading to activation of cell death pathways. The divergence between the increase in need for energy to repair tissue and mitochondrial dysfunction is thought to exacerbate cell death and secondary damage after TBI. Thus, mitochondrial damage following TBI is particularly unfavorable, as loss of mitochondrial function impedes normal cellular processes, and also diminishes the ability for the brain to endure secondary damage after injury. This section is intended to thoroughly review the current available literature for mechanisms contributing to mitochondrial dysfunction after TBI.

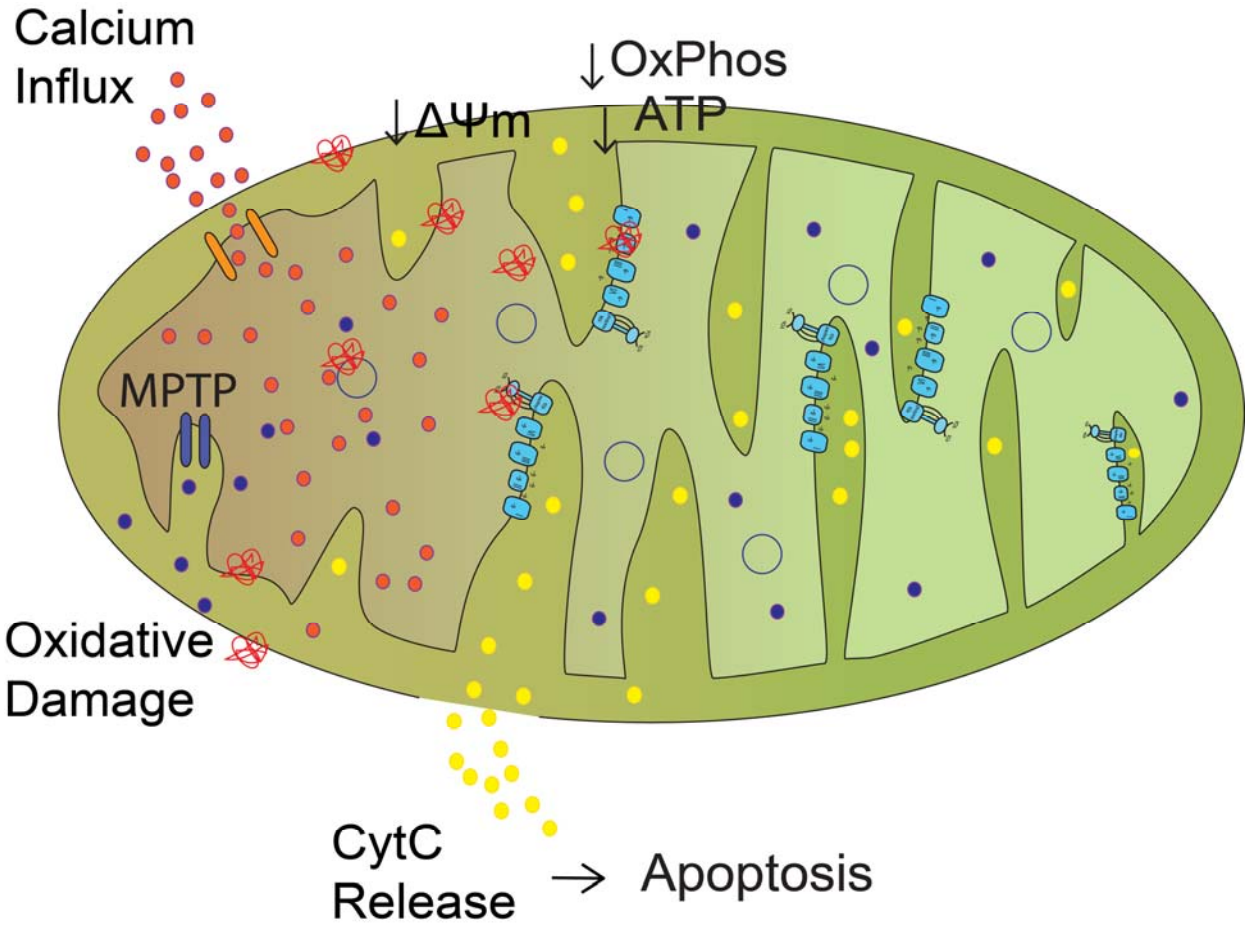

Figure 1.4 Mitochondrial damage after TBI.

Structural Damage. Mitochondrial structural damage can significantly impair the ability for mitochondria to adapt to the metabolic needs of the cellular environment and result in deficits in oxidative phosphorylation.(Pernas and Scorrano, 2015) After TBI, mitochondrial structural damage can occur from mechanical forces in the initial injury, as well as downstream dysregulation from aberrant biochemical cascades during secondary damage.(Lifshitz et al., 2004) Two-dimensional electron microscopy studies have observed severe structural damage to mitochondria after injury, including disruption of cristae organization and mitochondrial swelling in injured brain tissues as well as isolated mitochondria.(Colicos and Dash, 1996; Lifshitz et al., 2003; Okonkwo and Povlishock, 1999) Specifically, mitochondrial swelling is associated with increased mitochondrial calcium accumulation and mitochondrial permeability transition pore activation.(Lifshitz et al., 2003; Okonkwo and Povlishock, 1999; Sullivan et al., 1999) A recent study examining mitochondrial morphology in injured brain tissues from human patients also observed several additional morphological features of mitochondrial structural damage.(Balan et al., 2013) Mitochondria in damaged tissues near the site of injury were characterized, and described as mitochondrial "blebbing", spherical "fission" products, and mitochondrial accumulation in processes of neurons in the injured zones. This study also observed a gradation of mitochondrial structural damage, in which mitochondrial structural damage consistently appeared more severe near the area of impact in injured tissues. In areas immediately surrounding (penumbra), or further from the injury site, structural damage was less severe and normal mitochondria with "active" features, such as dense matrices and cristae, were more apparent. These studies suggest that precise structural features of mitochondria in injured tissues may be a key indicator of metabolic and recovery potential in the affected area after TBI.(Balan et al., 2013) Therefore, it may be possible to distinguish subpopulations of mitochondria that experience irreversible structural damage, from subpopulations that may be repairable after injury. Studies investigating the precise structural features of mitochondria in relation to their functional ability are necessary to determine the severity of damage and potential of recovery for mitochondrial function after TBI.

Oxidative Phosphorylation. As neurons have high metabolic needs and do not store excess energy, mitochondrial oxidative phosphorylation is critical for normal neuronal function and may be a pivotal determinant of cell survival after TBI. In experimental injury, defects in mitochondrial respiratory function have been observed as early as 3 hours, peaking at 72 hours, and persisting as long as 14 days post-injury.(Hill et al., 2016; Singh et al., 2006) This mitochondrial dysfunction is characterized by reduced membrane potential, decreased respiratory rates, and uncoupling of mitochondrial respiration and oxidative phosphorylation.(Lifshitz et al., 2003; Sullivan et al., 1999; Watson et al., 2014; Xiong et al., 1997) Some studies have also observed a biphasic response in mitochondrial dysfunction, with an initial decline and recovery within 24 hours after injury, and a secondary decline that peaks at 72 hours and remains reduced.(Hill et al., 2016; Singh et al., 2006) Interestingly, the same studies both observed a correlated increase in oxidative damage and lipid peroxidation in injured mitochondrial populations with the second decline in respiration. Thus, mitochondrial dysfunction appears to be an ongoing and oscillatory process within the first 72 hours after injury that may correspond with other mitochondrial insults. Defects in mitochondrial respiration are also accompanied by reduced membrane potential and overall decreases of ATP concentrations in whole injured tissues and isolated synaptosomes.(Lifshitz et al., 2003; Sullivan et al., 1999, 1998) Coordination and function of several mitochondrial and cytosolic factors are critical for mitochondrial respiration and oxidative phosphorylation, therefore defects at several rate limiting steps can result in disruptions of oxidative phosphorylation. Rate limiting factors can be at the mitochondrial compartment, such as respiratory complex function, membrane potential, TCA cycle function, substrate availability; or related to metabolic factors in the cytosol, such as glycolysis, or cellular glucose uptake. Several disruptions in both mitochondrial and cytosolic rate-limiting steps have been observed after TBI that can contribute to mitochondrial respiratory dysfunction. Most notably, experimental studies have observed reductions directly at the electron transport chain, as both complex-I driven, and complex-II driven respiration are reduced.(Hill et al., 2016; Singh et al., 2006; Watson et al., 2014) Accordingly, specific defects in the enzymatic

activity of Complex I, as well as Complex IV, have been observed following experimental injury.(Harris et al., 2001; Hovda et al., 1990; Lifshitz et al., 2003; Opii et al., 2007) Thus, initial entry of electrons into the electron transport chain and reduction of molecular oxygen are limited. Availability of matrix metabolites and defects in TCA cycle enzymes can also be rate limiting for oxidative phosphorylation. At the pivotal point between glycolysis and oxidative phosphorylation is Pyruvate Dehydrogenase (PDH) activity and the breakdown of Pyruvate into Acetyl-CoA for entry into the TCA cycle. PDH expression, and the expression of a critical catalytic subunit, PDH E1-∞, have both been observed to be reduced after experimental TBI, suggesting potential defects in PDH activity.(Clark et al., 1999; Sharma et al., 2009; Xing et al., 2009) Impairment in Pyruvate metabolism has recently been supported by a study using hyperpolarized [1-^{13}C] pyruvate magnetic resonance spectroscopic imaging in experimental injury.(Devience et al., 2017) In these studies, hyperpolarized [1-^{13}C] pyruvate was injected in the injured and non-injured hemispheres after injury. ^{13}C-bicarbonate signal was found to be significantly reduced compared to the uninjured hemisphere, along with significant decreases in hyperpolarized bicarbonate-to-lactate ratios. Therefore multiple factors at the mitochondrial compartment, including substrate import, can contribute to the reduction of mitochondrial respiration and oxidative phosphorylation. Defects in mitochondrial enzymatic activities in both the electron transport chain and upstream pathways have also been associated with oxidative damage after injury. A recent proteomic study investigating mitochondrial-specific oxidative damage found that several critical enzymes were found to be oxidized in mitochondria after injury including, complex I and IV, ATP synthase, and pyruvate dehydrogenase.(Opii et al., 2007) Oxidative damage of these complexes would severely limit mitochondrial respiration and ATP synthesis, therefore results from these studies present oxidative damage as a key player in mitochondrial dysfunction after injury.

Cytosolic alterations in cellular glucose handling can also affect downstream mitochondrial oxidative phosphorylation without mitochondrial-specific dysfunction. First, if

components at the mitochondrial level are all functional, alterations in the availability of cytosolic pyruvate can limit entry into the mitochondrial compartment and result in reduced oxidative phosphorylation. Pyruvate availability can be altered by preferential conversion to lactate by lactate dehydrogenase (LDH) or lactate production in the Pentose-Phosphate-Pathway. It is well known that lactate:pyruvate ratios are increased in injured brain regions after both experimental and clinical TBI, and are highly correlated with poor outcome. (Carpenter et al., 2015; Jalloh et al., 2015a) Studies have suggested potential mechanisms driving lactate production can result from increased glycolysis (both aerobic and anaerobic) and the preferential conversion of pyruvate to lactate via LDH due to 1) overburden of mitochondrial metabolism (inability to breakdown nutrient fast enough) or 2) mitochondrial dysfunction, as well as 3) potential shunting of Glucose-6-phosphate from the glycolytic pathway to the Pentose-Phosphate-Pathway (PPP), which could occur in a state of intact mitochondrial function.(Carpenter et al., 2015; Jalloh et al., 2015a, 2015b; Vespa et al., 2005) It is important to note, as lactate has also been debated as a primary nutrient source in the brain, the loss of lactate utilization in combination with one of the above mechanisms has also been suggested as a potential mechanism that could result in the observed increase of lactate.(Dienel, 2014; Glenn et al., 2015) Several clinical studies have provided evidence that supports these hypotheses, however the technical limitations to distinguish the contribution of each potential mechanism to lactate production has been challenging. A recent study was the first to directly compare lactate produced from glycolysis and the PPP using 1,2-$^{13}C_2$ glucose labeling and nuclear magnetic resonance analysis. Results from this study identified glycolysis as the major source of lactate production, with minimal lactate being produced from the PPP.(Jalloh et al., 2015b) Nonetheless, the precise upstream mechanisms that would result in the preferential conversion of pyruvate to lactate and glycolysis and whether overburden of mitochondrial metabolism or mitochondrial dysfunction is involved is currently unknown. Finally, a defect in glucose transport at the plasma membrane can result in the downstream reduction of mitochondrial respiration and oxidative phosphorylation. A study investigating plasma membrane transport in synaptosomal preparations from injured hippocampi

demonstrated reduced glucose uptake at a time corresponding to increases in a marker for lipid peroxidation (TBARS). Thus, synaptic-specific glucose transport can be altered potentially by oxidative damage after injury, and further result in reduced mitochondrial function.(Sullivan et al., 1998) In summary, several mechanisms, both mitochondrial and cytosolic, that are directly involved in oxidative phosphorylation have the potential to be disrupted after injury and contribute to reduced ATP synthesis after injury. The culmination of evidence for disruptions in these critical pathways indicate that it is probable many are occurring after injury and contributing to mitochondrial dysfunction and metabolic dysregulation in the injured brain. It will be necessary to determine the potential specificity of disruptions in these mechanisms at the regional, cellular, and subcellular levels to elucidate the precise contributions to overall metabolic regulation in the brain after injury.

Calcium Signaling. In steady state, extracellular and intracellular calcium levels in the brain are highly regulated to maintain membrane potential for proper neurotransmission.(Berridge, 1998) Intracellular calcium is an important second messenger for many signal transduction pathways; therefore several mechanisms have evolved to maintain calcium homeostasis in the cellular environment for normal cellular function and survival. Specifically, mitochondria, along with the endoplasmic reticulum, are efficient buffers of intracellular calcium and regulate calcium uptake and release through specific calcium transporters and ion-exchangers, respectively.(Rizzuto et al., 2012) TBI causes aberrant glutamate signaling and opening of voltage-gated and ligand-gated channels resulting in the continuous influx of calcium into the synaptic environment.(Guerriero et al., 2015) The rapid increase in intracellular calcium is thought to overwhelm the calcium buffering capacity of mitochondria as an accumulation of calcium in the mitochondrial compartment has been observed after experimental TBI.(Xiong et al., 1997) Increases of intramitochondrial calcium have been directly associated in the reduced respiratory capacity in isolated mitochondrial populations after injury.(Xiong et al., 1997) Additionally, efforts to reduce intracellular and intramitochondrial

calcium load have demonstrated improvements in respiratory capacity, indicating the potential for recovery of mitochondrial function to calcium-induced damage.(Verweij et al., 1997; Xiong et al., 1997) It was later observed that mitochondrial dysfunction in injured brain tissues can be reduced with specific blockers of N-type calcium channels, directly associating mitochondrial dysfunction with calcium influx from excitatory neurotransmission.(Verweij et al., 1997)

The accumulation of calcium in the mitochondrial matrix has also been associated with morphological swelling of mitochondria and Mitochondrial Permeability Transition after experimental TBI.(Buki et al., 1999; Lifshitz et al., 2003; Sullivan et al., 1999) Mitochondrial permeability transition (MPT) is an event in which the inner mitochondrial membrane becomes abruptly permeable to solutes larger than 1,500 kDa.(Bernardi and Di Lisa, 2015) This is accompanied by a structural swelling of the mitochondrial matrix, as well as loss of membrane potential and oxidative phosphorylation, calcium release, disruption of the outer membrane, and release of apoptogenic factors.(Rasola and Bernardi, 2011) Classic patch-clamping studies investigating MPT identified the regulatory role of a high conductance mitochondrial megachannel in the mitochondrial inner membrane (MIM), which has now been accepted as a pore in the MIM, termed the Mitochondrial Permeability Transition Pore (MPTP).(Petronilli et al., 1989) MPTP opens as a result of increases in matrix calcium concentrations that once a certain threshold is reached causes stabilization of the pore in an open conformation and subsequent Mitochondrial Permeability Transition.(Haworth and Hunter, 1979) Although the precise mechanisms underlying MPTP opening remain elusive, Cyclophilin D (CypD) is known to play an essential role in the calcium sensitivity of MPTP.(Basso et al., 2005) Thus, it has been observed that MPTP opening can be controlled by Cyclosporin A (CsA), an immunosuppressive that inhibits CypD activity.(Halestrap et al., 1997) The use of CsA has been essential in investigating the role of MPT in the pathophysiology of TBI. In the late 1990's and early 2000's, several studies demonstrated neuroprotection in cortical areas and axons with different methods of CsA treatment after TBI.(Albensi et al., 2000; Buki et al., 1999; Lifshitz et al., 2003; Okonkwo

and Povlishock, 1999; Okonkwo et al., 1999; Scheff and Sullivan, 1999; Sullivan et al., 1999, 2000) The neuroprotection was accompanied by improved mitochondrial pathologies, including reduced calcium-induced swelling of isolated mitochondria and mitochondria in injured tissues, reduced mitochondrial accumulation of calcium, reduced reactive oxygen species production, and increased mitochondrial membrane potential. Additionally, impairments in hippocampal long-term potentiation and long-term depression were improved, indicating decreased hippocampal damage and preservation of synaptic signaling.(Buki et al., 1999) More recently, improvements in cerebral metabolism in TBI patients after CsA treatment have also been observed.(Mazzeo et al., 2008) Therefore, both experimental and clinical studies indicate calcium-induced MPT as a major factor in mitochondrial damage and dysfunction after injury. The precise mechanisms involved in injury-induced mitochondrial calcium accumulation and MPTP are still unknown and thus require further investigation to determine the specific contribution to mitochondrial dysfunction and develop targeted approaches to reduced mitochondrial calcium after TBI.

Reactive oxygen species. Oxidative damage is significant contributor to secondary damage after traumatic brain injury.(O'Connell and Littleton-Kearney, 2013) Free radicals are highly reactive molecules containing unpaired electrons that promiscuously oxidize nearby substances, such as lipids, proteins, and DNA in an attempt to gain electrons. Free radicals, such as superoxide (O_2^-) and Nitric Oxide (NO•) are natural by-products from several cellular processes, and are important cellular signaling molecules in normal physiology.(Bolisetty and Jaimes, 2013) Mitochondria are a significant source of free radical production both during normal respiration and mitochondrial insult. Complex I and III have been identintified as the primary source of mitochondrial ROS, producing superoxides in the matrix and in the intermembrane space, respectively.(Murphy, 2009) Interactions of superoxides with other molecules produce a family of Reactive Oxygen Species (ROS), including Hydrogen peroxide (H_2O_2) and Hydroxyl radicals (•OH). Similarly, Reactive Nitrogen Species (RNS) can be produced; the most well known being the interaction of Nitric Oxide and Superoxide for the formation of Peroxynitrite

($ONOO^-$). Oxidation by ROS/RNS can cause significant cytotoxic damage, and are a major characteristic of cellular pathology after TBI. Production of Hydroxyl radicals and Peroxynitrite, in particular, are extremely reactive and are responsible for a cytotoxic damage after TBI.(Hall et al., 1993a, 1993b; Hill et al., 2016) Specifically, ROS/RNS production is an extremely acute event after, and has been observed to increase within minutes of experimental injury and persist for at least 90 minutes following experimental TBI.(Hall et al., 1993a, 1993b; Lewén et al., 2001; Marklund et al., 2001; Phillis and O'Regan, 2003) Accordingly, significant amounts of lipid peroxidation, protein oxidation, and nucleic acid oxidation in injured tissues after experimental TBI.(Cornelius et al., 2013; Hill et al., 2016; Shohami and Kohen, 2011; Singh et al., 2006) Sources of ROS/RNS production after TBI have been identified as both cytosolic and mitochondrial and are largely attributed to increases in intracellular calcium.(Hall et al., 2010) For example, activation of cytosolic enzymes by calcium can produce ROS/RNS in the cellular environment, including many phospolipases, nitric oxide synthase, and xanthine dehydrogenase.(Angeloni et al., 2015; Lewen et al., 2000; Mesenge et al., 1996; Tavazzi et al., 2005; Vagnozzi et al., 1999) Furthermore, intramitochondrial calcium accumulation after TBI has been implicated in the over-production of ROS/RNS either from the collapse of membrane potential and increase of electron transport or from the activation of mitochondrial-specific, calcium-activated enzymes, such as mitochondrial nitric oxide synthase.(Hall et al., 2005; Hill et al., 2016; Sullivan et al., 1999) As mitochondria are in close proximity to ROS/RNS, the mitochondrial membranes and several mitochondrial proteins critical for oxidative phosphorylation have been observed to suffer oxidative damage, including the TCA cycle enzymes and respiratory complexes.(Opii et al., 2007; Vagnozzi et al., 1999; Wada et al., 1998, 1999) A recent study has also shown that the time course of oxidative damage after experimental TBI parallels the induction of the second phase of mitochondrial dysfunction after injury with increases in peroxidation, nitrosylation, and protein oxidation at the same time of secondary decline.(Hill et al., 2016; Singh et al., 2006) Furthermore, along with mitochondrial calcium overload, ROS/RNS damage has been associated with mitochondrial structural swelling, and

therefore may either be directly associated with or a result of mitochondrial transition pore opening.(Sullivan et al., 1999) Thus, the production of ROS/RNS is a major source of mitochondrial damage and can result in specific dysfunction in oxidative phosphorylation after injury.

The significance of mitochondrial oxidative damage after injury further reveals itself as several studies have shown the potential for reducing oxidative damage using several different approaches to improve mitochondrial function and neuropathology after injury.(Cornelius et al., 2013; Hall et al., 2010; Kurutas, 2015; Wada et al., 1999) Cells have an antioxidant defense system in place to keep ROS/RNS at a healthy equilibrium for normal processes.(Kurutas, 2015) Cytosolic and mitochondrial Superoxide Dismutase (SOD) and Glutathione Peroxidase are robust scavengers of ROS and maintain normal free radical levels by rapidly reducing free radicals thereby rendering them inactive. A recent study has observed decreases in mitochondrial-specific glutathione levels acutely after injury, indicating a potential disruption in normal mitochondrial ROS maintenance.(Pandya et al., 2014) Administration of a the membrane, permeable form of the glutathione precursor N-Acetylcysteine Amide (NACA) after experimental injury increased mitochondrial glutathione levels and improved respiratory capacity, as well as reduced oxidative damage and tissue loss in injured brain areas. Accordingly, experimental injury studies using a transgenic mouse model expressing increased copper, zinc SOD activity observed improved mitochondrial function, attenuation of tissue damage, decreased blood brain barrier permeability, and improved motor dysfunction after injury.(Mikawa et al., 1996; Xiong et al., 2005) Thus, there is substantial evidence for the role of ROS/RNS production and mitochondrial oxidative damage in the contribution to mitochondrial dysfunction after injury, offering a potential target for therapeutic approaches that improve mitochondrial outcome after injury.

Cell Death. Mitochondria are critical regulators of cell death. At the nexus of the intrinsic pathway of apoptosis, mitochondria house essential upstream apoptogenic factors in the

signaling cascade of apoptotic cell death.(Tait and Green, 2010) Primary apoptogenic factors, such as Cytochrome *c* and Smac/DIABLO, reside within the intermembrane space and cristae compartments.(Martinou and Youle, 2011) Significant mitochondrial damage or the initiation of upstream regulatory pathways due to cellular insult can result in the release of these apoptogenic factors into cytosol and activation of the final stages in the apoptotic-signaling cascade irreversibly committing the cell to apoptosis. The release of Cytochrome *c*, specifically, binds with the cytosolic apoptotic protease-activating factor (APAF1) inducing a conformational change that forms the "apoptosome". The "initiator" Caspase 9 is recruited and activated by the apoptosome, in which it cleaves the "executioner" Caspases 3 and 7. Activation of the executioner caspases results in massive proteolysis that dismantles the cellular environment ultimately causing cell death. The release of Smac/DIABLO on the other hand, inhibits the X-linked inhibitor protein (XIAP) that inactivates its caspase inhibitory functions to allow for apoptosis to proceed. Apoptosis is a significant contributor to cell death after TBI, and has been detected using several methods in both experimental and clinical TBI.(Raghupathi et al., 2000) Characteristic morphological features of apoptosis, including pyknotic nuclei and dystrophic cell bodies, or positive staining for DNA degradation have been observed in neuronal layers of injured brain areas as early as 24 hours post-injury and as late as two weeks later.(Colicos and Dash, 1996; Colicos et al., 1996; Newcomb et al., 1999; Rink et al., 1995) Thus, apoptosis is an ongoing process in the brain after TBI. More specifically, increases in the detection of processed forms of the "initiator" Caspase 9, as well as the "executioner" Caspase 3 have been observed as early as one hour and up to 72 hours after experimental TBI.(Beer et al., 2000; Clark et al., 2000; Keane et al., 2001; Knoblach et al., 2002) Furthermore, pharmacological inhibitors of Caspase 3 administered after experimental TBI have demonstrated improvements in neuropathological outcome.(Clark et al., 2000; Knoblach et al., 2002; Yakovlev et al., 1997) Thus, caspase-dependent activation of the intrinsic cell death pathway is a significant mechanism of cell death after experimental TBI. Detection of cleaved caspases in injured brain tissues and serum in TBI patients has also been correlated with increased rates of mortality after injury, and is therefore a

significant factor in human TBI outcome as well.(Clark et al., 1999; Lorente et al., 2015) Evidence for mitochondrial-specific activation of caspases and the intrinsic cell death pathway has also been demonstrated by the observation of Cytochrome *c* release from mitochondria early as four hours after experimental injury.(Hu et al., 2009; Lewén et al., 2001; Robertson et al., 2007; Sullivan et al., 2002) Lewen et al. (2001), also demonstrated that the mitochondrial release of cytochrome c was partly inhibited after injury in a manganese superoxide dismutase (mnSOD) deficient mouse, indicating that Cytochrome *c* release is associated with oxidative damage.(Lewén et al., 2001) Thus, substantial evidence in both experimental and clinical TBI has demonstrated the contribution of mitochondrial-driven activation of the intrinsic pathway of apoptosis. Two primary events occur at the mitochondrial membranes that can result in the release apoptogenic factors, mitochondrial permeability transition, and mitochondrial outer membrane permeabilization. Calcium-mediated mitochondrial permeability transition has been observed to disrupt the outer mitochondrial membrane and release Cytochrome *c*.(Rasola and Bernardi, 2011) As mitochondrial permeability transition is a major factor in mitochondrial pathology after experimental TBI, it is possible that MPT contributes to apoptosis induction after injury via Cytochrome *c* release, however there is no direct experimental evidence connecting apoptosis and MPT currently in the literature. On the other hand, Mitochondrial Outer Membrane Permeabilization (MOMP) is mediated by the BCL-2 family of pro- and anti-apoptotic proteins that are upstream in the intrinsic pathway of apoptosis.(Martinou and Youle, 2011) Few experimental studies have observed acute alterations in protein and mRNA expression of BCL-2 family members in injured brain tissues, however these studies report widely inconsistent findings and should be interpreted with caution.(Clark et al., 1997; Raghupathi et al., 2008; Strauss et al., 2004) Similar to the role of MPT in apoptosis, there is no experimental evidence directly connecting BCL-2 mediated Cytochrome *c* release and apoptotic cell death after experimental injury. Therefore, although activation of critical caspases and mitochondrial Cytochrome *c* release have been observed after injury, the direct connection between these two

events, as well as upstream initiating mechanisms require further investigation to determine the contribution of mitochondrial regulation of cell death pathways in neuropathology after TBI.

Conclusions. The potential for mitochondrial damage due to both the initial injury and in secondary damage after TBI is overwhelming. Structural damage, dysregulated calcium signaling, and oxidative damage all converge on mitochondrial populations causing substantial dysfunction in respiratory capacity and oxidative phosphorylation. The resulting loss of mitochondrial function perpetuates cellular dysfunction and death, and impedes the ability for the brain to recover after brain injury. Evidence of the various insults on mitochondria after TBI suggests that mitochondrial-associated pathologies are heterogeneous and may be specific to different brain regions, cell-types, and subcellular locations. Directed investigation into the precise mechanisms involved in the injury-induced insults that cause and contribute to mitochondrial damage and dysfunction are necessary to understand the pathophysiology of TBI. Elucidation of these mechanisms and their contribution to overall neuropathology will be critical in developing targeted therapeutic approaches to improve mitochondrial function.

1.3 Mitochondria are Dynamic Organelles

Only in the past 20 years has it been accepted that mitochondria, are not just "bean-shaped" organelles, but are actually morphologically dynamic and interconnected organellar network formed throughout the cell. To ensure quality control of the network and maintain functional homeostasis, mitochondria are constantly fusing together and dividing apart (fusion and fission), moving throughout the cell (transport), selectively removing damage from the network (mitophagy), generating new mitochondrial contents (biogenesis), and interacting with other organelles.(Chen and Chan, 2009; Labbé et al., 2014) These dynamic behaviors act in concert, creating a morphologically plastic and functionally adaptive mitochondrial compartment to efficiently meet the metabolic needs of the cell. Morphology of the mitochondrial network is governed by delicately balanced processes of fusion and fission.(Pernas and Scorrano, 2015) These processes are integral to the dynamic nature of the mitochondrial network and serve two critical functions: 1) as a quality control mechanism to maintain functional homeostasis within the network, and 2) to allow flexibility of the mitochondrial compartment for functional adaptations to metabolic changes in the cellular environment.(Mishra and Chan, 2016) To achieve these goals, mechanisms of mitochondrial fusion and fission intimately linked with the machinery of other dynamic mitochondrial behaviors, such as mitophagy and transport. To maintain quality and health of the network, fusion promotes the distribution of mitochondrial DNA (mtDNA) and mixing of matrix contents throughout the mitochondrial compartment to counteract the accumulation of damage and preserve function.(Westermann, 2012; Youle and van der Bliek, 2012) Mitochondrial fission, on the other hand, offers quality control by segregating and removing damaged portions of the mitochondrial network to facilitate degradation through mitophagy.(Twig et al., 2008a) By maintaining health of the mitochondrial network, mitochondrial fusion and fission thus ensure functional homeostasis within the network.

The balance of fusion and fission also allows flexibility of the mitochondrial compartment to efficiently adapt mitochondrial function to metabolic changes in the cellular

environment.(Mishra and Chan, 2016) Thus, mitochondrial morphology is also intimately linked to the metabolic status of the cell and a key indicator of dynamic functional adaptations of the mitochondrial network. For example, mitochondrial networks in cells exhibiting high metabolic activity typically demonstrate elongated, “hyperfused” mitochondrial networks with increased mitochondrial respiratory rates and oxidative phosphorylation.(Westermann, 2012) On the other hand, cells in nutrient excess, or during cellular dysfunction typically demonstrate a fragmented mitochondrial network with low respiratory activity. Additionally, fission controls mitochondrial distribution throughout the cell by allowing mitochondria to be divided from the network and transported to local areas with high metabolic needs. At steady state in most cells, fusion and fission counterbalance each other, with equal contributions to morphological regulation of the mitochondrial network and reciprocal cellular metabolic adaptations.

The delicate balance between mitochondrial fission and fusion is crucial for mitochondrial health and function, and is therefore essential for cellular survival. The disruption of either mitochondrial fission or fusion tips the balance, resulting in over-activity of the other, and impeding the ability of dynamic responses of the mitochondrial network.(Detmer and Chan, 2007b) Growing evidence in the literature has suggested that disruptions in the balance of fusion and fission, whether unopposed fission or unopposed fusion, cause metabolic dysregulation and contribute to cell death and disease progression. In this chapter, I will discuss the molecular machineries of mitochondrial fission and fusion, how each process regulates cellular function, how the cell regulates each dynamic behavior, as well as the consequences of disruptions in the balance of mitochondrial fission and fusion.

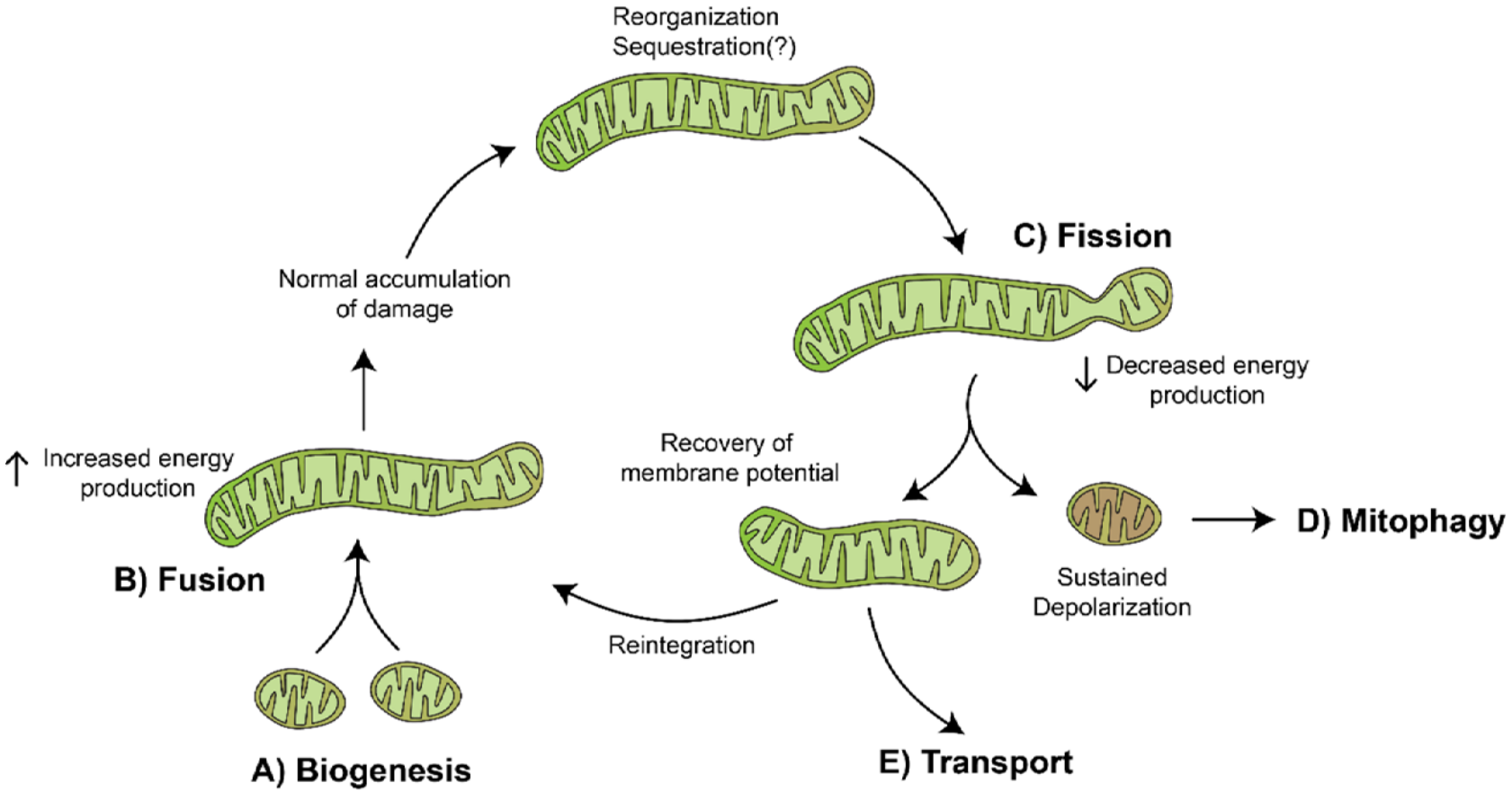

Figure 1.5 Dynamic behaviors of the mitochondrial network.

Mechanisms of mitochondrial fusion. Mitochondrial fusion is regulated by three conserved large dynamin-family GTPases, the Mitofusins (Mfn1 and Mfn2) and Optic Atrophy 1 (Opa1).(van der Bliek et al., 2013) Recently, another component of mitochondrial outer membrane fusion has been discovered, Mitochondrial phospholipase D (mitoPLD), and has been implicated in modulation of phospholipids during the membrane fusion process; however, further investigation is required to fully elucidate the precise role of mitoPLD in relation to the other fusion proteins for mitochondrial membrane fusion.(Wai and Langer, 2016) Fusion of the mitochondrial membranes is generally thought of as a two-step process, with Mitofusins mediating outer membrane fusion, and Opa1 subsequently mediating inner membrane fusion (**Figure 1.6**). As the fusion GTPases are mechanistically distinct, recent work has however elucidated the possibility for differential control of these mechanisms to mediate inner and outer membrane actions separately from the canonical mitochondrial fusion process. Although precise structural and functional coordination is required between these mechanisms to mediate successful mitochondrial fusion, the molecular and temporal events underlying such coordination are currently unresolved. Therefore, the potential functional regulation of the fusion proteins is far reaching, and is still currently being elucidated.

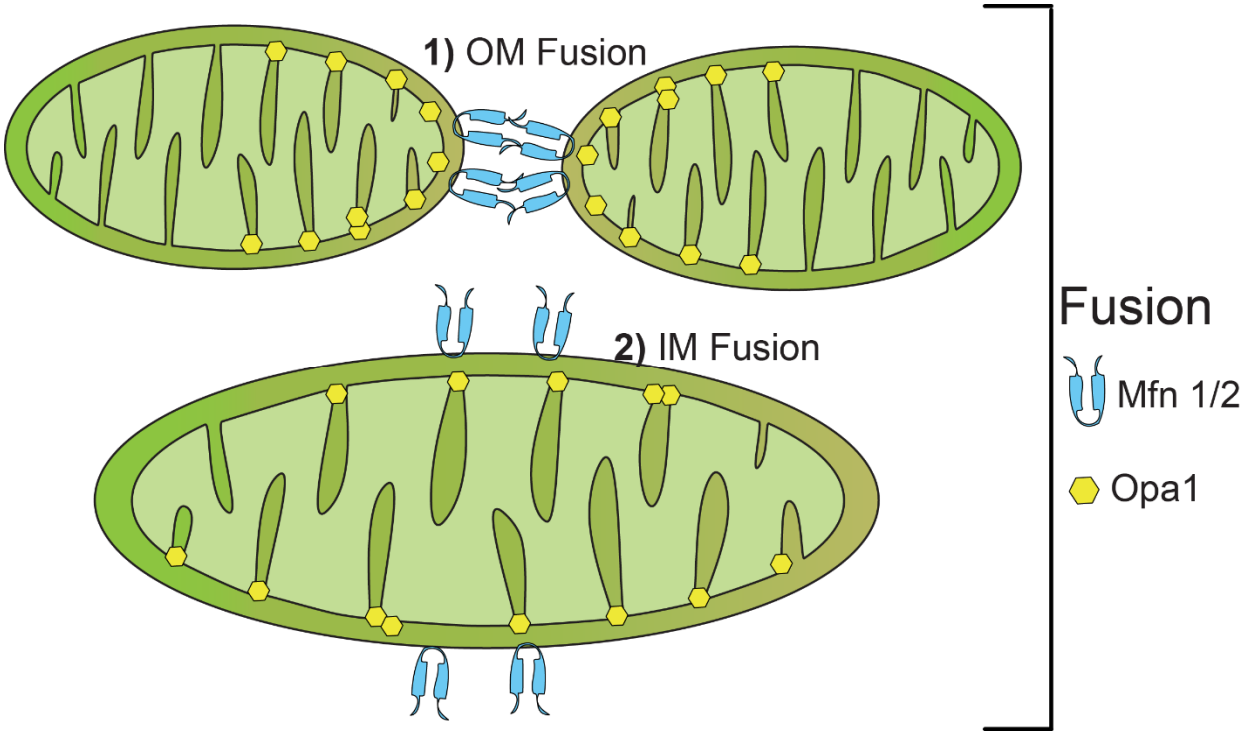

Figure 1.6 Mechanisms of mitochondrial fusion

Mitofusins. Mitofusins are integral outer membrane proteins that are anchored by a C-terminal transmembrane domain in the MOM.(Schrepfer and Scorrano, 2016; Westermann, 2008) The mitofusins share 80% structural similarity, and both contain essential amino-terminal GTPase domains and two coiled-coil domains that are exposed to the cytosol.(Santel et al., 2003) Current models propose that the Mitofusins are maintained in an “active”, extended conformation that is structurally permissive to tethering and efficient outer membrane fusion.(Franco et al., 2016) Although the precise mechanisms to which the Mitofusins mediate outer membrane fusion are still relatively unclear, it has been observed that Mfn1 and Mfn2 form both homo- and hetero-oligomeric complexes on neighboring mitochondria by tethering their coiled-coil domains in *trans* to drive membrane fusion.(Koshiba et al., 2004) The hetero-oligomeric complexes appear to be particularly critical to ensure complementation between the two to mediate fusion.(Detmer and Chan, 2007a)

Expression levels of both Mfn1 and Mfn2 vary in tissue and cell-type specificity.(Santel et al., 2003) Accordingly, mutations of Mfn1 or Mfn2 *in vivo* exhibit tissue and cell-type specific deficits, however complete deletion of either is embryonically lethal.(Chen et al., 2003a) Conditional deletion of Mfn1/2 results in fragmentation of the mitochondrial network, mitochondrial dysfunction, accumulation of mtDNA mutations, as well as mtDNA depletion, thus supporting the critical role of mitochondrial fusion for tolerance and distribution of mtDNA damage. (Chen et al., 2010) Although Mfn1 and Mfn2 appear to be somewhat functionally redundant, each exhibit key differences indicating specialization of function.(Chen et al., 2003a; Detmer and Chan, 2007a) For example, Mfn1 has been observed to be more efficient for MOM fusion, demonstrating higher GTPase activity than Mfn2 and resulting in greater fusion of the mitochondrial network when overexpressed.(Ishihara et al., 2004) Additionally, coordination of outer membrane fusion with Opa1 inner membrane fusion appears to be more dependent on Mfn1 function, rather than Mfn2, as well. Cipolat et *al.* (2004) demonstrated that coordination between Opa1 and Mfn1 is indispensable for mitochondrial fusion and membrane fusion is

preserved without Mfn2. Interestingly, Mfn2 appears to be more functionally diverse, and is involved in many processes in addition to MOM fusion. For example, the presence of a proline-rich, protein binding region on Mfn2, indicates that it may be regulated through protein-protein interactions.(Schrepfer and Scorrano, 2016) In fact, Mfn2 has been implicated in Bcl-2 family-mediated apoptosis by interacting with Bax/Bak on the outer membrane to mediate pore formation and the release of Cytochrome *c,* potentially to inhibit fusion and promote fission.(Hoppins et al., 2011; Karbowski et al., 2002) Mfn2 has also been found to be involved in mitochondrial transport through direct interactions with the molecular motor complex, Miro1/Milton, to tether mitochondria to microtubules.(Misko et al., 2012) Furthermore, Mfn2 has been found to be involved in endoplasmic reticulum (ER) mitochondrial associated membranes (MAMs) to mediate ER-mitochondrial interactions and calcium regulation.(de Brito and Scorrano, 2008) Most notably, mutations of Mfn2 cause Charcot-Marie-Tooth type 2A (CMT2A) disease, which results in specific degeneration of motor axons, peripheral neuropathy, and muscular atrophy.(Züchner et al., 2004) The specific degeneration of motor axons, supports the cell-type specificity and function of Mitofusins. Interestingly, Mfn1 mutations modeling CMT2A in vitro reveal that Mfn2 mutations can be complemented with wild-type Mfn1 to rescue fusion through heterotypic Mfn1-Mfn2 interactions, where as wild-type Mfn2 homotypic interactions do not rescue fusion in these mutations.(Detmer and Chan, 2007a) These results reveal the functional importance of heterotypic interactions between Mfn1 and Mfn2, as well as the potential consequences of differences in expression between the two in certain tissues or cell types that may be vulnerable to defects in MOM fusion. Most importantly, the elucidation of Mfn1 complementation in Mfn2 mutations in CMT2A provide a potential target to restore mitochondrial fusion, reduce pathology and improve disease outcomes in CMT2A.

The Mitofusins and MOM fusion have also been found to be regulated through post-translational modifications, therefore MOM fusion can be mediated by signaling in the cellular environment. Both Mfn1 and Mfn2 are substrates of Parkin ubiquitination, and have been found

to be degraded through the proteasome in cytotoxic conditions, presumably to inhibit fusion of damaged mitochondria back into the network.(Cunningham et al., 2015; Escobar-Henriques and Langer, 2014) However, stabilizing Mitofusins and antagonizing Parkin ubiquitination preserves fusion and has demonstrated benefits for cell viability during cellular stress.(Cunningham et al., 2015) Therefore, inappropriate regulation by the cellular environment may cause dysregulation of mitochondrial fusion by altering the activity of the Mitofusins. A few phosphorylation sites have also been identified on both Mfn1 and Mfn2 that can either promote or inhibit MOM fusion with different cellular stimuli, further supporting the potential for environmental regulation to mediate fusion events.(Leboucher et al., 2012)

Optic Atrophy 1 (Opa1). Opa1 is located at the mitochondrial inner membrane (MIM) and is anchored by an N-terminal transmembrane domain.(MacVicar and Langer, 2016; Westermann, 2008) The majority of the structure, including the GTP binding and GTPase effector domains, face the inter membrane space. At steady state, Opa1 exists in two isoforms, the long form, L-Opa1 and, the short form S-Opa1. The soluble S-Opa1 is generated by proteolytic cleavage at sites near the transmembrane domain of L-Opa1, and thus resides in the inter membrane space partly associated with the MIM.(MacVicar and Langer, 2016) The balance between the concentrations of each isoform is critically important for the dynamic regulation of the balance of fusion and fission and recent research has demonstrated potential differential regulation of L-Opa1 and S-Opa1 that drives either mitochondrial fusion or fission.(Anand et al., 2014; Rainbolt et al., 2016; Wai et al., 2015) Evidence from these studies indicates that L-Opa1 may be the primary mediator of fusion, while S-Opa1 and accumulation within the inter membrane space is important for environmental regulation and mediation of fission during changes in the cellular metabolic status.

Opa1 is ubiquitously expressed in all tissues, however multiple forms generated from alternative splicing at the transcriptional level are found to be tissue and cell-type specific.(Akepati et al., 2008; MacVicar and Langer, 2016; Wai and Langer, 2016) Similarly to

Mitofusins, homozygous knockouts are embryonically lethal, and heterozygous knockouts or conditional mutations of Opa1 exhibit tissue-specific pathologies. Mitochondrial phenotypes of Opa1 mutation or knockout are characterized by fragmentation of the mitochondrial network, mtDNA mutation and depletion, and defects in oxidative phosphorylation.(Alavi et al., 2006; Chen et al., 2012) Most notably, Opa1 mutations are the underlying cause of Autosomal Dominant Optic Atrophy, a progressive neuropathy exhibiting specific degeneration of the optic nerve and vision loss.(Delettre et al., 2000) Mitochondrial pathologies from mutations of Opa1 are due to both the lack of mitochondrial fusion, as well as defects in other critical mitochondrial processes regulated by Opa1, such as cristae formation and remodeling.(Frezza et al., 2006; Olichon et al., 2003) Specifically, Opa1 has been observed to be critical in the formation and stabilization of cristae junction through oligomeric interactions between L-Opa1 and S-Opa1.(Anand et al., 2014; Cogliati et al., 2013; Mishra et al., 2014) Cristae junctions allow the formation of the cristae compartment by sequestering the necessary components in the inter membrane space for efficient oxidative phosphorylation, therefore Opa1 is indispensable for mitochondrial function.(Frezza et al., 2006) Accordingly, Opa1-deficient mitochondria contain disorganized ultrastructure and cannot maintain oxidative phosphorylation.(Frezza et al., 2006) As respiratory chain complexes are integral to the MIM, the formation of cristae compartments is also critical for respiratory chain complex assembly.(Cogliati et al., 2013) Thus, a later study found that Opa1 is also critical for respiratory chain complex assembly, as detection of respiratory super complexes was reduced in a conditional knockdown of Opa1, and increased during Opa1 overexpression. However, whether this is direct mediation by Opa1, or secondary to the role Opa1 plays in cristae formation and shape has not been resolved. Interestingly, studies investigating Opa1 overexpression in steady state have demonstrated variability in Opa1 regulation of mitochondrial morphologies, suggesting differential responses due to the metabolic requirements of mitochondrial dynamics for specific cell types. Studies investigating cell types, such as neurons, COS cells, and HeLa cells, undergo mitochondrial fragmentation upon Opa1 expression, whereas different lines of fibroblasts exhibit the expected tubulation of mitochondrial

network upon Opa1 overexpression.(Cipolat et al., 2004; Griparic et al., 2004; Li et al., 2004; Misaka et al., 2002; Olichon et al., 2002) These studies not only demonstrate the importance of cell-type specificity, but also reflect the metabolic implications of Opa1 expression, reflecting the significance in the processing required for adaptability of the inner membrane and efficient functional responses to the environment. Furthermore, in situations of cellular stress, overexpression of Opa1 has been observed to be protective by restoring mitochondrial morphology, inhibiting Cytochrome *c* release, and improving mitochondrial function during cellular insult *in vitro* and more recently in mouse models deficient in respiratory function.(Civiletto et al., 2015; Frezza et al., 2006; Jahani-Asl et al., 2011) These studies underscore the balance between fusion and fission, and the potential for improving cellular health and survival by countering the dysregulated process and restoring the balance between fusion and fission.

As Opa1 maintains closure of the cristae compartment, remodeling of cristae during apoptosis and the facilitation of Cytochrome *c* release has been also been directly associated with Opa1 regulation of cristae junction sites.(Arnoult et al., 2005a; Frezza et al., 2006; Scorrano et al., 2002; Varanita et al., 2015) Cytochrome *c* release from the mitochondrial compartment during cellular insult is the critical step in activation of downstream effectors that the cell to the intrinsic pathway of apoptosis.(Martinou and Youle, 2011) 85% of Cytochrome *c* is found sequestered in cristae compartments that are tightly formed by Opa1 and other complexes at the cristae junction. Therefore opening of cristae junctions would be required for the complete release of Cytochrome c from the mitochondrial compartment to initiate apoptosis.(Scorrano et al., 2002) Additionally, it has been observed that Opa1 is co-released with cytochrome c release during apoptosis, effectively promoting mitochondrial fission by inhibiting fusion.(Arnoult et al., 2005a) Thus, the incredible cellular regulation of the balance between fusion and fission is further elucidated in the response of the mitochondrial network to cell death. Evidence from these studies and studies investigating the role of Drp1-mediated mitochondrial fission during apoptosis

suggest that coordination of fission mechanisms with Opa1-dependent fusion mechanisms may be required for the complete release of Cytochrome c to initiate apoptosis. However, the complementation of mechanisms of mitochondrial fusion and fission requires further investigation to fully elucidate the precise mechanisms and temporal relationship between these events in the facilitation of Cytochrome *c* release and apoptosis by mitochondrial dynamic processes.

Opa1 function and MIM fusion can also be regulated by post-translational modification, particularly through proteolytic processing by inner membrane peptidases. In fact, the proteolytic processing of Opa1 has been an exciting area of research in the mitochondrial dynamics field due to implications of cellular metabolic control of mitochondrial function.(Cogliati et al., 2016; Mishra and Chan, 2016) Cleavage of L-OPA1 is known to occur at primary two sites (S1 and S2) located above the transmembrane domain.(Ishihara et al., 2006; MacVicar and Langer, 2016) Each site is differentially processed by two metalloproteases resident to the inner membrane that contain exposed catalytic sites to the inter membrane space. Site S1 is conditionally regulated by the *m*-AAA protease OMA1, and site S2 is constitutively regulated by the *i*-AAA ATPase, YME1L.(Anand et al., 2014; Käser et al., 2003; Song et al., 2007; Wai and Langer, 2016) Both of these peptidases are activated by different stimuli associated with changes in the cellular metabolic environment.(Anand et al., 2014; Rainbolt et al., 2016; Wai et al., 2015) OMA1 is conditionally activated in response to depolarization of the MIM, in which it completely cleaves L-Opa1, resulting in an accumulation of S-Opa1 in the intermembrane space.(Anand et al., 2014; Wai et al., 2015) Accordingly, the complete processing of every L-Opa1 into S-Opa1 ablates the ability for MIM fusion, and therefore induces unopposed fission and aids in the segregation of damage mitochondria from the network. Therefore, the stress-induced processing of Opa1 by OMA1 plays a key role in mediating quality control of the mitochondrial network by shifting the balance toward fission in response to mitochondrial dysfunction or cellular insult. Interestingly, in humans, most of L-Opa1 contain site S1, however only ~50% contain S2; therefore, only OMA1 activity can result in complete processing of L-Opa1 in the mitochondrial network, supporting the

role of OMA1 in maintenance of mitochondrial health during damage.(MacVicar and Langer, 2016; Song et al., 2007) Activation of YME1L, on the other hand, is driven by oxidative phosphorylation and stimulates inner membrane fusion during instances of high metabolic demand, such as cellular starvation.(Anand et al., 2014; Mishra et al., 2014; Rainbolt et al., 2016) Therefore, cleavage of L-Opa1 at S2 by YME1L couples mitochondrial inner membrane fusion to cellular metabolism and is critical for functional adaptation of the mitochondrial network to meet the metabolic demands of the cell. Additionally, further mechanistic regulation has been elucidated between these two proteolytic events, as each is reciprocally degraded in response to cellular changes to facilitate the activity of the other.(Rainbolt et al., 2016) Thus, the precise mechanistic regulation of Opa1 is integral to the balance of fusion and fusion and the dynamic functional adaptability of the mitochondrial compartment.

Mechanisms of mitochondrial fission. Mitochondrial fission is regulated by the cytosolic GTPase, Dynamin-related Protein 1 (Drp1), and several receptors located on the mitochondrial outer membrane (MOM).(Westermann, 2008) Drp1 is a mechanoenzyme that is structurally similar to Dynamins, containing an N-terminal GTP-binding domain, a middle domain, and a C-terminal GTPase effector domain.(Smirnova et al., 1998, 2001) However, Drp1 lacks the proline-rich domain and the pleckstrin homology domain necessary for lipid binding, instead Drp1 contains a variable domain (also known as insert B) that is not well characterized.(Strack and Cribbs, 2012) Although the specific mechanisms of mammalian Drp1 machinery remain elusive, insights have been made based on the crystal structure and assembly of Dynamins and the yeast homologue DNM1.(Fröhlich et al., 2013) Models based on the structural assembly of Dynamins predict Drp1 to exist in a T-shaped dimer, with the middle and the GTP effector domains forming the stalk and the GTP binding domain forming the head.(van der Bliek et al., 2013; Francy et al., 2015; Fröhlich et al., 2013; Westermann, 2008) Drp1-mediated mitochondrial fission occurs in three critical steps: 1) recruitment and translocation, 2) self-assembly, and 3) membrane constriction and division (**Figure 1.7**). Fission is initiated by translocation of Drp1 from

the cytosol to the mitochondrial outer membrane via recruitment by membrane-bound receptors.(van der Bliek et al., 2013) Of note, other mechanisms have been elucidated to be involved in Drp1 recruitment to the MOM, including endoplasmic reticulum (ER) mitochondrial associated membrane (MAM) contact sites.(Rowland and Voeltz, 2012) However, these mechanisms are not well characterized, and it is unknown whether receptor-mediated recruitment is also a contributing factor. Once located at the MOM, GTP-bound Drp1 oligomers assemble forming a spiral structure that circumscribes the mitochondrion. The spiral structure is thought occur by the formation of stable dimers between the stalks of Drp1. The interaction of the GTP effector domains at the stalks then mediate reciprocal hydrolysis of GTP at neighboring stalks to drive a conformational change that constricts the spiral structure in a “corkscrew” motion and provides the forces necessary for membrane division.(Fröhlich et al., 2013; Ingerman et al., 2005; Mears et al., 2011) Recently, there has been question in the literature whether Drp1 spirals alone have the dynamic range to complete membrane scission of all four membranes, which has yielded investigation into other possible mechanisms that would aid in constriction.(van der Bliek et al., 2013; Lee and Voeltz, 2015) Most notably, the observation of Drp1 foci at ER MAM contact sites suggests that the initial constriction by the ER might aid in both the recruitment of Drp1 and subsequent division by allowing appropriate membrane curvature amenable for fission.(Rowland and Voeltz, 2012) However, the causal relationship of ER MAM constriction to Drp1-mediated fission has not yet been elucidated, as fission can occur with disruption of ER MAM contacts.(Rowland and Voeltz, 2012) Additionally, recent evidence has shown that Dynamin 2 (Dyn2) is critical for mammalian mitochondrial fission, in which Dyn2 is transiently recruited to Drp1 foci and assembles around the mitochondrion for secondary constriction and completion of membrane scission.(Lee and Voeltz, 2015) The molecular machinery required for Drp1-mediated mitochondrial membrane division is thus incredibly complex and requires further characterization to elucidate the precise coordination of events that mediate complete outer and inner membrane division.

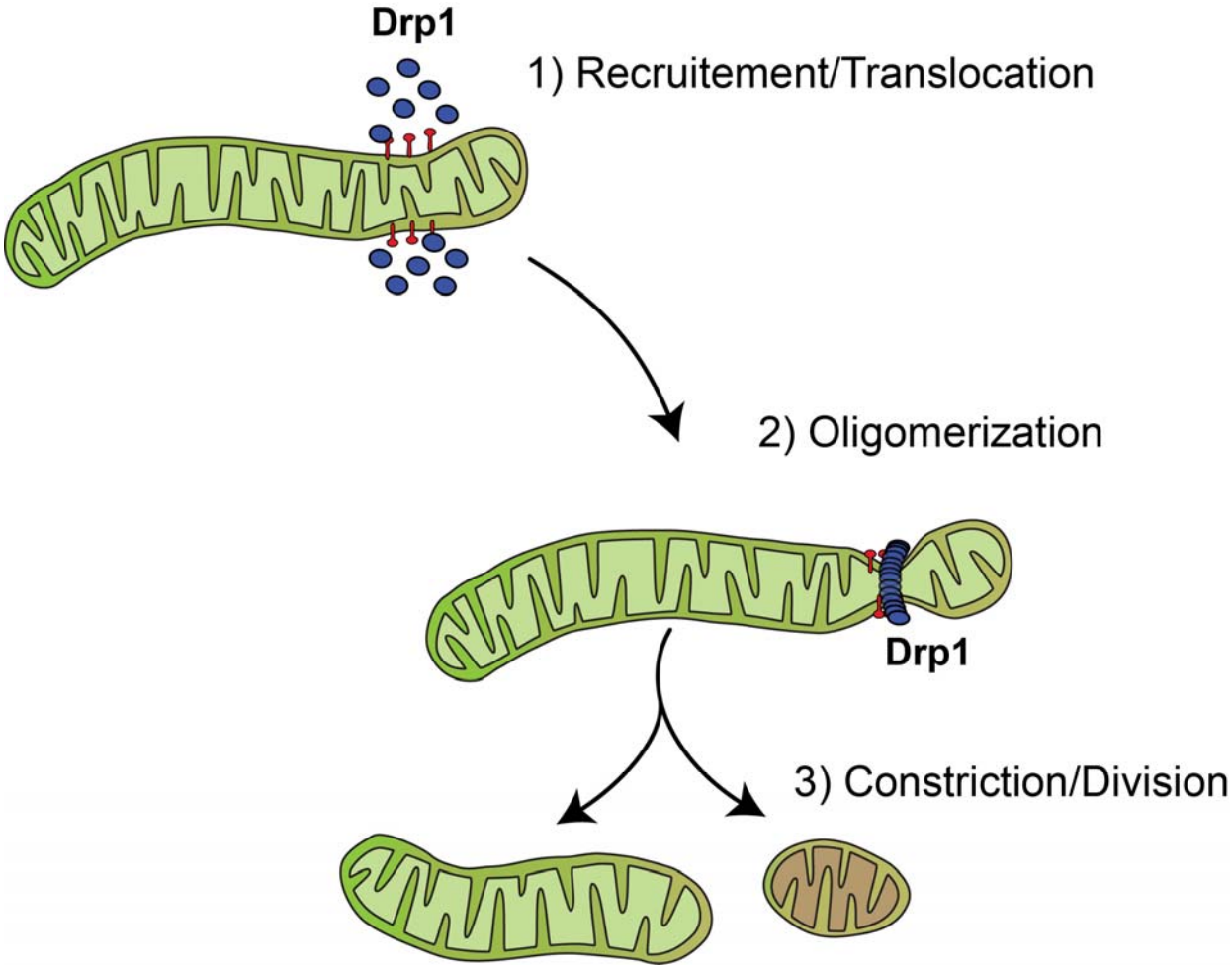

Figure 1.7 Mechanisms of mitochondrial fission.

Drp1-receptor mediated interactions. Drp1 primarily resides in the cytosol and therefore requires precise regulation of recruitment and translocation to the mitochondrial outer membrane (MOM) to mediate fission events. The mechanisms driving Drp1 recruitment and translocation to the MOM have been an area of intense investigation, and very precise recruitment by specific Drp1 receptors has been recently elucidated.(Smirnova et al., 1998, 2001) Translocation and recruitment of Drp1 from the cytosol is thought to occur via recruitment by functionally distinct receptors anchored to the MOM. Four receptors have been identified, Fis1, MFF, MiD49, MiD51 that all appear to play specific roles in mitochondrial fission by selective recruitment of Drp1.(Losón et al., 2013; Otera and Mihara, 2011) Mitochondrial fission protein 1 (Fis1) was the first identified and thought to be the primary receptor for Drp1, due to the role of its homologue in yeast.(Yoon et al., 2003) However, mammalian Fis1 does not contain the adaptor proteins that are necessary for mitochondrial fission in yeast (Mdv1 and Caf4), and there have been inconsistent reports in Fis1 knockdown and overexpression studies that question whether Fis1 has a significant role in Drp1-mediated fission events.(Lee et al., 2004; Otera and Mihara, 2011; Otera et al., 2013) Mitochondrial Fission Factor (MFF) appears to be the primary receptor candidate involved in Drp1 recruitment and fission. Knockdown studies of MFF have consistently demonstrated mitochondrial elongation and decreased Drp1 mitochondrial localization, whereas overexpression consistently increases Drp1 translocation and mitochondrial fission.(Otera et al., 2010) On the other hand, the role of the Mitochondrial Dynamics proteins of 49 and 51 (MiD49/51) has been less clear due to contradictory results in overexpression and knockdown studies.(Palmer et al., 2011; Zhao et al., 2011) Specifically, multiple groups have demonstrated that knockdown of MiD49 and MiD51, both together and individually, result in reduced mitochondrial recruitment of Drp1 and mitochondrial elongation.(Liu and Chan, 2015; Losón et al., 2013; Palmer et al., 2011; Zhao et al., 2011) Expectedly, overexpression studies from the same reports increase Drp1 recruitment; however, unexpectedly, mitochondrial elongation is observed, indicating reduced mitochondrial fission. Only recently have potential mechanisms been elucidated in two elegant studies, elucidating the

perplexing regulation of Drp1-mediated fission through MiD49/51 receptors.(Liu and Chan, 2015; Losón et al., 2013) Loson et *al.* (2013) first observed that although overexpression of MiD49/51 increased Drp1 recruitment to the mitochondrial outer membrane, inhibitory phosphorylation of Drp1 at serine site 637 was also increased, therefore promoting mitochondrial fusion. The removal of phosphorylation via site-directed mutagenesis allowed for normal mitochondrial fission to proceed, indicating its direct role in the mitochondrial elongation observed with MiD49/51 overexpression. These results reveal the potential for differential regulation of Drp1-mediated fission via MiD49/51 depending on the modification of Drp1. Intriguingly, these studies suggest potential fission receptor regulation of the physiological state of the cell as phosphorylation at Ser637 is mediated by Protein Kinase A (PKA), a critical kinase in many cellular metabolic pathways. A later study by the same group further elucidated precise mechanisms involved by identifying that each of the known receptors for Drp1 exhibits preferential recruitment and affinity depending on the conformational state of Drp1.(Liu and Chan, 2015) In the cytosol, Drp1 can be found in many conformational states, including dimer, tetramers, and higher-order assemblies.(Zhu et al., 2004) Lui et al. (2015) observed that MiD49/51 receptors preferentially bind dimeric, inactive forms of Drp1, unlike the other primary receptor, MFF, which only has a high affinity for active, tetramer and higher-order assemblies of Drp1. Taken together, the proposed model is that while MFF preferentially binds a subpopulation of fission-competent Drp1, MiD49/51 binds inactive forms of Drp1 that require an additional stimulus to initiate oligomerization and fission events. This stimulus may be dependent on modifications of Drp1 and the physiological state of the cell. Thus, Drp1 recruitment and translocation from the cytosol is complex process that is only beginning to be precisely resolved.

Drp1 regulation of cellular metabolism. Drp1 and mitochondrial fission are critical to cellular metabolism, and thus display a repertoire of diverse functions, including mitochondrial quality control and distribution, apoptosis, cell cycle regulation, and peroxisomal division.(Otera et al., 2013) Similar to the fusion proteins, homozygous knockouts of Drp1 are embryonically

lethal, and expression levels vary in different tissues and cell types.(Ishihara et al., 2009; Wakabayashi et al., 2009) Accordingly, the cell-type specificity of Drp1 expression appears to be of particular importance, as cellular consequences to loss of Drp1 are widely varied. The cell type specificity of Drp1 and mitochondrial fission has been well-characterized *in vitro* with mutation and overexpression studies, but has more recently revealed itself *in vivo* in homozygous knockout animals. (Ishihara et al., 2009; Wakabayashi et al., 2009) For example, lethality of Drp1 KO mice has been attributed to defects in placental and cardiomyocyte development, cells with high Drp1 expression; however cells with lower Drp1 expression such as, mouse embryonic fibroblasts (MEFs) appear to be largely unaffected.(Ishihara et al., 2009) Drp1 KO MEFs exhibit some features of Drp1 loss, such as elongation of mitochondria, but otherwise have normal ATP levels, respiratory activity, and mtDNA distribution.(Ishihara et al., 2009; Wakabayashi et al., 2009) Thus, some phenotypic features are demonstrated in Drp1 KO MEFs, but not all, supporting variability in cellular responses even within a cell-type. As mitochondrial morphology is intimately linked to cellular metabolism, the cell-type specificity of Drp1 expression and cellular responses to Drp1 ablation is likely reflective of metabolic characteristics and requirements of the cell type. Nonetheless, in affected cell-types, disruption of Drp1 function generally results in similar phenotypes of the mitochondrial network. Studies using knockout, knockdown, or mutation of Drp1 *in vivo* and *in vitro* have demonstrated mitochondrial elongation and increased network interconnectivity, increased mitochondrial oxidative damage, mtDNA nucleoid clustering and depletion, as well as defects in oxidative phosphorylation.(Ishihara et al., 2009; Parone et al., 2008; Sesaki et al., 2014) Thus, loss of Drp1 and unopposed fusion cause severe mitochondrial damage and dysfunction. Accordingly, due to the integral role of Drp1-mediated fission in the mechanistic interaction and function of other mitochondrial dynamic behaviors, such as mitophagy and transport, these mechanisms are also disrupted with Drp1 ablation. Specifically, to ensure quality control of the network, Drp1-mediated fission segregates and removes damaged portions of the mitochondrial network to be selectively degraded through mitophagy.(Twig et al., 2008a) Twig et al. found that at basal state, 85% of fission events resulted

in “asymmetrical” division, in which one daughter mitochondria is depolarized, and one is hyperpolarized.(Twig et al., 2008b) The hyperpolarized mitochondrion remains fusion-competent, however the depolarized daughter exhibits reduced Opa1 and is later selectively degraded by autophagosome engulfment. These studies demonstrate the importance and reciprocal nature of the balance between fusion and fission, as fusion proteins are disrupted in the damaged portions thereby further ensuring their segregation from the network. Mutation of Drp1 results in decreased degradation of mitochondria, accumulation of damage in the mitochondrial network without increased reactive oxygen species production, and reduced respiratory capacity within cells.(Frank et al., 2012; Parone et al., 2008; Twig et al., 2008b) Additionally, overexpression of Drp1 appears to facilitate mitophagic degradation of damaged mitochondria during significant cellular stress and damage.(Arnoult et al., 2005b; Twig et al., 2008b) Accordingly, there is also evidence of functional interplay between Drp1 and the Pink1-Parkin pathway to mediate coordinated detection of mitochondrial damage, segregation, and ubiquitination of damaged mitochondria for subsequent degradation. However, opposing results in studies from drosophila, cell culture, and mammalian models have proven difficult to resolve the precise mechanisms involved in the coordination between fission and mitophagy.(Shirihai et al., 2015) Nonetheless, Drp1-mediated fission is essential for removal of mitochondrial damage incurred during normal activity, sparing the mitochondrial network from further dysfunction.(Frank et al., 2012; Kageyama et al., 2012, 2014; Twig et al., 2008b) Additionally, as a mediator of mitochondrial distribution throughout the cell, Drp1-mediated fission is also critical for mitochondrial transport.(Chen and Chan, 2009; Saxton and Hollenbeck, 2012) This is particularly important for neurons, as mitochondria are required to be transported long-distances down axons to provide metabolic assistance in synapses.(Fukumitsu et al., 2016; Li et al., 2004; Verstreken et al., 2005) Accordingly, Drp1 knockdown in neurons results in aggregation of mitochondria in the soma and reduced mitochondrial presence in dendrites and axons.(Li et al., 2004; Verstreken et al., 2005) This results in severe defects in synaptic growth and activity demonstrated *in vivo* and *in vitro*, indicating the importance for mitochondrial distribution in neuronal development and

synaptic transmission. Results from these studies reveal a critical role for Drp1 in synaptic development and plasticity, and highlight the diverse functions of Drp1-mediated fission in cell types with high metabolic requirements. Lastly, it is important to also note that in the diverse set of cellular regulatory functions of Drp1, it has also been been found to mediate peroxisomal division, as well; however, the role of Drp1-mediated fission events in peroxisomal activity and function has not been well characterized.(Koch et al., 2003)

Fragmentation of the mitochondrial network is a canonical characteristic of apoptosis.(Martinou and Youle, 2011) Frank et *al.* (2001), found that Drp1 and mitochondrial fission play a critical role in apoptosis by controlling the release of Cytochrome *c* from the mitochondrial compartment, revealing Drp1-mediated fission at the crux of cell death by controlling a necessary step required for final commitment to apoptosis.(Frank et al., 2001) Since these groundbreaking studies, later findings from the same group and others, have further elucidated precise interaction between the Bcl2 family members, and several fission/fusion factors, including Drp1, Mfn2, and Opa1, that coordinate to control Cytochrome *c* release and apoptosis.(Arnoult et al., 2005a, 2005b; Estaquier and Arnoult, 2007; Karbowski et al., 2002, 2004; Parone et al., 2006; Scorrano et al., 2002) During significant cellular stress or damage, Bax, a pro-apoptotic Bcl-2 family member, translocates to the mitochondrial outer membrane where it coalesces into foci with Bak, a Bcl-2 family member resident to the MOM.(Martinou and Youle, 2011) These Bax/Bak foci are also known to co-localize with Mfn2 and potentially promote fission by inhibiting fusion.(Karbowski et al., 2002) Bax/Bak foci form a pore that permeabilizes the membrane (MOMP) allowing for release apoptogenic factors, such as cytochrome c and SMAC/Diablo, into the cytosol. Further activation of caspases by Cytochrome *c* and disinhibition of caspases by Smac/DIABLO commits the cell to proteolytic processing and death. Drp1 was found to be recruited to the MOM by post-translational modification in a Bax/Bak dependent matter and assembles at sites of Bax/Bak foci on the MOM.(Wasiak et al., 2007) Subsequent Drp1-mediated mitochondrial fission then divides the membrane at the Bax/Bak complex and

either prior to, or concomitantly with, allows for Cytochrome *c* to be released. Accordingly, mutants of Drp1 block mitochondrial fragmentation, cytochrome c release, and caspase activation, therefore demonstrated a direct role for Drp1 and the control of apoptosis.(Estaquier and Arnoult, 2007; Parone et al., 2006)(Frank et al., 2001; Karbowski et al., 2002; Szabadkai et al., 2004) Later studies have, however, questioned the role of Drp1 in apoptosis, as further observations demonstrated that downregulation of Drp1 does not completely block apoptosis.(Estaquier and Arnoult, 2007; Parone et al., 2006) These studies revealed that inhibition of Drp1 selectively blocks the release of Cytochrome *c*, but does not block the release of Smac/DIABLO and other apoptogenic factors.(Estaquier and Arnoult, 2007; Parone et al., 2006) Drp1-mediated fission therefore appears to facilitate apoptosis by controlling Cytochrome c release, but is not required for apoptosis. These perplexing observations have led to a model that incorporates the fusion proteins, in which Drp1-mediated fission and Opa1-mediated cristae remodeling coordinate to completely release Cytochrome *c* from the cristae compartment with subsequent release from the outer membrane.(Cipolat et al., 2004; Yamaguchi et al., 2008) Further investigation is required to resolve the precise mechanistic role of Drp1 and mitochondrial fission, as well as the temporal relationship of these events, in the control of the intrinsic pathway of apoptosis.

The culmination of evidence gathered since the mammalian isoform was discovered 20 years ago supporting the diverse cellular responses to Drp1 function and mitochondrial fission ago is insurmountable. Therefore, as Drp1-mediated mitochondrial fission is intimately linked to many critical processes, proper balance and regulation of Drp1 is essential to maintain mitochondrial health and metabolic homeostasis for cellular function and survival.

Metabolic regulation of Drp1. As Drp1 is primarily cytosolic (97%), its activity is sensitive to modifications by environmental signaling to communicate changes in the metabolic status of the cell and regulate the rate of mitochondrial fission.(Chang and Blackstone, 2010; Smirnova et al., 1998, 2001) Communication from the cellular environment is essential for proper dynamic

responses of the mitochondrial network, thus several post-translational modifications (PTMs) are known to mediate the critical steps in Drp1-mediated fission and control the balance of fusion and fission in response to environmental signaling.(Cereghetti et al., 2008; Chang and Blackstone, 2007a, 2010; Cho et al., 2009; Harder et al., 2004; Karbowski et al., 2007; Santel and Frank, 2008) Phosphorylation of Drp1 is the most well-characterized PTM, and has been of particular interest due to its role in the regulation of mitochondrial fission in response to changes in the metabolic status of the cell. Additionally, SUMOylation, ubiquitination, and s-Nitrosylation have all been identified to modify the activity of Drp1 and regulate mitochondrial fission. These modifications are less well-characterized, but have been associated with dysregulation of Drp1 and mitochondrial fission in neurodegenerative diseases.

Two serine sites in the GTPase effector domain (GED) of Drp1, Serine 616 and Serine 637 (656 in rats), have been identified as primary sites of phosphorylation.(Chang and Blackstone, 2010) As both sites reside in the GED, it is proposed that phosphorylation at these sites alter intramolecular interactions to modulate Drp1 activity, however the precise structural changes have not been elucidated. Ser616 is phosphorylated by CDK1/Cyclin b and is primarily important for mitochondrial fission and the transfer of mitochondria to daughter cells during mitosis.(Taguchi et al., 2007) The potential for post-mitotic regulation at this site has not been elucidated. Rather the most well-characterized site Ser637 due to its regulation of mitochondrial adaptation to changes in cellular metabolic status. Both phosphorylation and dephosphorylation mechanisms have been identified in the regulation of Drp1 at Ser637 and mitochondrial fission.(Cereghetti et al., 2008; Chang and Blackstone, 2007a; Cribbs and Strack, 2007) Phosphorylation by cyclic-AMP (cAMP)-dependent protein kinase A (PKA) inhibits Drp1 GTPase activity by blocking intra-molecular interactions necessary for GTP hydrolysis, thereby reducing mitochondrial fission and permitting mitochondrial elongation due to unopposed fusion.(Chang and Blackstone, 2007a; Cribbs and Strack, 2007) PKA is known to be targeted to the MOM via a complex with the A Kinase Anchorin Protein 1 (AKAP), and therefore offers direct access to

phosphorylation of Drp1 to regulate fission.(Dickey and Strack, 2011) During cellular starvation, activation of PKA and increased phosphorylation of Drp1 ser637 inhibits fission and promotes mitochondrial “hyperfusion”.(Gomes et al., 2011) The resulting elongation is accompanied by increases in cristae formation, ATP synthase activity and oxidative phosphorylation, thereby providing direct evidence for metabolically linked regulation of mitochondrial dynamics and function. Additionally, the starvation-induced mitochondrial elongation mediated by phosphorylation of Drp1 at ser637 blocks starvation-induced autophagy, sparing mitochondria from degradation, and improving cell viability. Therefore, phosphorylation of this site is a critical pro-survival signal that increases mitochondrial function to maintain cellular homeostasis during mild nutrient stress. Conversely, dephosphorylation by the calcium/calmodulin serine-threonine phosphatase, Calcineurin (CaN; also known as protein phosphatase-2B or PP2B), increases mitochondrial fission by specifically enhancing Drp1 translocation to the MOM.(Cereghetti et al., 2008; Cribbs and Strack, 2007) Although phosphorylation at 637 in basal conditions is relatively low, dephosphorylation results in fragmentation of the mitochondrial network, indicating potential subpopulations of Drp1 that remain in a phosphorylated state.(Cereghetti et al., 2008; Cribbs and Strack, 2007) As calcineurin is mediated by intracellular calcium levels, studies have also demonstrated direct association of dephosphorylation to sustained increases in intracellular calcium, which can be blocked by Cyclosporin A (CsA), a pharmacological inhibitor of calcineurin. Thus, intracellular calcium regulation directly modulates Drp1-mediated mitochondrial fission. Accordingly, dephosphorylation at Ser637 by calcineurin sensitizes cells to apoptotic stimuli, indicating that calcineurin may regulate Drp1-mediated apoptosis as well. Interestingly, phosphorylation/dephosphorylation at this site has also been directly implicated in the neuronal development by inhibiting and promoting fission in association with alterations in intracellular calcium levels during dendritic growth and synaptic plasticity.(Dickey and Strack, 2011) Thus, environmental regulation of Drp1 via phosphorylation is essential in several cellular mechanisms, as well as the adaptive mitochondrial responses to changes in the metabolic needs of the cell.

Recently it has also been observed that MFF, the primary receptor of fission-competent Drp1, can also undergo post-translational modification to modulate fission. Phosphorylation of MFF by AMPK, a key energy sensing kinase, has been demonstrated to promote fission, and potentially mediate fission in states of energy stress. Thus, it appears that environmental modification can target several molecular machineries to mediate mitochondrial fission in response to changes in cellular metabolism. Given the diverse functions of Drp1 and its critical role in several cellular processes, it is not surprising that Drp1 and mitochondrial fission are sophisticatedly regulated by cellular signaling pathways.

Drp1 in neuronal development and plasticity. The differences in dynamic mitochondrial features across different tissues and cell types support variability in metabolic characteristics and requirements of a cell. Neurons, in particular, are high energy consuming cells, and display very morphologically heterogeneous and active mitochondrial populations that are diversely distributed throughout soma, axons, dendrites, and synapses. Thus, the balance of mitochondrial fission and fusion has demonstrated critical importance in several neuronal processes including neuronal and synaptic development, synaptic ATP supply, neuorotransmission, and neuroplasticity.(Bertholet et al., 2016) These observations have lead intense investigations into the contribution of mitochondrial fission and fusion processes in brain development and function. Although significant discoveries have been made revealing the role of Drp1 and mitochondrial fission in neuronal function and development *in vitro*, this section will focus on the most recent insights that have been revealed *in vivo* using Drp1 knockout mouse models.(Dickey and Strack, 2011; Li et al., 2004; Verstreken et al., 2005)

Recent advances in knockout models of Drp1 have been essential in elucidating the fundamental role of Drp1 and mitochondrial fission in synaptic development and neurotransmission. The brain exhibits high Drp1 expression, but the cell-type specificity further extends to neuronal populations, as variability in responses to Drp1 ablation in these models have been observed.(Smirnova et al., 1998) For example, knockout of Drp1 specifically in the

cerebellum and midbrain regions (EN1-Cre) die within 36 hours with specific defects in mitochondrial morphology and proliferation of purkinje cells; however, granule cells in this model exhibit normal mitochondrial morphology.(Wakabayashi et al., 2009) Accordingly, granule cells have demonstrated lower levels of Drp1 expression, which likely accounts for this cell-type specificity.(Lein et al., 2007) Whether the lack of dependence of Drp1 and mitochondrial fission for normal cellular activity in granule cells reflects the metabolic requirements or dependence on mitochondrial function is unknown. Another study using a Nes-Cre Drp1 KO, affecting several brain regions, showed specific deficits in forebrain development.(Ishihara et al., 2009) Neurons in the forebrain of Nes-Cre Drp1 KO mice exhibited defects in synaptic growth, aggregation of mitochondria and reduced distribution in processes, calcium sensitivity, and increased apoptosis. Results from both of these studies, revealed Drp1-mediated mitochondrial fission to be essential for development, although Drp1 expression intriguingly appears to be critical for some neuronal cell-types, and not others. Furthermore, studies investigating a heterozygous Drp1 KO mice observed normal growth and neural development, normal synaptic and dendritic markers, and normal levels of mitochondrial function and ATP levels in mitochondria from cortical and cerebellar tissues.(Manczak et al., 2012) These studies suggest that normal brain and neuronal development can persist even with less available Drp1; however, Drp1 protein expression in examined brain tissues was only partially reduced and not statistically significant, therefore interpretations should be met with caution. Nonetheless, at least partial reduction of Drp1 in the brain does not yield in observable development differences, indicating sufficiency of function providing Drp1 is present. Studies using developmental knockout models of Drp1 have been paramount to our knowledge of Drp1-mediated mitochondrial fission in vivo, and the critical role in mitochondrial distribution, quality control, and development in the brain. However, due to the lethality of Drp1 during development investigating the post-mitotic functions of Drp1 and the potential role in neurodegeneration has been limiting. Only in the last few years have inducible Drp1 knockout models been developed to observe the functional consequences of Drp1 in the mature brain. A study using a post-mitotic KO model of Drp1 in purkinje neurons (L7-Cre)

revealed several interesting insights, that underscore the importance of Drp1-mediated fission in quality control of the mitochondrial network and neuronal survival.(Kageyama et al., 2012) Firstly, progressive cerebellar degeneration was observed with 90% loss of purkinje neurons by 6 months post-KO. Prior to major degeneration, mitochondria were found to be swollen, and had increased oxidative damage, exhibited deficits in respiratory complex activity, and were decorated with ubiquitin and mitophagy markers. Administration of two antioxidants, N-*acetylcysteine* and MitoQ, in vitro at the time of infection inhibited mitochondrial swelling and preserved morphology. Additionally, antioxidant treatment of the L7-Cre Drp1 KO animals with a high CoQ10 diet restored mitochondrial morphology in purkinje neurons and reduced degeneration. Thus, loss of Drp1 and mitochondrial fission disrupt the ability for mitochondria to segregate damage from the network, specifically from reactive oxygen species, and results in dysfunction and degeneration. These studies provide critical evidence for the potential for antioxidants to mitigate damage and dysfunction due to imbalances of mitochondrial fusion and fission. Furthermore, two studies using a floxed Drp1 knockout mouse model ($Drp1^{lox/lox}$) crossed with an inducible Cre under the control of the CaMKIIa neuronal promoter (Drp1cKO), observed similar mitochondrial morphological features in cortical and hippocampal neurons, however cell-type specificity of Drp1 function was further identified.(Oettinghaus et al., 2016; Shields et al., 2015) Development of the phenotype differed between the two cell types, as cortical neurons demonstrated mitochondrial fragmentation and aggregation at three days post-ablation, and this phenotype was not observed in hippocampal neurons until 10 days to 4 weeks later. However, only one study observed reduced mitochondrial presence in CA1 hippocampal synapses, indicating the potential for normal transport and distribution without Drp1. This could potentially be due to differences in technique (ultrathin tissue section vs immunofluorescence) or in the particular brain region CA1 synapses were examined (not specified vs entorhinal cortex). Mitochondrial dysfunction and reduced ATP content was also observed in the hippocampus of Drp1cKO mice, and was interestingly accompanied by increased expression of glycolytic proteins. Thus, loss of mitochondrial ATP production due to Drp1 ablation may be compensated

by increased glycolysis. These results reveal incredible cellular metabolic regulation and the compensatory mechanisms in neurons between glycolysis and mitochondrial oxidative phosphorylation to maintain homeostasis and health. Furthermore, significant neuronal death or degeneration was not observed in the hippocampus for at least one year after ablation, although hippocampi were atrophied and dendrites were significantly shorter. Autophagy markers and reactive oxygen species were also not significantly increased, in contrast to observations in the purkinje neuron-specific knockout mouse.(Kageyama et al., 2012) Most notably, both studies observed deficits in synaptic transmission in CA1 pyramidal neurons of Drp1cKO mice, however only in circumstances in which synaptic activity was challenged by frequent stimulation. Therefore, Drp1 is dispensable for normal neurotransmission, but essential for increases in synaptic signaling, and therefore potentially synaptic plasticity. These functional deficits in the hippocampus were further supported by cognitive deficits in Drp1cKO mice, elucidated in behavioral assessments specific for hippocampal-dependent function. Studies using Drp1 conditional knockout models have yielded comprehensive insight into the fundamental role of Drp1 in brain development and function, in addition to revealing mechanisms of mitochondrial dynamics in the metabolic regulation of synaptic plasticity.

Conclusions. In summary, the dynamic processes of mitochondrial fission and fusion are critical for mitochondrial health and function, and reciprocally regulate cellular metabolism for efficient adaptation of mitochondrial function to meet the needs of the cell. Thus, these dynamic behaviors of the mitochondrial compartment are essential for several cellular processes, including proliferation and development, metabolic regulation, autophagy, and apoptosis. The underlying mechanisms and the environmental regulation of mitochondrial fission and fusion are incredibly complex, and only beginning to be precisely elucidated. Nonetheless, the balance of these processes is perhaps the most important characteristic, as the inactivation of one, causes over-activity of the other, and most certainly results in metabolic dysregulation and cellular dysfunction. As neurons have high temporal and spatial metabolic demands, this balance is

particularly critical for neuronal function and survival. Disruption in the balance of mitochondrial fission and fusion has thus been an area of intense investigation, especially in the pathogenesis and progression of neurodegenerative diseases.

1.4 Hypothesis

Traumatic Brain Injury (TBI) causes major biochemical, physiological, and metabolic changes in the normal cellular environment of the brain. The inappropriate activation and propagation of critical cellular pathways can lead to cascading secondary damage that results in further downstream dysregulation. Mitochondrial dysfunction and loss of ATP synthesis is at the center of secondary damage, creating a vicious cycle that perpetuates damage after injury. Mitochondrial health and function is regulated by tightly balance processes of fission and fusion. These processes allow for efficient functional adaptation to changes in the cellular environment and are therefore critical for metabolic homeostasis and cell survival. Dysregulation of mitochondrial fission and fusion can disrupt the balance between these two processes, causing mitochondrial dysfunction, metabolic dysregulation, and cell death. The pathological characteristics of imbalances in mitochondrial fission and fusion, in which there is an imbalance towards excessive fission are remarkably similar to mitochondrial damage and metabolic dysfunction observed after TBI. These studies investigate whether dysregulation of mitochondrial fission is a pathological feature of TBI, and whether inhibiting Drp1, the primary protein regulator of mitochondrial fission, improves neuropathological outcome after injury. Therefore, my working **hypothesis** is that *TBI causes dysregulation of Drp1 and increases mitochondrial fission in the hippocampus, and inhibiting Drp1 will reduce mitochondrial dysfunction, reduce neuronal damage, and improve cognitive function after injury* (**Fig. 1.8**).

These studies provide an innovative perspective on mechanisms of mitochondrial dysfunction after TBI by 1) employing a combination of biochemical techniques and electron microscopy to investigate specific changes in mitochondria relating to fission after injury, and 2) evaluating whether inhibiting mechanisms of mitochondrial fission reduces mitochondrial dysfunction, neuropathology, and deficits in learning and memory after injury. Additionally, by investigating mitochondrial fission after TBI, these studies may lead to novel mitochondrial-targeted therapeutic approaches that improve outcome and quality of life after brain injury.

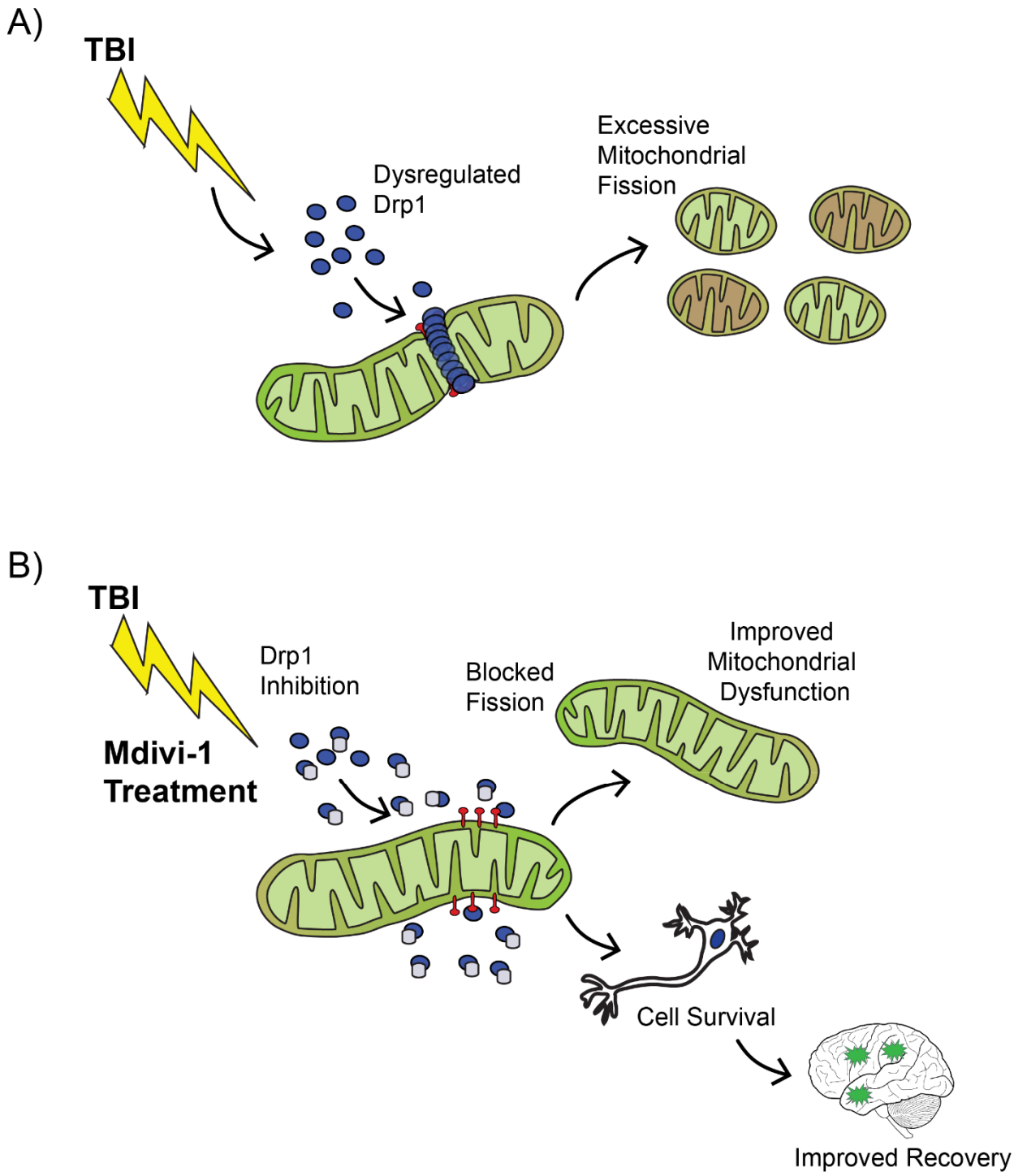

Figure 1.8 Hypothesis model

Chapter 2. Methodology

2.1 Controlled Cortical Impact (CCI) Injury

All surgeries described in these studies were completed by Dr. Jing Zhao and procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Adult, male Sprague Dawley rats (300-400 g) were purchased from Charles River Laboratories (Wilmington, MA). An electromagnetic controlled cortical impact (CCI) device was used for experimental TBI as previously described.(Edward Dixon et al., 1991) Animals were anesthetized using 5% isofluorane with a 1:1 O_2/N_2O mixture and then mounted on a stereotaxic frame with anesthesia maintained with 2.5% isofluorane in 1:1 O_2/air. Bilateral 6-mm craniectomies were produced midway between the bregma and lambda (offset 0.5 mm from midline) and a single impact (2.5 mm deformation) was given at a velocity of 5 meters/sec to the right parietal cortex. Sham-operated animals received all surgical procedures described above excluding the craniectomies and impact. Recovery of pain reflexes and restoration of the righting response were recorded immediately after surgery to ascertain consistency in the injury. For mitochondrial isolation experiments, animals underwent all described procedures, except the TBI was delivered using an Impact One™ Leica Biosystems CCI device with a 2.8 mm deformation.

2.2 Drug Preparation and Administration

Mitochondrial Division Inhibitor, Mdivi-1, was purchased from Tocris Bioscience (Bristol, UK). Mdivi-1 was dissolved in DMSO to a concentration of 25 mg/ml, after which it was diluted to a working concentration of 1.5 mg/ml. For all drug studies, injured animals were randomly assigned to either vehicle (50% DMSO) or 3 mg/kg Mdivi-1 groups. This dosage of Mdivi-1 was based on previous studies that demonstrated neuroprotection in an ischaemic injury model with Mdivi-1 treatment.(Grohm et al., 2012) For behavioral testing, injured animals were randomly assigned to two groups and injected (i.p.) at 30 minutes, 24 hours, and 48 hours post-injury with Mdivi-1 or an equivalent volume of vehicle. For immunohistochemistry studies, animals received

treatment at 30 minutes post-injury and again at 8 hours post-injury. For biochemistry studies, animals were treated at 30 minutes, 24 hours, and 48 hours post-injury, and received an additional a dosage two hours prior to dissection.

2.3 Isolation of Mitochondria from Rat Hippocampi and Cortices

To isolate mitochondria from brain tissues, Percoll density gradient centrifugation procedures were employed as previously described.(Sims and Anderson, 2008) One brain structure (hippocampus or cortex) from two different animals per experimental group were pooled together (equivalent to one sample) to increase the quantity of starting material. Hippocampi and cortices from sham and injured (ipsilateral to the injury) animals were rapidly dissected and homogenized in a 5% tissue wet wt/vol in ice-cold homogenization buffer (320 mM Sucrose, 30 mM Tris pH 7.4, 3 mM EDTA, 12% Percoll solution, 1 mM Sodium Fluoride, 1 mM Sodium Molybdate, 100 nM Okadaic Acid, 1 mM PMSF and 10 µg/ml leupeptin) with a Dounce homogenizer using four strokes with the loose pestle A followed by eight strokes with the tight pestle B. A small fraction (100 µl) of each homogenate was removed for determination of protein content. The remaining homogenate was then layered onto a discontinuous Percoll gradient (26% and 40% Percoll) and centrifuged for 10 minutes (30,700*g* at 4°C). The enriched mitochondrial fraction was removed from the 26/40% interface, transferred to individual centrifuge tubes, and diluted (1:4) with isolation buffer (320 mM Sucrose, 30 mM Tris pH 7.4, 3 mM EDTA). Fractions were then pelletized by centrifugation (16,700*g* at 4°C) for 10 minutes. The supernatant was discarded and the samples were either immediately prepared for further analysis or stored at -80°C. Mitochondrial isolation from brain tissue resulted in ~5x enrichment of mitochondrial specific proteins (**Fig. 2.2**). Additionally, percoll density centrifugation resulted in a pure mitochondrial fraction (free of other organelles) with 94% of all structure positively labeled for TOMM20, a mitochondrial outer membrane marker, detected using immunogold labeling and electron microscopy analysis (**Section 2.5**; **Fig. 2.6**).

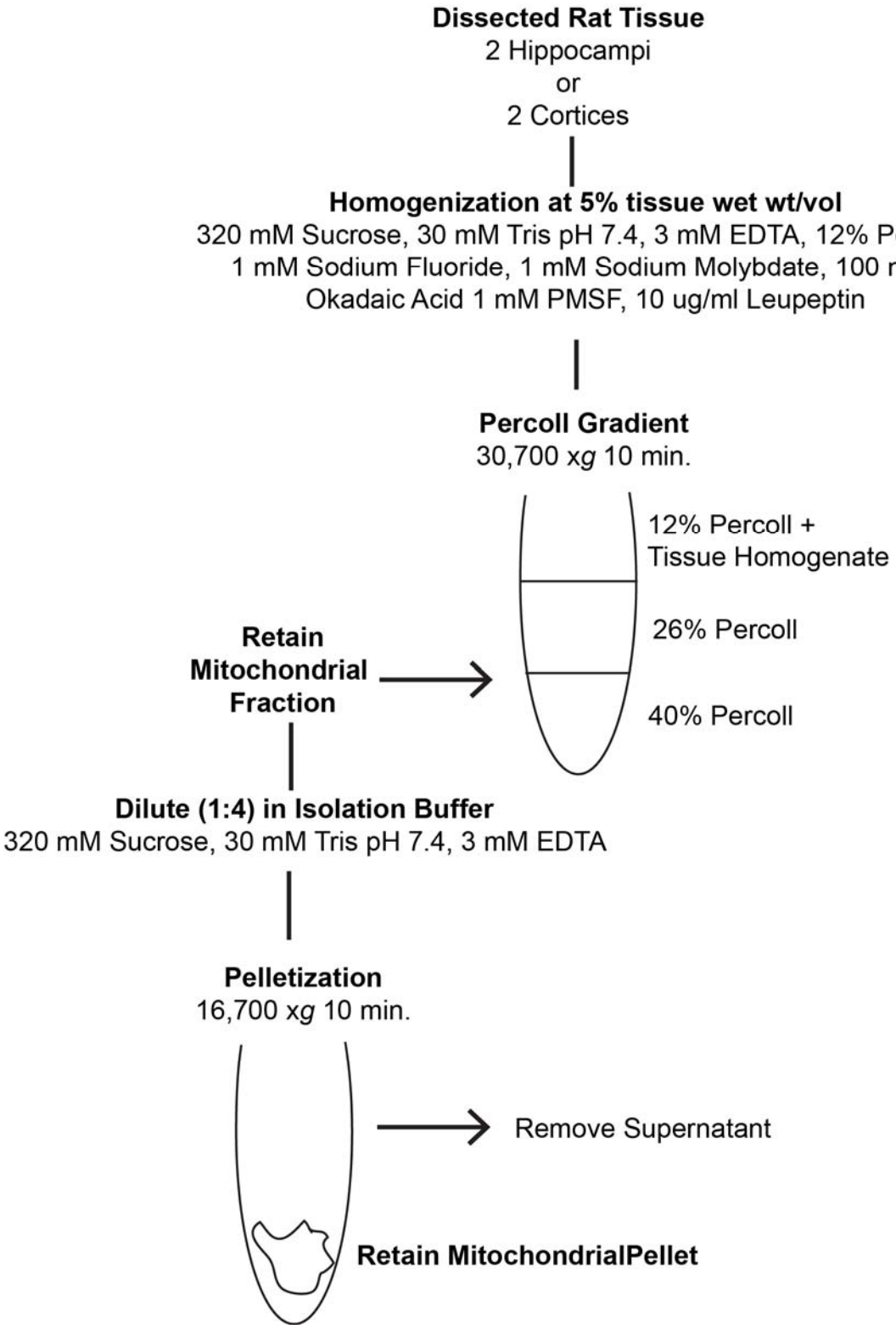

Figure 2.1 Mitochondrial isolation procedures.

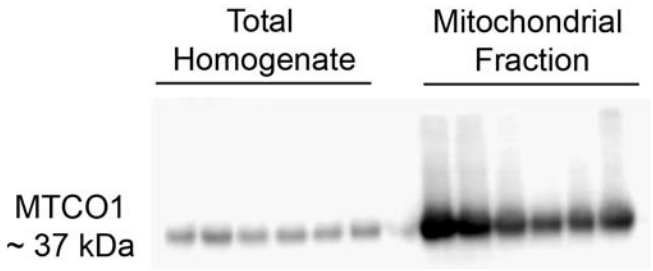

Figure 2.2 Percoll density centrifugation results in enriched mitochondrial fractions from rat brain homogenate. Equal protein from total homogenates and mitochondrial fractions from individual samples were probed for MTCO1 (cytochrome c oxidase, complex IV subunit), a mitochondrial-specific protein. A large increase in immunoreactivity (~5x) was detected in the mitochondrial fraction indicating enrichment for mitochondrial-specific proteins.

2.4 Protein Assays and Western Blotting

Protein assays. Protein concentrations of the total homogenate and the mitochondrial fractions were determined using a Bicinchoninic Acid (BCA) protein assay (Thermo Scientific™ Protein Biology). Serial dilutions of a Bovine Serum Albumin (BSA) standard were prepared for each assay. Samples were diluted in respective buffers and analyzed in duplicate. Protein amounts per well were determined via normalization to the standard BSA curve using a nonlinear regression of a four-parameter logistic curve analysis. Determined protein concentrations for duplicates were then averaged for a final protein concentration (µg/µl) for each sample.

SDS-Page. Total homogenates and mitochondrial fractions were reduced in 4x SDS buffer (NuPAGE® LDS sample buffer and 0.06% β-mercaptoethanol) and denatured at 65°C for 10 minutes. Stock samples were prepared at equal amounts of protein and stored at -20°C until use. Equal amounts of protein for each sample were separated using 4-12% Bis-tris gels (NuPAGE® Novex®) in 1x NuPAGE® MES SDS Running Buffer. Separated proteins were then transferred in 1x NuPAGE® Transfer Buffer to Immobilon-P membranes (Millipore) for 90 minutes at a constant voltage (30 V, 150 mA).

Western blotting. Antibodies used for western blot analysis were as follows: Anti-Drp1 (#8570, Cell Signaling Technology), Anti-TOMM20 (ab56783, Abcam), Anti-GAPDH (ab9485, Abcam). Membranes were blocked overnight at 4°C with SuperBlock™ (TBS) Blocking Buffer (ThermoFisher Scientific) and then incubated in primary antibody solutions (Drp1, 1:1000; TOMM20, 1:500; GAPDH, 1:1000) for three hours at room temperature. The membrane was then washed and incubated with species-specific, horseradish peroxidase-conjugated, secondary antibodies for one hour. Immunoreactivity was detected using SuperSignal™ West Pico chemiluminescent substrate (ThermoFisher Scientific) and exposure to Kodak XAR5 film (Rochester, NY). Relative optical density of each band was analyzed using ImageJ (NIH) for quantification of immunoreactivity. Optical densities for each antibody were normalized to optical densities of standard markers (GAPDH and TOMM20) prior to statistical analysis.

ProteinSimple Wes™. All reagents were used from the ProteinSimple Wes™ separation and detection modules. Mitochondrial fractions were reduced and denatured in DTT (200 mM) and 5x Sample Buffer at 65°C for 10 minutes. Stock samples were prepared at equal amounts of protein and stored at -20°C until use. Stock samples were diluted in 5x Fluorescent Master Mix to obtain a 1x mixture and heated at 65°C for 10 minutes prior to each assay. Primary antibodies used were as follows: Anti-Cytochrome C (#4272S, Cell Signaling) and Anti-TOMM20 (ab56783, Abcam), Anti-OPA1 (D7C1A, #67589, Cell Signaling), and Anti-MFN1 (ab57602, Abcam). Samples at equal amounts of protein, Wes Antibody Diluent 2, primary antibodies (1:50), Streptavidin-HRP, Wes Secondary Antibodies, and Luminol-Peroxide Mix were loaded into the Wes microplate prior to automated capillary electrophoresis. Data were analyzed using the Compass software (ProteinSimple). Areas under the curve for detected immunoreactivity were normalized to areas of standard markers (TOMM20) prior to statistical analysis.

2.5 Immunogold Labeling and Electron Microscopy

Negative stain transmission electron microscopy. Immediately after isolation, mitochondria from rat hippocampi were applied to freshly glow-discharged (30 seconds) carbon-coated copper grids, blotted, and then fixed with 4% paraformaldehyde for 15 minutes on a chilled plate (**Fig. 2.3**). Grids were washed in 1x HEPES Buffered Saline (HBS) and then either prepared for immunogold labeling (described below), negative staining, or stored at room temperature. For negative staining, grids were washed three times with Milli-Q H_2O and incubated in methylamine vanadate (Nanovan, Nanoprobes; **Fig. 2.4**) for 20 seconds. Grids were then blotted and dried for 10 minutes prior to imaging. CCD images of isolated mitochondria were taken on a JEOL1400 transmission electron microscope running at 120kV with a Gatan Orius SC1000 camera. For immunogold labeling, structures were randomly selected for high magnification imaging (30k-150k) to detect positive gold labeling. For mitochondrial morphology measurements, four to six grid squares were selected based on optimal density of structures within the grid square (**Fig.**

2.5A). A series of high magnification images (15k) of the entire grid square were then taken to capture morphological features of the mitochondrial population within the grid square.

Isolated mitochondrial populations from rat brain revealed large morphological heterogeneity (**Fig. 2.5**), not typical of previous reports. Isolated mitochondria also demonstrated canonical mitochondrial features, including outer membranes, electron dense matrices, and cristae. Heterogeneity of internal structure was also observed within the population, as these features were not uniformly evident. It is unknown whether this is a biological phenomenon or whether variability in negative staining may mask internal structure.

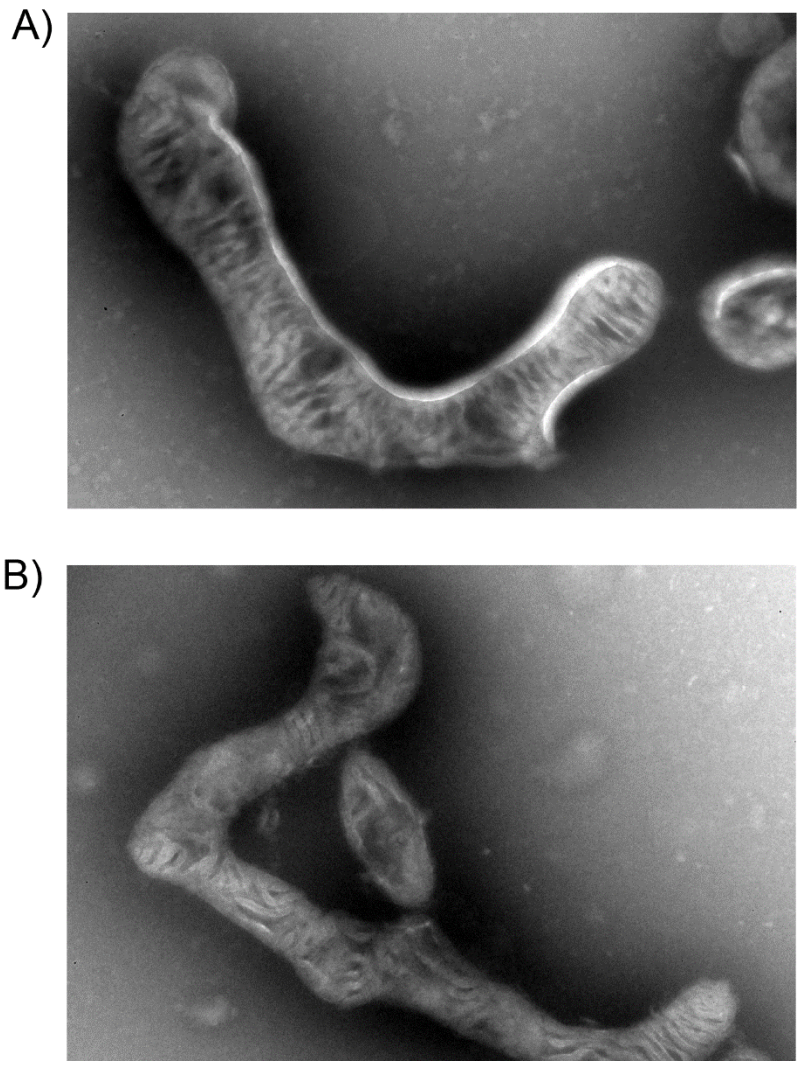

Figure 2.3 Paraformaldehyde fixation of isolated mitochondrial fractions on EM grids preserves morphological features. A) Freshly isolated mitochondrial fractions incubated on carbon-coated copper grids and negatively stained with 2 mM ammonium molybdate. B) Freshly isolated mitochondria fixed with 4% paraformaldehyde an negatively stained with 2 mM ammonium molybdate.

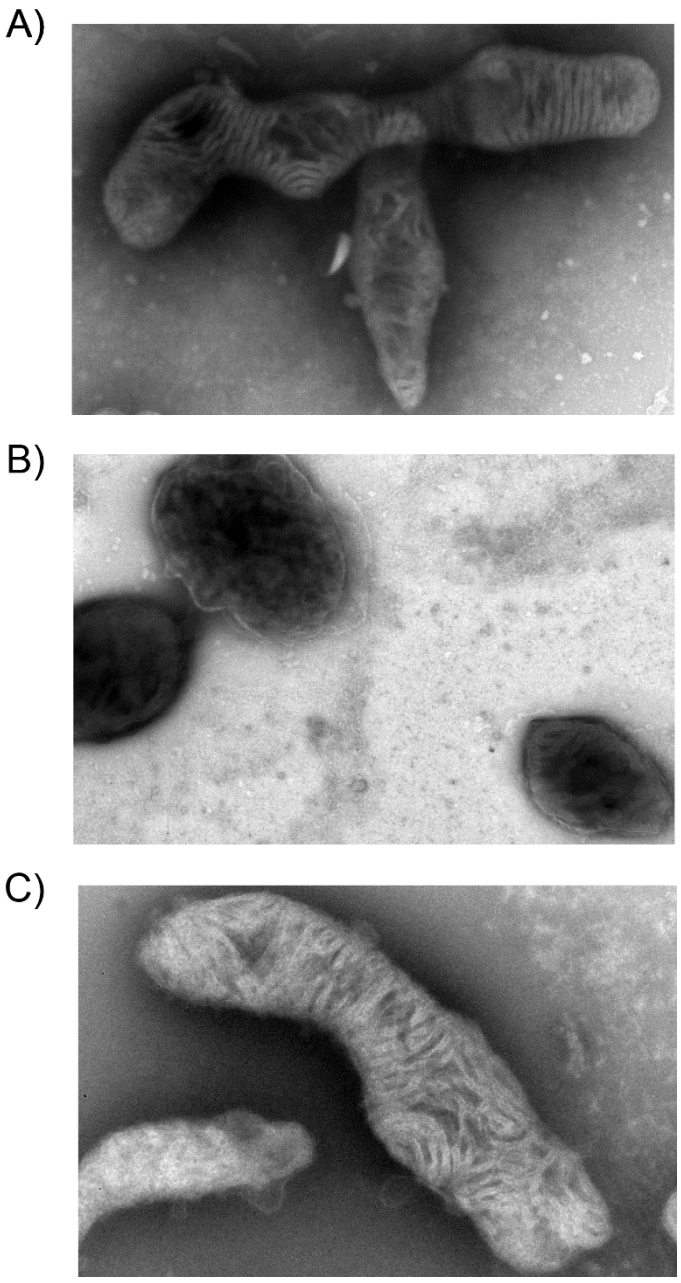

Figure 2.4 Comparison of negative staining procedures. Isolated mitochondrial fractions were fixed with 4% paraformaldehyde on carbon-coated copper grids and negatively stained with (A) 2 mM ammonium molybdate, (B) methylamine tungstate with bacitracin, (C) methylamine vanadate (Nanovan). Nanovan was determined to be optimal in signal-to-noise for immunogold labeling.

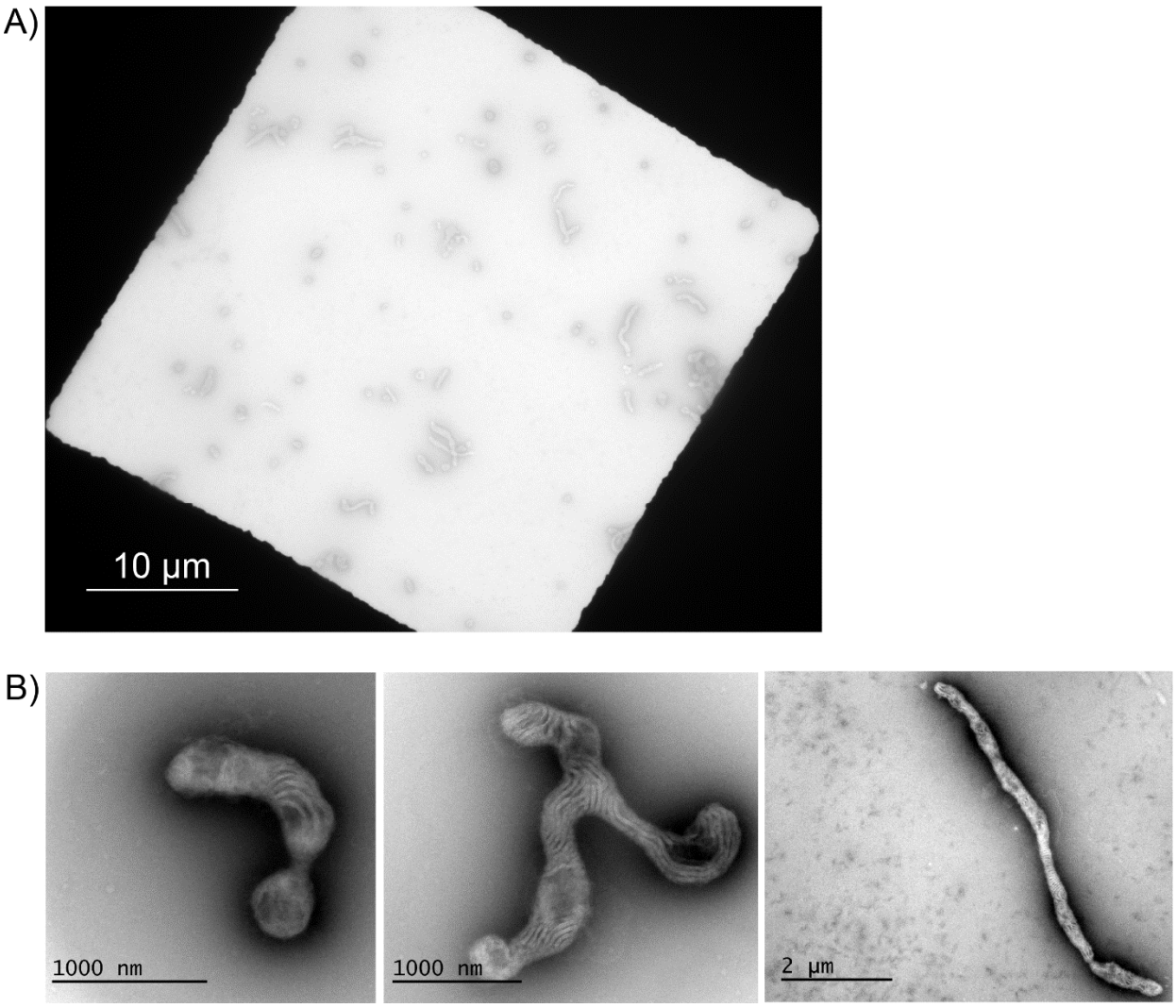

Figure 2.5 Electron micrographs of isolated mitochondrial preparations from brain tissues. A) Representative electron micrograph of a square on a carbon-coated copper grid at low magnification (3k) showing a morphologically heterogeneous population of isolated mitochondria. B) Representative EM micrograph of isolated mitochondria varying in gross morphological features at higher magnifications. Canonical mitochondrial features, such as the electron dense matrix and the cristae, can be seen.

Immunogold labeling. Antibodies used for immunogold labeling were as follows: Anti-TOMM20 (ab56783, Abcam) and Anti-Drp1 (ab56788, Abcam). Grids were blocked for 15 minutes sample-side down on a 50 µL drop of blocking buffer (5% BSA, 1x HBS). Grids were then floated on a drop of primary antibody (1:25) for 30 minutes and washed before incubation in 12-nm gold-conjugated secondary antibody (1:50; Jackson Immunoresearch) for 30 minutes. After immunogold staining, grids were washed and negative stained in methylamine vanadate (Nanovan, Nanoprobes), washed again, blotted, and then air dried. Structures identified as mitochondria were first labeled for mitochondrial outer membrane markers (TOMM20) to confirm mitochondrial identity. **Figure 2.6** shows representative images of gold labeling for TOMM20. Positive gold labeling can be seen decorating the outer membranes of structures. 200 structures were analyzed for positive labeling of TOMM20. Results show ~94% of structures in mitochondrial fractions are positive for TOMM20.

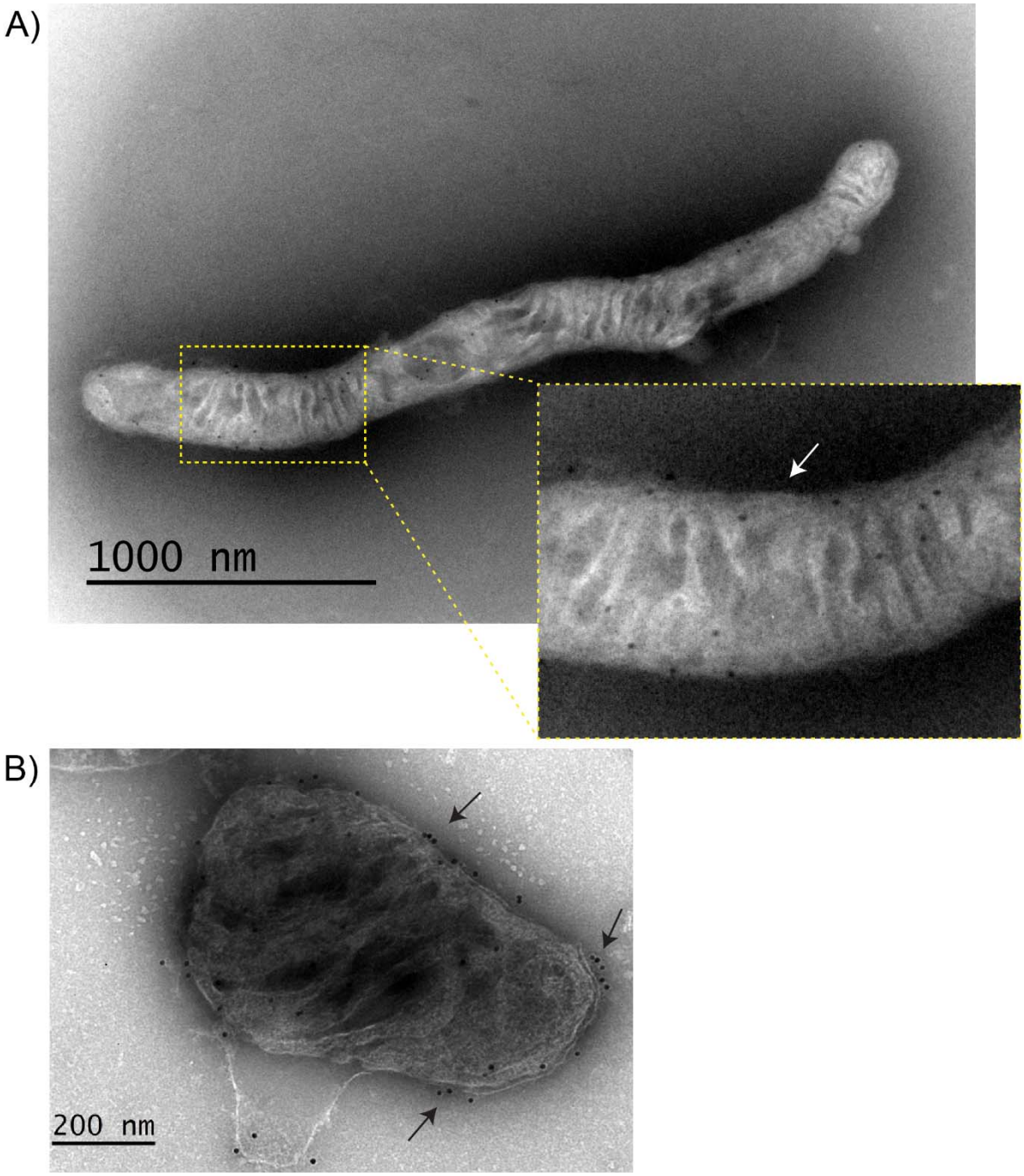

Figure 2.6 Immunogold labeling of isolated mitochondria from rat brain. (A and B) Representative electron micrographs of immunogold labeling for TOMM20 on structures varying in gross morphological features.

Cryo-electron microscopy. All grid preparation and cryo-electron microscopy imaging described in these studies was performed by Dr. M. Neal Waxham. Freshly isolated mitochondria from rat hippocampi were immediately applied to freshly glow-discharged (30 seconds) 2/2 Quantifoil on 200 mesh copper grids. After 30 sec, excess buffer was blotted and the sample was immediately plunged into ethane cooled to liquid N_2 temperature. Cryo-preserved grids were stored in liquid N_2 until use. Cryo-electron microscopy was performed on a FEI Polara G2 equipped with a Gatan K2 Summit direct electron detector. Multiple areas of the grid were chosen at random and 8 x 8 images were collected at 4700x in low dose/photon counting mode prior to montage creation using SerialEM. To remove potential biases, Dr. M. Neal Waxham was blinded to all experimental groups and samples. Representative cryo-electron micrographs at low magnification and high magnification can been seen in **Figure 2.6**. High magnification cryo-electron micrographs of isolated mitochondria demonstrate observable mitochondrial features, including the outer membrane, inner membrane, intermembrane space, cristae, and matrix. Heterogeneity within internal structures (cristae, matrix, intermembrane space) is also apparent within the isolated mitochondrial populations (**Fig. 2.7B**).

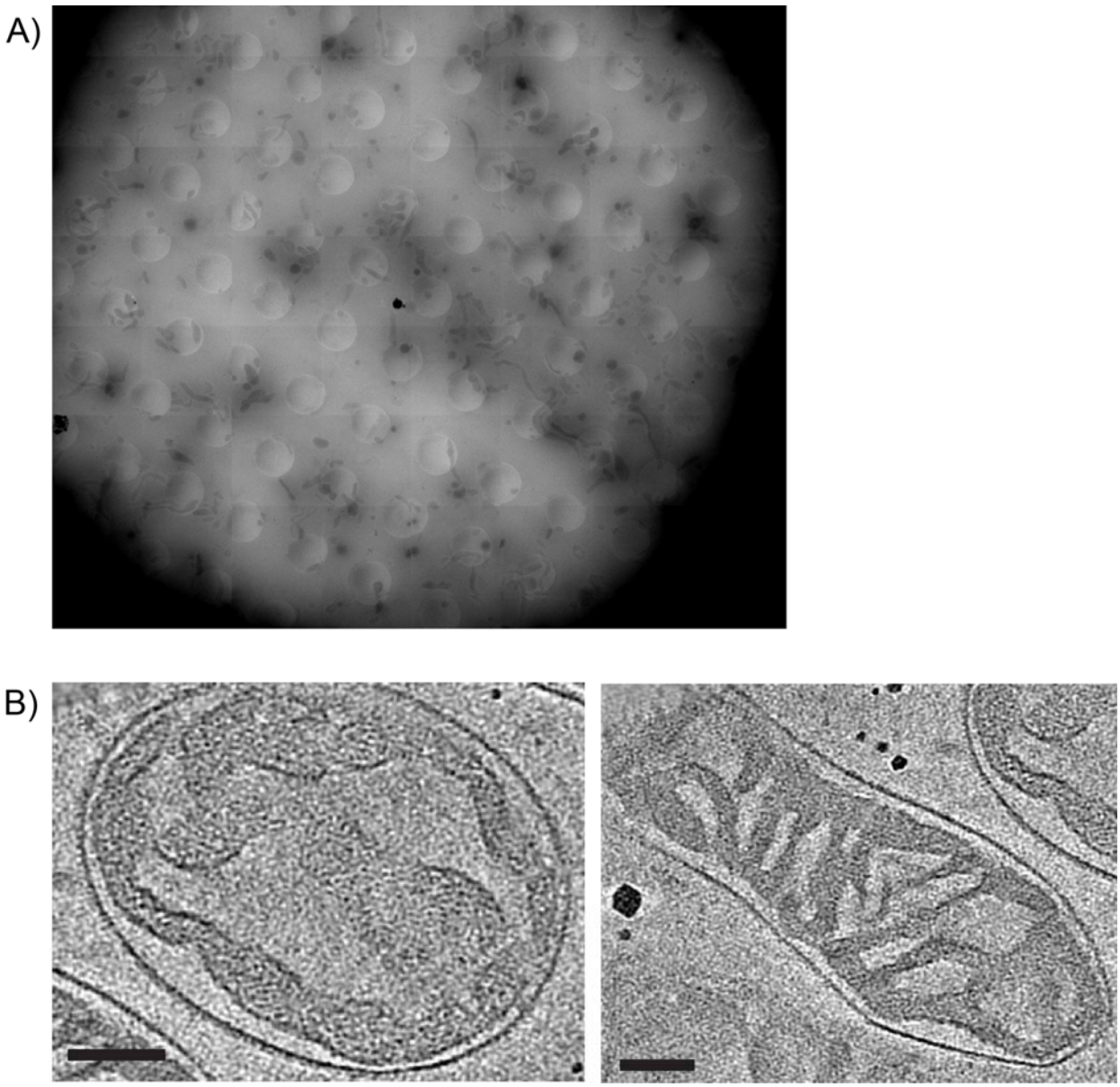

Figure 2.7 Representative cryo-electron micrographs of isolated mitochondria from rat brain. A) Montage of images collected at 4700x in low dose/photon counting mode. A morphologically heterogeneous population of isolated mitochondria can be seen across the grid. B) High magnification cryo-electron micrographs of isolated mitochondria. Scale bar = 100 nm. Images used with permission from Dr. Neal Waxham.

2.6 Mitochondrial Morphological Measurements

To quantify the length of mitochondria in cryo-electron microscopy images, montages were displayed in IMOD and a line was drawn along the major and minor axes of each mitochondrial structure and stored in a model file for each montage.(Kremer et al., 1996) To quantify mitochondrial length for negative stain TEM images, CCD images were displayed in FIJI (NIH) and a line was drawn along the major axis of each mitochondrion. Length measures of the major axes and aspect ratios (major/minor axis) were used for further analysis. A demonstration of these measurements can be seen in **Figure 2.8.** For both electron microscopy data sets, measures were extracted for 200 mitochondria, imported into Excel and the data displayed by separating the lengths into 500 nm bins. To remove potential biases, the experimenter quantifying the length of individual mitochondria was blinded to the sample identities.

Several comparisons of mitochondrial lengths in different conditions were made from collected data sets in different studies. Significant differences were observed in mitochondrial lengths from cryo-preserved and fixed (4% PFA) preparations (n = 200 mitochondria/condition, n = 2 hippocampi/condition; U = 14121, p = <0.001; **Fig. 2.9A**). Therefore, cryo-preservation was determined as the preferred technique to preserve native morphology and investigate differences due to experimental manipulations (i.e., sham vs CCI Injury). No differences of mitochondrial length were found when comparing mitochondria isolated from rat hippocampi (n = 4 samples/group, n = 200 mitochondria/sample, n = 2 hippocampi/sample) and mouse hippocampi (n = 6 samples/group, n = 200 mitochondria/sample, n = 2 hippocampi/sample; **Fig. 2.9B**). Additionally, no differences were found when comparing lengths of isolated mitochondria from mouse hippocampus (n = 4 samples/group, n = 200 mitochondria/sample, n = 2 hippocampi/sample) and mouse cortex (n = 4 samples/group, n = 200 mitochondria/sample, n = 2 cortices/sample; **Fig. 2.9C**).

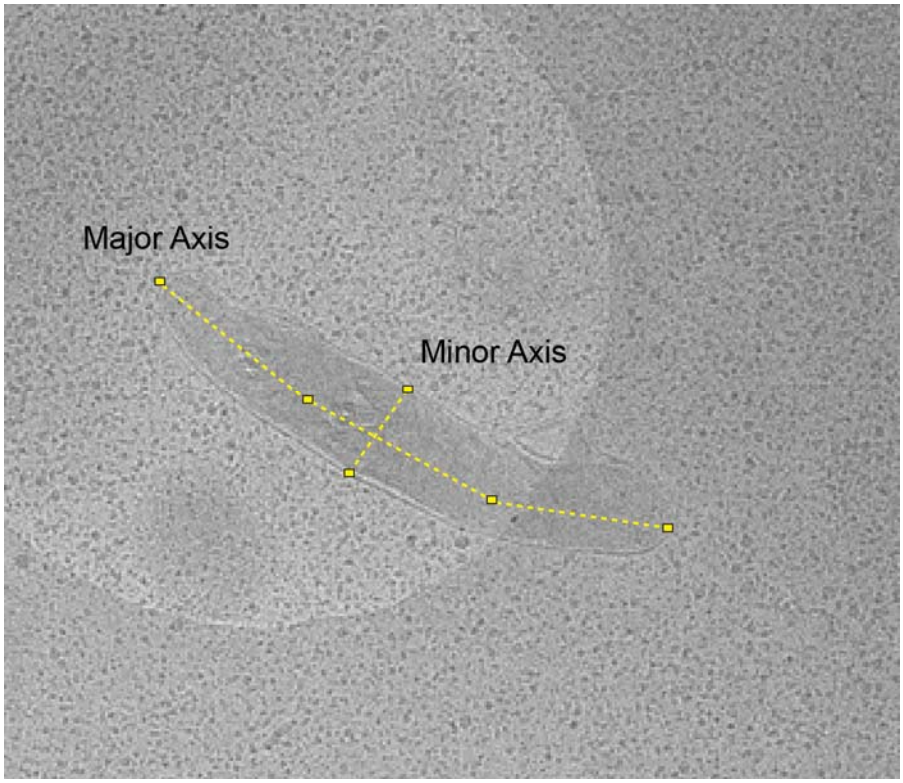

Figure 2.8 Demonstration of mitochondrial length measurements in isolated mitochondrial preparations from brain tissues. Lengths of the major and minor axes of each mitochondrial structure were measured using the segmented line tool in IMOD or FIJI.

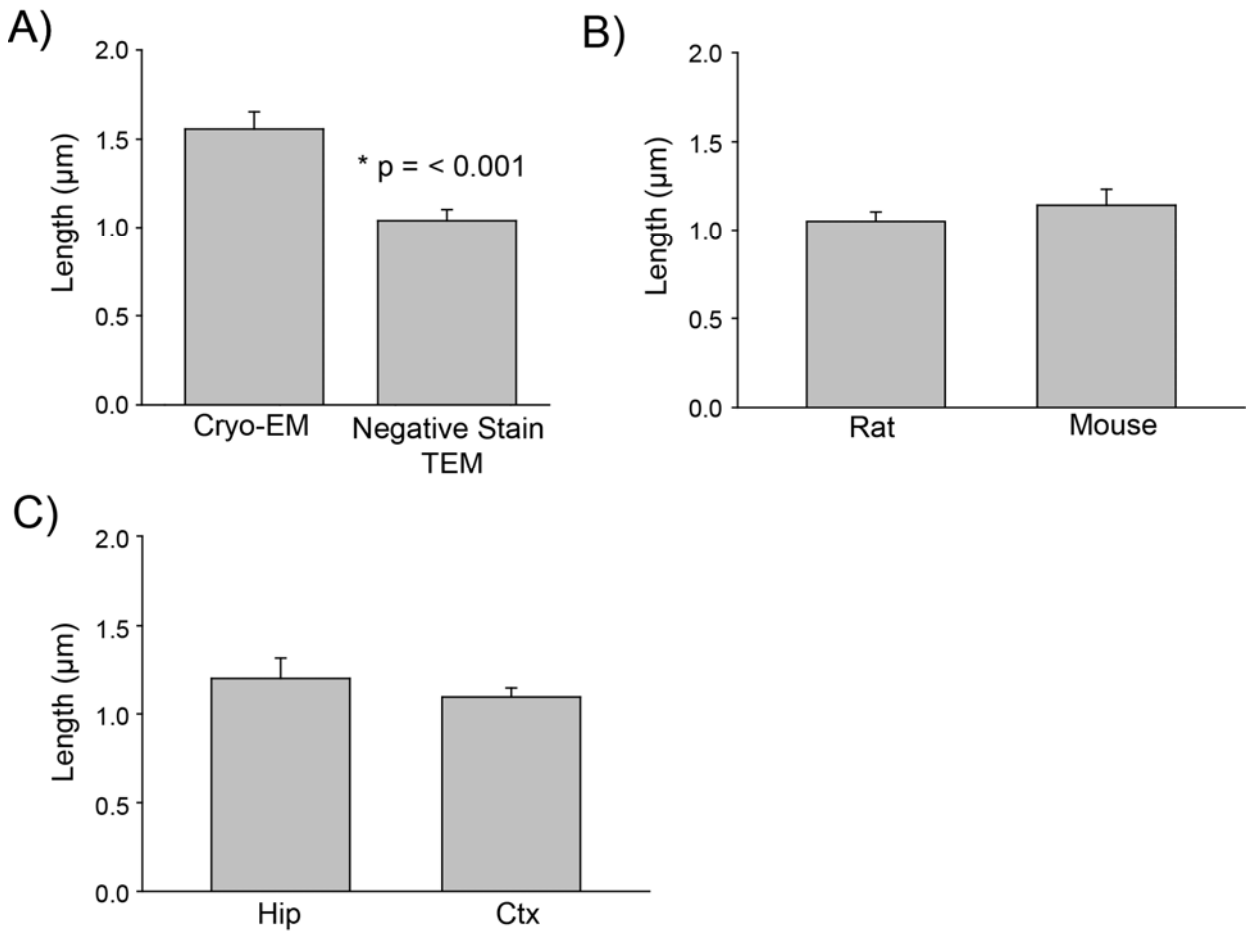

Figure 2.9 Comparisons of mitochondrial length measurements. (A) Differences in mitochondrial lengths from cryo-preserved and fixed (4% PFA) isolated mitochondria (n = 200/condition). Fixation and negative stain procedures appears to significantly reduce mitochondrial lengths in comparison to cryo-preservation methods. Cryo-preservation of isolated mitochondria was therefore preferred gross morphological measurements. B) Comparison of mitochondrial lengths from Rat and Mouse hippocampi (n = 200 mitochondria/condition. C) Comparisons of mitochondrial lengths from Mouse Hippocampus and Mouse Cortex (n = 200 mitochondria/condition).

2.7 Mitochondrial Respiration

Mitochondrial respiration of isolated mitochondria was measured using the Seahorse XFe96 Analyzer (Agilent) and an adapted protocol from Rogers *et al*. (2011).(Rogers et al., 2011) Metabolically active mitochondria were freshly isolated from hippocampi and immediately diluted in ice-cold Mitochondrial Assay Solution (MAS; 70 mM Sucrose, 220 mM Mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA, 10 mM Pyruvate, 5 mM Malate, 0.2% (w/v) Fatty Acid-free BSA, pH 7.2). Mitochondrial samples were diluted to ~ 0.05 mg/ml and were loaded into a Seahorse XF96 Cell Culture Microplate at 1 ug per well with 10 replicates per sample. Optimal protein concentrations for respiratory analysis were determined prior to the assay in establishment of conditions and procedures (**Fig. 2.10**). The plate was then transferred to a centrifuge equipped with a swinging-bucket adaptor, and centrifuged at 2,000x*g* for 20 minutes at 4°C. After centrifugation, MAS plus 4 mM ADP was added to each well and the plate was incubated at 37°C for ten minutes prior to Seahorse XFe analysis. Measurements of oxygen concentrations were detected and analyzed across time throughout the assay to determine oxygen consumption rates (pmol/min).

A classical coupling assay was employed to determine coupling of electron transport to oxidative phosphorylation in isolated mitochondrial populations (**Fig. 2.11A**). During the assay, an initial respiration measure was obtained in the presence of respiratory substrates (pyruvate and malate) and ADP. After the initial measures, various electron transport inhibitors and uncouplers were sequentially injected into the wells (Oligomycin (2.5 µg/ml), FCCP (4 µM), and Antimycin A/Rotenone (4 µM, 2 µM), respectively) and changes in oxygen consumption rates were detected in each condition (**Fig. 2.11A**). Mitochondrial oxygen consumption rates for each well were normalized to protein amount per mitochondrial fraction (pmol/min/ug) and used for further analysis. Data were analyzed using the Wave software (Agilent).

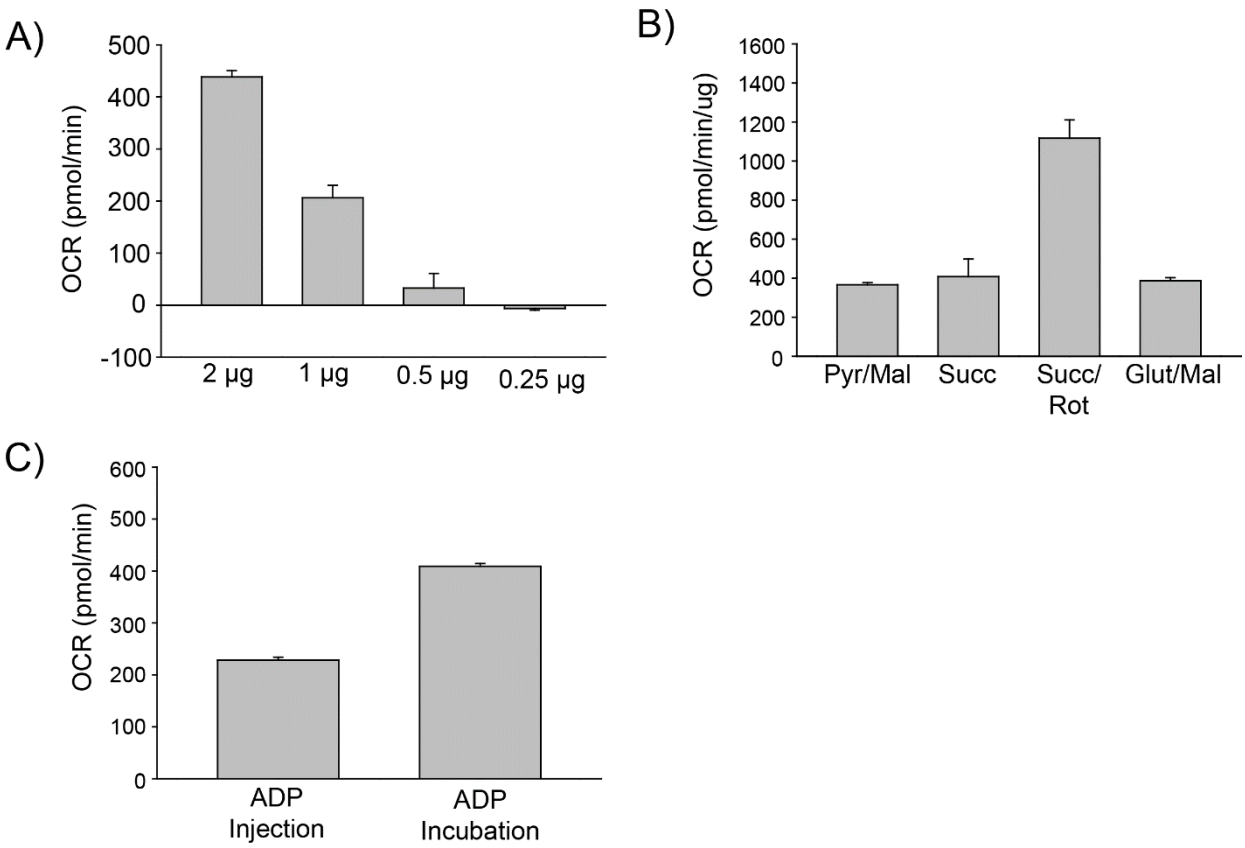

Figure 2.10 Establishment of Seahorse procedures for isolated mitochondria. Optimal parameters of respiration in isolated mitochondrial populations from brain were established prior to experimental testing. A) Demonstration of mitochondrial protein titration and oxygen consumption rates in the presence of TCA substrates and ADP (State III-ADP). B) Comparisons of oxygen consumption with different TCA cycle substrates. C) Comparisons of oxygen consumption rates with an injection of ADP during the assay or a short incubation with ADP prior to assay measurements. Oxygen consumption rates were higher and more stable with the incubation procedure.

Respiratory state definitions. Figure 2.11B represents measures of respiratory states in different conditions during the mitochondrial coupling assay. Respiratory states as defined by Chance and Williams (1955) were calculated to characterize “coupling” or the differences between non-phosphorylating and phosphorylating oxygen consumption within isolated mitochondrial populations.(Chance and Williams, 1955) State III-ADP occurs in the presence of all necessary substrates needed for electron transport and ADP phosphorylation (TCA substrates and ADP, P_i), and therefore reflects “phosphorylating” respiration and represents a “coupled” state of mitochondrial oxygen consumption and ATP synthesis. State IV can be defined in two ways: 1) in the presence of TCA substrates without ADP, or 2) in the presence of TCA substrates, ADP, and Oligomycin (ATP synthase inhibitor). State IV reflects the limitation of electron flow by ATP synthesis and the electrochemical gradient in the inner mitochondrial membrane. In these assays, State IV in the presence of oligomycin was used for analysis, and is hereby referred to as State IV-oligomycin throughout this dissertation. Additionally, measures of “proton leakage” can be calculated in State IV during oligomycin conditions as it also reflects potential electron flow due to unregulated proton release across the inner mitochondrial membrane. State III-uncoupled is defined as “uncoupled” respiration as it is reflective of electron flow and oxygen consumption without the limitation of the electrochemical gradient and ATP synthase activity. This state occurs in the presence of TCA cycle substrates, ADP, and FCCP (ionophore). Finally, “non-mitochondrial” respiration can be calculated in the presence of Antimycin A (Complex III inhibitor) and Rotenone (Complex I inhibitor), which completely arrests mitochondrial oxygen consumption. Therefore any remaining oxygen consumption cannot be attributed to mitochondrial respiration.

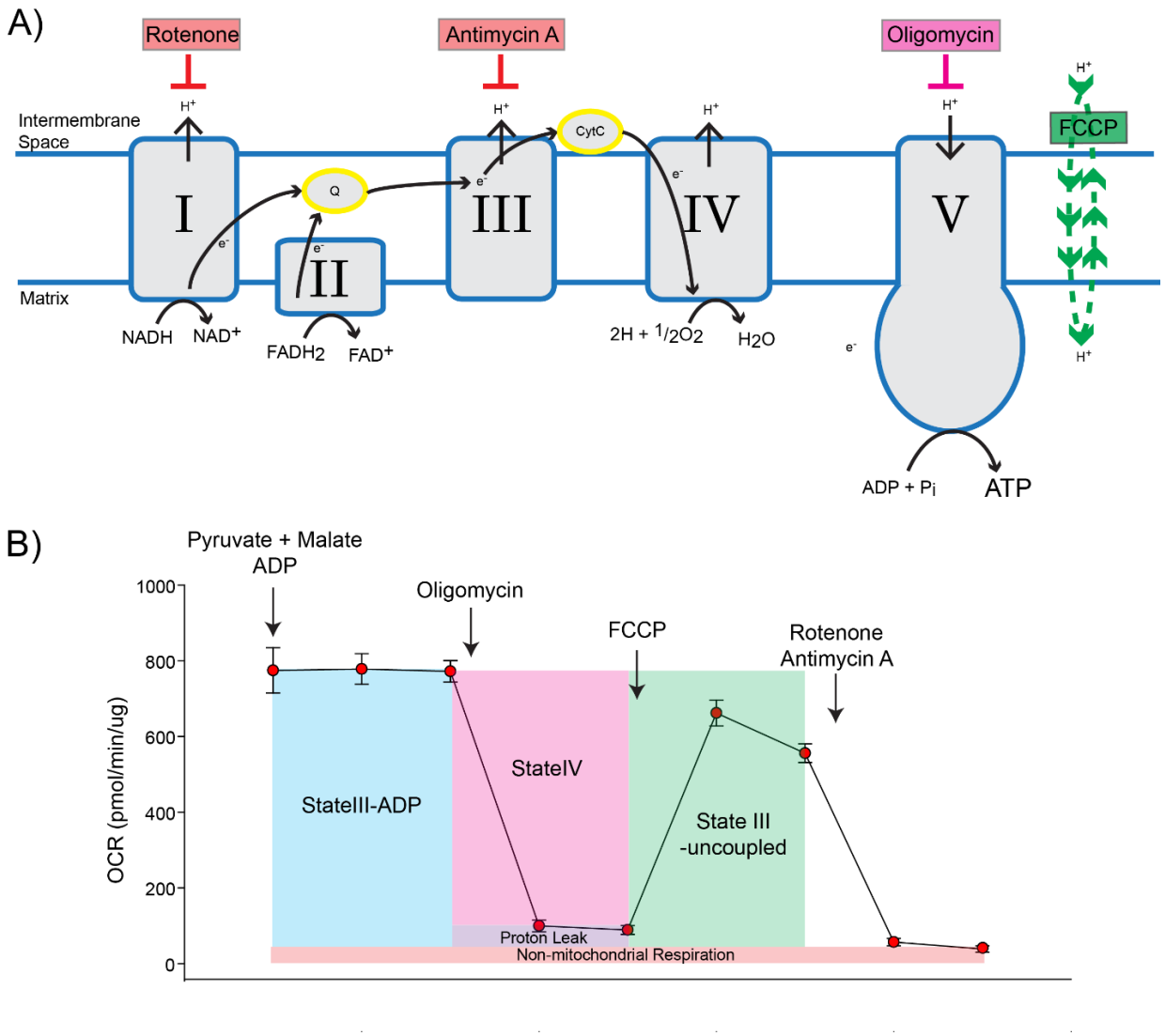

Figure 2.11 Classic mitochondrial coupling assay experimental design. A) Representative cartoon of oxidative phosphorylation via electron transport chain complexes and ATP synthase in the inner mitochondrial membrane. Mechanisms and targets of mitochondrial poisons used in the mitochondrial coupling assay are also demonstrated. B) Time-course and characteristic oxygen consumption rate measures of isolated mitochondria with sequential poison administration during the mitochondrial coupling assay. Respiration state measures during in each condition are also shown.

Respiratory state calculations. **Table 2.1** outlines the calculations for each respiratory state measure. Each period of measurement is the average between all well replicates per sample. Three periods of measurement are obtained in the State III-ADP condition and the last measurement is used for analysis. For the State IV and State III-uncoupled-conditions, two measurement periods are obtained and the first measurement after injection (oligomycin or FCCP, respectively) is used for analysis. For all calculations, the oxygen consumption rates from the Antimycin A/Rotenone conditions were subtracted to remove contributions of "non-mitochondrial" oxygen consumption. A Respiratory Control Ratio (RCR) can further be used by normalizing the State III-uncoupled and State IVo measures (State III-uncoupled:State IVo). The RCR reflects coupling between electron flow/oxygen consumption and ADP phosphorylation.

Table 2.1 Calculations for respiratory state measures from the classical coupling assay.

Parameter	Rate Measurement Equation Used for Analysis
Non-mitochondrial Respiration	Minimum rate measurement after Rotenone/Antimycin A injection
State III-ADP	(Last rate measurement before first injection) – (Non-mitochondrial respiration)
State IVo	(First rate measurement after injection of Oligomycin) – (Non-mitochondrial respiration)
State III-uncoupled	(First rate measurement after injection of FCCP) – (Non-mitochondrial respiration)
Respiratory Control Ratio (RCR)	State III-ADP/State IVo

2.8 Histopathology

Animals were sacrificed using an overdose of sodium pentobarbital and exsanguination with ice cold phosphate-buffered-saline (PBS) followed by 4% paraformaldehyde. Brains were collected and post-fixed in 4% PFA for 24 hours before step-wise cryopreservation in sucrose (15% to 30% in PBS) at 4°C. Brains were cut into 40 µm-thick coronal sections using a cryostat at chilled to -20°C. Tissue sections containing the dorsal hippocampus were selected for both immunohistochemistry and immunofluorescence.

Immunohistochemistry. Antibodies used for immunohistochemistry were as follows: Anti-Doublecortin (#4604, Cell Signaling). Tissues were permeabilized in PBS containing 0.6% Hydrogen Peroxide and 0.25% Triton-X100 for 30 minutes, blocked for 1 hour in blocking buffer (2.5% BSA and 2.5% normal goat serum), then incubated in primary antibody solutions overnight at 4°C. After extensive washing, tissues were incubated in a species-specific biotinylated secondary antibody for three hours followed by incubation in Avidin-Biotin solution (Vectastain ABC kit, Vector Laboratories) for 45 minutes. Tissues were washed, then developed in DAB solution (3,3'-diaminobenzidine; Vector Laboratories). After development, tissues were washed, mounted on gelatin-subbed slides, and allowed to dry overnight. Tissues were then dehydrated in an alcohol series, delipidated in xylenes, and coverslipped with Permount.

Cell Counting. Doublecortin-positive cells were identified along the dentate gyrus of the hippocampus and number of positively stained cells were counted using a Zeiss *Axiovert* microscope. The length of the dentate gyrus was measured (mm) using StereoInvestigator (MFB Bioscience) and the number of identified doublecortin-positive cells per mm was calculated and used for analysis. Measurements were performed by an experimenter blind to sample identity.

Immunofluorescence. Antibodies used for immunofluorescence were as follows: NeuN (MAB377, Millipore), GFAP (AB5804, Millipore), and Map2 (AB5622, Millipore). Tissues were permeabilized in PBS containing 0.25% Triton-X100 for 30 minutes, blocked in blocking buffer (2.5% BSA and 2.5% normal goat serum) for 1 hour, and then incubated in primary antibody solutions overnight at 4°C. After extensive washing, tissues were incubated in a species-specific

secondary antibody (Alexa Fluor® 488 or 568, Abcam) for three hours. Tissues were washed, slide mounted and dried overnight, and finally coverslipped with Fluoromount-G. Images were collected on a Zeiss Axiovert microscope with either a MagnaFire camera using Q-Capture Pro 7 software (QImaging) or an Andor Zyla 4.2 camera using MetaMorph software (Molecular Devices).

2.9 Rodent Behavioral Assessment

Motor performance. On days one through three post-injury, the Foot Fault task was used to assess motor skills (paw placement) and the Beam Balance task was used to assess vestibulomotor performance. For the Foot Fault task, animals were placed on a wire grid (opening size 2x2 cm) and number of foot faults was recorded for both the left and right front paws. A foot fault was defined as a paw falling below the plane of the grid. Three trials of 50 total faults were recorded and the average between trials for each day was used for analysis. For the Beam Balance task, animals were placed on a narrow metal beam (1.5 cm wide) and allowed to establish balance for three seconds prior to start of recording time. If the animal fell within the first three seconds, they were placed back on the beam and allowed to repeat the trial. The duration of time the animal was capable of balancing on the beam was recorded in a 60 second time period. Three 60 second trials were measured each day and the average between trials for each day was used for analysis.

Novel Object Recognition. The Novel Object Recognition (NOR) task is a hippocampal/entorhinal cortex-dependent learning and memory task that assesses novel versus familiar object recognition (**Fig. 2.12A**).(Antunes and Biala, 2012; Ennaceur and Meliani, 1992) On day one, animals were placed in the testing chamber (100 x 100 cm box; **Fig. 2.12B**) and allowed to habituate for two, 10 minutes sessions. On the second day, two identical objects were placed in the box and the animal was allowed to explore the objects for one, 10 minute familiarization session. Twenty-four hours later, a new object of the same color and size, but different shape replaced one of the objects and the animal was allowed to again explore the

objects for one, 10 minute testing session. The time spent exploring each object was recorded by a blind experimenter on both the familiarization and testing days. The difference in percent time exploring the novel versus the familiar object ((time spent exploring object/total time exploring) x 100) on the testing day was calculated and used as a measure of recognition memory.

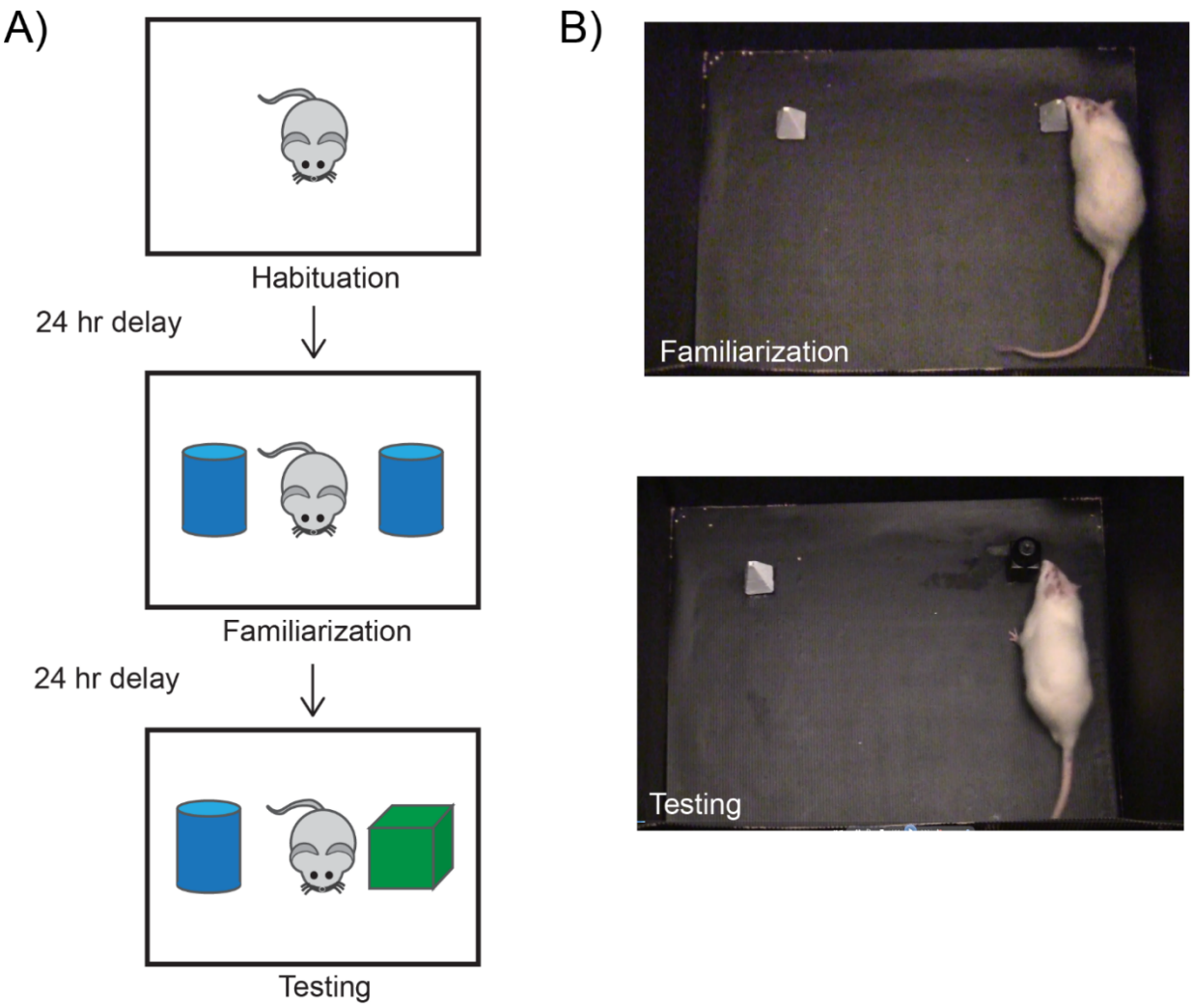

Figure 2.12 Novel object recognition paradigm. A) Cartoon representation of Novel Object Recognition (NOR) Procedure. A 24 hour delay was maintained between habituation, familiarization, and testing to model long-term learning and memory. B) Images of the Dash Lab NOR setup. A rat can be seen "exploring" an object in the familiarization phase and exploring the novel object in the testing phase.

Contextual Fear Conditioning. Contextual Fear Conditioning studies presented in this dissertation were administered by Dr. Michael Hylin. Contextual Fear Conditioning is a hippocampal-dependent task that employs classical conditioning approaches to associate a negative stimulus with an environment of particular context.(Curzon et al., 2009) A One-Trial Contextual Fear Conditioning procedure was adapted from Wiltgen, *et al.* (2006) and Drew *et al.* (2010) (**Fig. 2.13A**).(Drew et al., 2010; Wiltgen, 2006) The training chamber included only visual contextual cues, and no auditory stimuli (i.e. a tone; **Fig. 2.13B**). The protocol did not include a pre-exposure period before training to ensure no prior association with the context. On the first day, the animal was placed in the training chamber and allowed to familiarize itself with the context for 150 seconds before receiving a mild foot shock (2 sec, 0.7 mA). Thirty seconds after the shock, the animals were removed from the training chamber and returned to their home cage. Twenty-four hours later, the animal was placed back in the training chamber for three minutes (without shock). Freezing behavior (defined as the absence of movement except that needed for respiration) was recorded for both the training and the testing day every two seconds within the three minute time period by an experimenter who was unaware of the treatment groups. The percent of total time the animal remained frozen was calculated and used as an index of fear memory. It is important to note that data presented in these studies from the Contextual Fear Conditioning task were obtained from a separate cohort of injured/treated animals using the same experimental design and treatment schedule. Animals from this study were consistently assessed on all described behavioral tasks, and exhibited similar behavioral results from experimental injury and Mdivi-1 treatment.

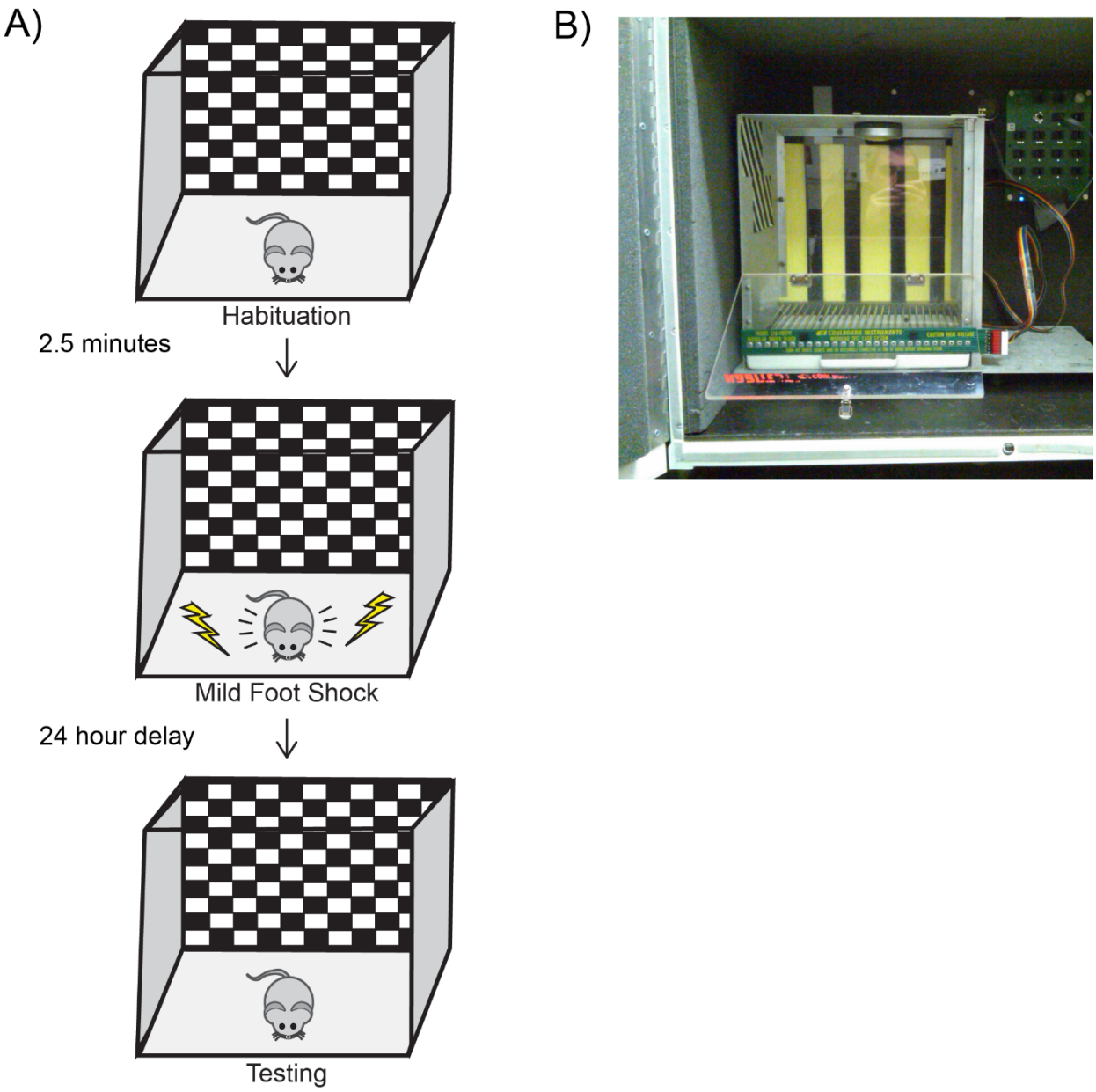

Figure 2.13 Contextual fear conditioning paradigm. A) Cartoon representation of Contextual Fear Conditioning task procedure. A 24 hour delay was maintained between training and testing days to model long-term learning and memory. B) Images of the Dash Lab Contextual Fear setup. The fear chamber environment displays visual cues as contextual features. The image in 2B was obtained with permissions from Dr. Michael Hylin.

Morris Water Maze. The Morris Water Maze task is an assessment of hippocampal-dependent spatial learning and memory by training an animal to navigate to the location of a hidden platform in a swimming arena based on distal visual cues in the environment.(Vorhees and Williams, 2006) These studies use a standard training and long-term memory probe procedure of the hidden platform version of the Morris Water Maze.(Morris, 1984) For six days, rats were trained to locate a hidden platform in an open swimming area based on distal visual cues around the perimeter of the pool. Each training day entailed four subsequent trials with 5-10 minute inter-trial intervals. For each trial, animals were given 60 seconds to find the hidden platform in the swimming arena. If the platform was not found, the animal was led to the platform by the experimenter and allowed to remain on the platform for an additional 30 seconds before removal from the pool. Upon trial completion, animals were removed from pool and placed in a warming chamber in between trials. Twenty-four hours after the final training session, spatial memory was assessed in a long-term memory probe trial by removing the platform from the swimming arena and allowing the animals to search for the platform for 60 seconds. Latency to platform location was recorded by the experimenter for each trial during the training sessions and during long-term memory probe. The number of platform crossings during the probe trial was also recorded by the experimenter.

2.10 Statistical Analysis

For western blotting and mitochondrial respiration, data were analyzed using a one-way analysis of variance (ANOVA), with a Bonferroni method for *post-hoc* analysis. For mitochondrial morphology, data were analyzed using the Kruskal-Wallis Analysis of Ranks with Tukey's method for *post-hoc* comparisons and the Mann-Whitney Rank Sum Test. For behavioral assessments and cell population quantifications, data were analyzed using a Student's t-test. Results were considered significant at $p < 0.05$. Data are presented as the mean +/- standard error of the mean (SEM).

Chapter 3. Experimental TBI Increases Drp1 Translocation and Alters Mitochondrial Morphology in the Injured Brain

Under normal conditions, mitochondria maintain cellular energy homeostasis and health via balanced processes of fusion and fission, continuously dividing and fusing to form an interconnected network throughout the cell.(Labbé et al., 2014) Processes of fission and fusion govern mitochondrial morphology, and are critical for quality control of the mitochondrial network, as well as mitochondrial adaptation to changes in cellular energy demands for efficient metabolic maintenance.(Mishra and Chan, 2016) Mitochondrial fusion is regulated by the inner- and outer-membrane GTPases Optic Atrophy 1 (Opa1) and Mitofusins 1/2 (Mfn1/2) and is important for maintenance of the network by complementation of mitochondrial DNA (mtDNA) and matrix metabolites and maximization of ATP production during high metabolic activity. Fission is mediated by Dynamin-related protein 1 (Drp1), a cytosolic GTPase that self-assembles and translocates to the mitochondrial outer membrane (MOM) to constrict and divide the membrane.(van der Bliek et al., 2013; Smirnova et al., 2001) Fission is important for maintaining the mitochondrial network by segregating damaged mitochondria for subsequent degradation through mitophagy, and dividing mitochondria to be distributed throughout the cell based on energy needs. An imbalance of fusion and fission, particularly an excess of mitochondrial fission, has been associated with mitochondrial dysfunction, apoptosis, and neurodegeneration.(Knott et al., 2008; Manczak and Reddy, 2012) These pathological fission states are characterized by fragmentation of the mitochondrial network, mitochondrial dysfunction, and the release of pro-apoptotic factors, such as cytochrome c, from the mitochondrial compartment. Dysregulation of Drp1 activity specifically has been implicated as a driving mechanism of pathological fission states in many neurodegenerative models.(Arnoult et al., 2005a; Chen et al., 2005; Cho et al., 2009; Detmer and Chan, 2007b; Frank et al., 2001; Knott et al., 2008; Manczak and Reddy, 2012; Manczak et al., 2011; Reddy et al., 2011; Song et al., 2011) Mitochondrial dysfunction and cell death are key characteristics of neuropathology after TBI; however, whether Drp1-mediated

mitochondrial fission is altered after injury has yet to be determined. Therefore, I hypothesized that TBI may cause dysregulation of Drp1 and excessive mitochondrial fission in the injured brain.

3.1 CCI Injury Does Not Alter Total Drp1 Levels, but Increases Mitochondrial Association of Drp1

Drp1 activity and the rate of fission is known to be regulated by several intracellular signaling pathways that allow for the mitochondrial network to dynamically respond to changes in the cellular environment.(Chang and Blackstone, 2010; Otera et al., 2013) Dysregulation of Drp1 can be described as 1) changes in Drp1 protein levels (e.g., transcription/translation or degradation) 2) changes in translocation to the MOM, and/or 3) changes in GTPase activity.(Cereghetti et al., 2008; Mai et al., 2010; Manczak et al., 2011; Song et al., 2011; Uo et al., 2009) The balance of fusion and fission can be mediated by expression levels of the key regulators involved in each process.(Mai et al., 2010; Sesaki et al., 2014; Uo et al., 2009; Wakabayashi et al., 2009; Wu et al., 2011) To determine whether experimental TBI alters Drp1 protein levels after injury, Drp1-specific antibodies were used to probe for protein immunoreactivity in sham and injured hippocampal and cortical tissues via western blotting. With assistance from Dr. Jing Zhao, rats were injured using a controlled cortical impact (CCI) injury device and ipsilateral hippocampi and cortices were dissected and homogenized at 24 hours and 72 hours post-injury (n = 3 samples/group, n = 2 hippocampi or cortices/sample). These time points were chosen as they represent periods of acute and sub-acute mitochondrial dysfunction in this model.(Gilmer et al., 2009; Hill et al., 2016; Lifshitz et al., 2004; Singh et al., 2006; Xiong et al., 1997) A group of sham-operated animals was also simultaneously prepared and used as baseline controls. **Figures 3.1A** and **3.2A** show representative western blot images indicating an immunoreactive band at approximately 80 kDa in total hippocampal and cortical homogenates. Results showed no significant changes in overall hippocampal Drp1 immunoreactivity after injury

in hippocampal tissues ($F(2,6) = 0.0435$, $p = 0.958$; **Fig. 3.1A**) or cortical tissues ($F(2,4) = 0.390$, $p = 0.700$; **Fig. 3.2A**).

The rate of mitochondrial fission can be altered by modulation of the critical steps in the execution of Drp1-mediated fission, such as translocation to the mitochondrial outer membrane, Drp1 assembly, or GTPase activity.(Cereghetti et al., 2008; Chang and Blackstone, 2007b, 2010) Increases in Drp1 translocation have been shown to induce excessive fission events and mitochondrial fragmentation, therefore mitochondrial-associated Drp1 was assessed after experimental TBI. At 24 and 72 hours after CCI injury, mitochondrial were rapidly isolated from sham and CCI injured hippocampal and cortical tissues (n = 3 samples/group, n = 2 hippocampi or cortices/sample) and probed for mitochondrial associated Drp1-immunoreactivity via western blotting. **Figures 3.1B** and **3.2B** show representative western blot images from mitochondrial fractions, demonstrating a time-dependent increase of Drp1 immunoreactivity at 24 hours and 72 hours post-injury compared to sham controls. Quantification of Drp1 optical densities (**Fig. 3.1B**) revealed that Drp1 levels after CCI are significantly increased compared to sham controls in the hippocampal mitochondrial fractions ($F(2,6) = 13.059$, $p = 0.007$), with a non-significant increase at 24 hours ($p = 0.05$) and a significant increase at 72 hours post-injury ($p = 0.007$). Although there appeared to be a marginal increase of Drp1 immunoreactivity in cortical mitochondrial fractions at both 24 hours and 72 hours post-injury, quantification proved to be non-significant ($F(2,6) = 2.478$, $p = 0.164$; **Fig. 3.2B**). Therefore, although total Drp1 levels do not increase in the injured hippocampus or cortex, mitochondrial-associated Drp1 levels significantly increase in the hippocampus after experimental injury.

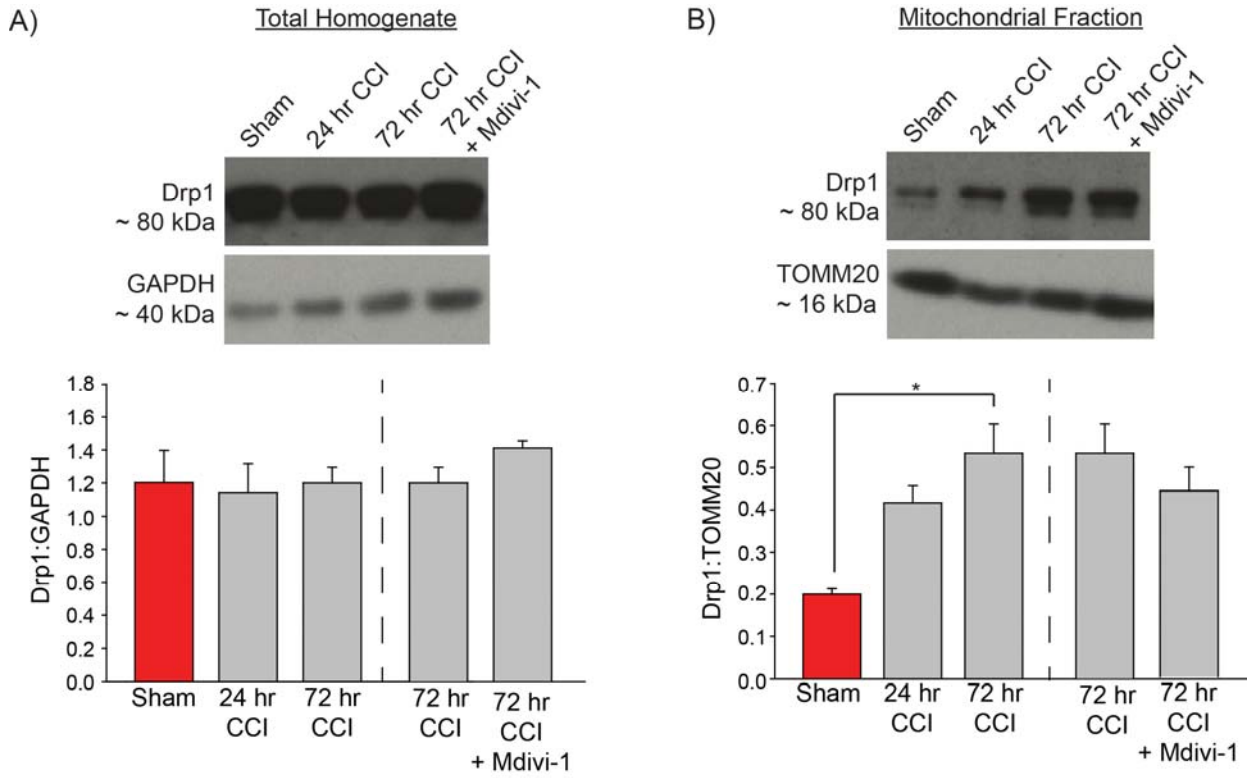

Figure 3.1 CCI injury increases Drp1 levels in mitochondrial fractions from injured hippocampus. A) Top Panel - Representative western blots of Drp1 and GAPDH immunoreactivity in ipsilateral hippocampal homogenate from sham and CCI injured (24 and 72 hours) animals, as well as 72 hour injured, Mdivi-1 treated animals (n = 3 samples/group, n = 2 hippocampi/sample); Bottom Panel – Quantifications of Drp1 immunoreactivity. Average Drp1 optical densities were normalized to GAPDH optical densities. B) Top Panel - Representative western blots of Drp1 and TOMM20 immunoreactivity in isolated ipsilateral hippocampal mitochondrial fractions from sham and CCI injured (24 and 72 hours) animals, as well as 72 hour injured, Mdivi-1 treated animals (n = 3 samples/group, n = 2 hippocampi/sample). Bottom Panel – Quantifications of Drp1 immunoreactivity. Average Drp1 optical densities normalized to TOMM20 optical densities. Data are presented as the mean +/- standard error of the mean. *p < 0.05.

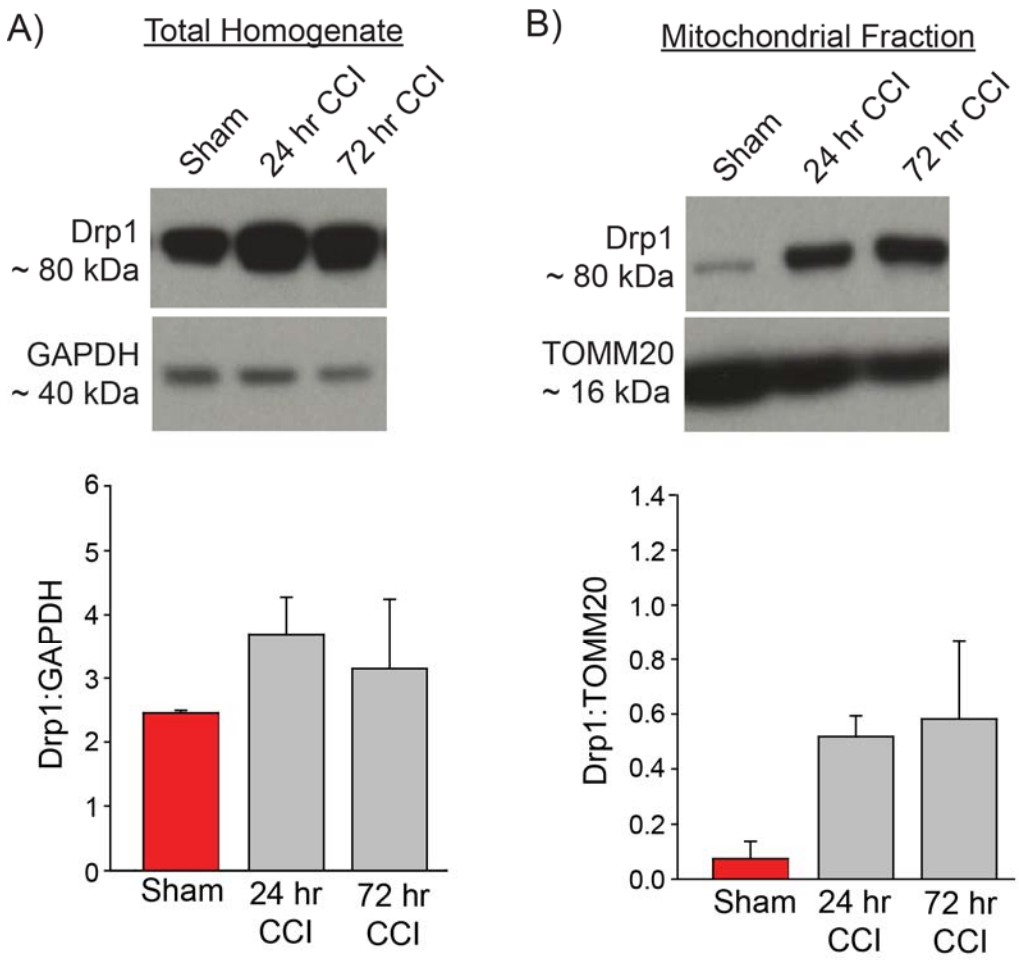

Figure 3.2 Drp1 levels in mitochondrial fractions from injured cortex. A) Top Panel - representative western blots of Drp1 and GAPDH immunoreactivity in ipsilateral cortical homogenate from sham (n = 2 samples/group, n = 2 cortices/sample), CCI injured (24 hours) (n = 2 samples/group, n = 2 cortices/sample), and CCI injured (72 hours) (n = 3 samples/group, n = 2 cortices/sample). Bottom Panel - average Drp1 optical densities were normalized to GAPDH optical densities. B) Top Panel - representative western blots of Drp1 and TOMM20 immunoreactivity in isolated ipsilateral cortical mitochondrial fractions from sham and CCI injured (24 and 72 hours) animals (n = 3 samples/group, n = 2 cortices/sample; Top Panel). Bottom Panel - average Drp1 optical densities were normalized to TOMM20 optical densities. Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

3.2 Pharmacological Inhibition of Drp1 Does Not Reduce Mitochondrial Association of Drp1 after Injury

Mdivi-1 is a pharmacological inhibitor of Drp1 that attenuates self-assembly of Drp1 into the oligomeric, rope-like structure required for membrane division.(Cassidy-stone et al., 2008) To determine whether Mdivi-1 treatment inhibits Drp1 translocation after experimental TBI, animals were treated with 3 mg/kg (i.p) starting at 30 minutes post-injury, subsequently at 24 and 72 hours post-injury, and then finally, two hours prior to dissection. Mitochondria were isolated from injured hippocampi at 72 hours post-injury (n = 3 samples/group, n = 2 hippocampi/sample) as in the previous studies. Results showed no significant differences in Drp1 immunoreactivity detected in the total homogenate ($t(4) = -1.982$, $p = 0.118$; **Fig. 3.1A – right panel**) or in the mitochondrial fraction ($t(4) = 1.072$, $p = 0.344$; **Fig. 3.1B – right panel**). Therefore, Mdivi-1 treatment does not appear to reduce Drp1 increases in the mitochondrial fraction after experimental TBI.

3.3 Drp1 is Found Localized on Outer Membranes of Isolated Mitochondria

Although Drp1 immunoreactivity was found to be increased in isolated mitochondrial fractions after CCI injury, it was still unresolved whether the increase reflected association of Drp1 to the outer membranes of mitochondria within the fraction. To determine whether the Drp1 observed in mitochondrial fractions was spatially localized on outer mitochondrial membrane, isolated mitochondrial fractions were applied and fixed onto carbon-coated copper grids for immunogold labeling and electron microscopy analysis. Immunogold labeling for mitochondrial markers was first performed to confirm isolated structures were positively labeled for mitochondria (TOMM20; **Chapter 2.4**; **Figure 2.5**). Separate grids from the same preparation were subsequently incubated with primary antibodies for Drp1 and gold-conjugated secondaries, and then negatively stained using to visualize mitochondrial structures at high magnification

transmission electron microscopy. **Figure 3.3** shows an electron micrograph of a negatively stained mitochondrion positive for Drp1 immunogold labeling. Localization of gold particles (black dots) can be seen on the outer membranes of isolated mitochondria from brain. A majority of the mitochondrial structures were positive for Drp1, however at different densities and distributions on the surface area. Gold particles were also found in various organizations on the mitochondrial membranes, with some found spatially distributed, in small clusters (2-4 gold particles), or in larger clusters (15+ gold particles). Very little gold labeling was observed in the background. Therefore, Drp1 in isolated mitochondrial fractions is associated with the outer mitochondrial membrane.

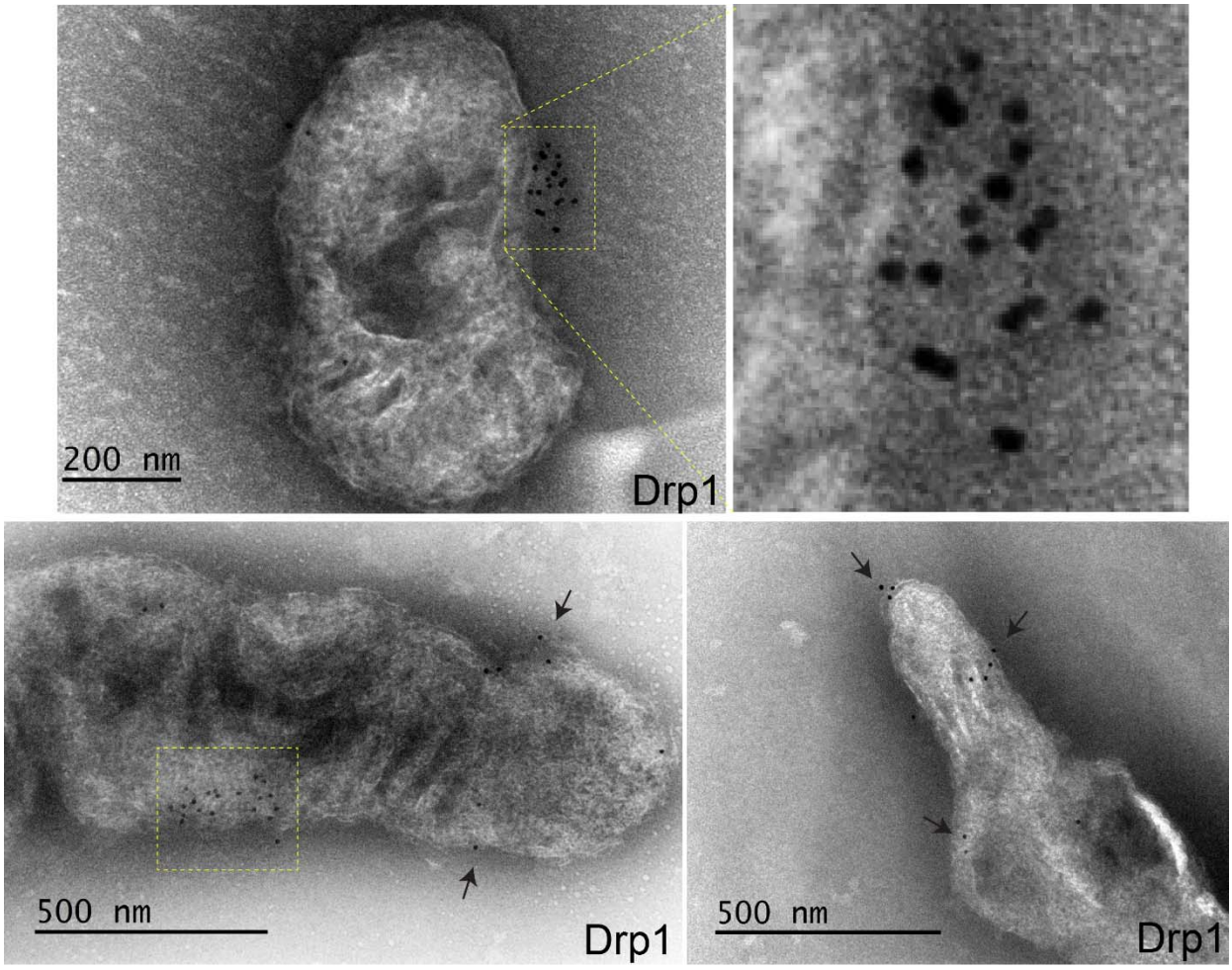

Figure 3.3 Representative electron micrographs of Drp1 immunogold labeling in isolated mitochondrial fractions. Isolated mitochondrial fractions were applied to carbon-coated copper grids and fixed with 4% paraformaldehyde for immunogold labeling and electron microscopy. Gold labeling for Drp1 in isolated mitochondrial fractions was found associated with the mitochondrial outer membranes. A heterogeneous distribution of Drp1 gold labeling on mitochondrial outer membranes was observed, often occurring as single gold particles, in small clusters (2-4 gold particles), and in large clusters (15+). Gold particles indicated by black arrows.

3.4 Mitochondrial Fusion Protein Levels are Not Significantly Altered after CCI Injury

Disruption of fusion proteins can induce unopposed fission events, leading to fragmentation of the mitochondrial network and mitochondrial dysfunction.(Burté et al., 2014; Chen et al., 2005; Detmer and Chan, 2007b) Additionally, recent evidence has suggested excessive fission can cause loss of fusion proteins, resulting in the inability of mitochondria to fuse back into the network and exacerbation of pathological fission.(Arnoult et al., 2005a; Jahani-Asl et al., 2011; Leboucher et al., 2012; Sanderson et al., 2015) For example, complete proteolytic cleavage of the inner membrane fusion protein, Opa1, into the soluble short form (S-Opa1) and release into the cytosol has been identified as an underlying mechanism contributing to stress induced mitochondrial fragmentation. (Arnoult et al., 2005a; Jahani-Asl et al., 2011; Sanderson et al., 2015) Additionally, ubiquitination and degradation of the outer membrane fusion proteins Mfn1/2 has also been observed to contribute to pathological fission states.(Cunningham et al., 2015; Gegg et al., 2010; Leboucher et al., 2012) To determine whether experimental TBI alters fusion protein levels, mitochondrial fractions from sham and CCI injured hippocampi were probed for both Opa1 and Mfn1 immunoreactivity at 24 and 72 hours post-injury (Opa1 - n = 3 samples/group, n = 2 hippocampi/sample; Mfn1 - n = 4 samples/group, n = 2 hippocampi/sample). **Figure 3.4A** and **3.4B** demonstrates representative bands from Wes analysis with molecular weights corresponding to Opa1 and Mfn1 (~100 kDa and ~84 kDa, respectively). Although results showed a slight reduction in immunoreactivity at 24 hours post-injury for both Opa1 and Mfn1, neither yielded statistical significance in a One-way ANOVA (Opa1 – $F(2,6) = 0.778$, $p = 0.501$; Mfn1 – $F(2,7) = 2.199$, $p = 0.182$). Therefore, experimental TBI does not appear to significantly alter fusion protein levels in isolated mitochondria post-injury.

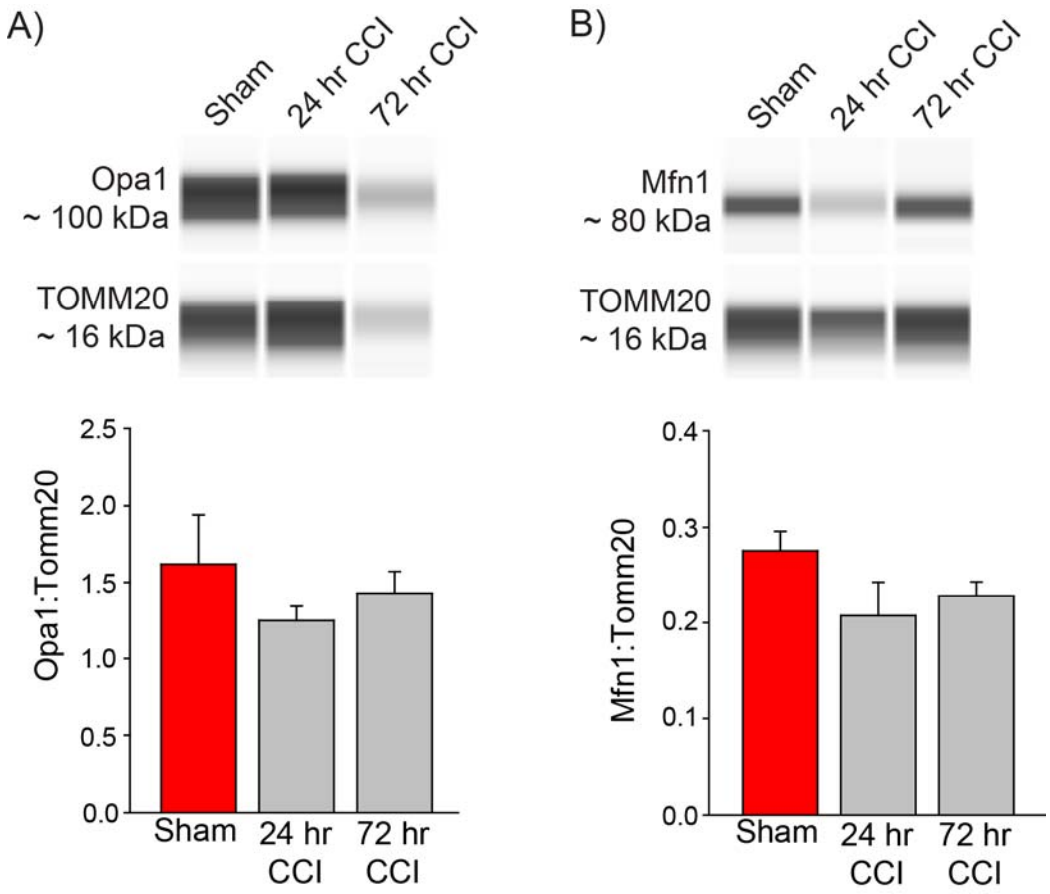

Figure 3.4 No differences of fusion protein levels in mitochondrial fractions from injured hippocampus. A) Top Panel - Representative Wes® images of Opa1 and TOMM20 immunoreactivity in isolated ipsilateral hippocampal mitochondrial fractions from sham and injured (24 and 72 hours) animals (n = 3 samples/group, n = 2 hippocampi/sample) Bottom Panel - Average Opa1 optical densities were normalized to TOMM20 optical densities. B) Top Panel - Representative Wes® images of Mfn1 and TOMM20 immunoreactivity in isolated ipsilateral hippocampal mitochondrial fractions from sham (n = 4 samples/group, n = 2 hippocampi/sample) and injured (24 and 72 hours) animals (n = 3 samples/group, n = 2 hippocampi/sample). Bottom Panel - Average Mfn1 optical densities normalized to TOMM20 optical densities. Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

3.5 CCI Injury Causes Acute Changes in Mitochondrial Morphology after Injury

Excessive mitochondrial fission causes fragmentation of the mitochondrial network that is morphologically characterized by smaller, rounder mitochondria. Therefore, to determine whether experimental TBI alters mitochondrial shape consistently with increased fission events, lengths of the long axis and short axis were measured from sham and CCI injured mitochondria isolated from hippocampus. As fixation may cause size changes of isolated organelles due to dehydration (refer to **Chapter 2, Figure 2.8A**), isolated mitochondria were cryo-preserved in ethane to better preserve the native structure for morphological analysis. Low magnification cryo-EM maps of isolated mitochondria on grids were captured to obtain length measurements from a large population of mitochondria. **Figure 3.5A** shows representative cryoEM micrographs of mitochondria isolated from sham and CCI injured hippocampal tissues at 24 and 72 hours post-injury. A varied distribution of mitochondrial lengths can be seen in all groups, demonstrating the heterogeneity in morphology of isolated mitochondria from brain. Quantification of mitochondrial lengths (n = 200) in each group (n = 1 sample/group, n = 2 hippocampi/sample) revealed significant changes at both time points after CCI injury compared to sham controls ($H = 71.413$, $p = < 0.001$; **Fig. 3.6A**). An acute increase of mitochondrial length is observed at 24 hours post-injury (diff. of ranks = 28.922, $p = < 0.05$), followed by a significant decrease at 72 hours post-injury (diff. of ranks = 18.514, $p < 0.05$). Aspect ratios (long axis/short axis; **Fig. 3.6B**) of mitochondria also showed consistently significant changes at both time-points ($H = 60.115$, $p = < 0.001$). A significant decrease at 72 hours post-injury represents a more round, fragmented population (diff. of ranks = 1.415), however the observed increase at 24 hours post-injury was not statistically significant. To examine differences in distribution of mitochondrial size, lengths were binned (binning = 0.5 μm/bin) and the number of mitochondria per bin was recorded. **Figure 3.6C** shows that in sham animals, 63.5% of the mitochondria are less than 1.5 μm in length. Twenty-four hours after CCI injury, there is a modest decrease in the number of mitochondria less than 1.5 μm (-14%) with a shift in the distribution in favor of mitochondria with

lengths between 2 μm and 4 μm (+13.5%), compared to sham animals. By 72 hours post-injury, majority of mitochondria are less than 1.5 μm and smaller (86%), with 26% less than 0.5 μm. Very few mitochondria were observed to be longer than 2 μm 72 hours post-injury (8%). Therefore, experimental injury alters mitochondrial morphology after injury, eventually resulting in a delayed decrease in size acutely after injury.

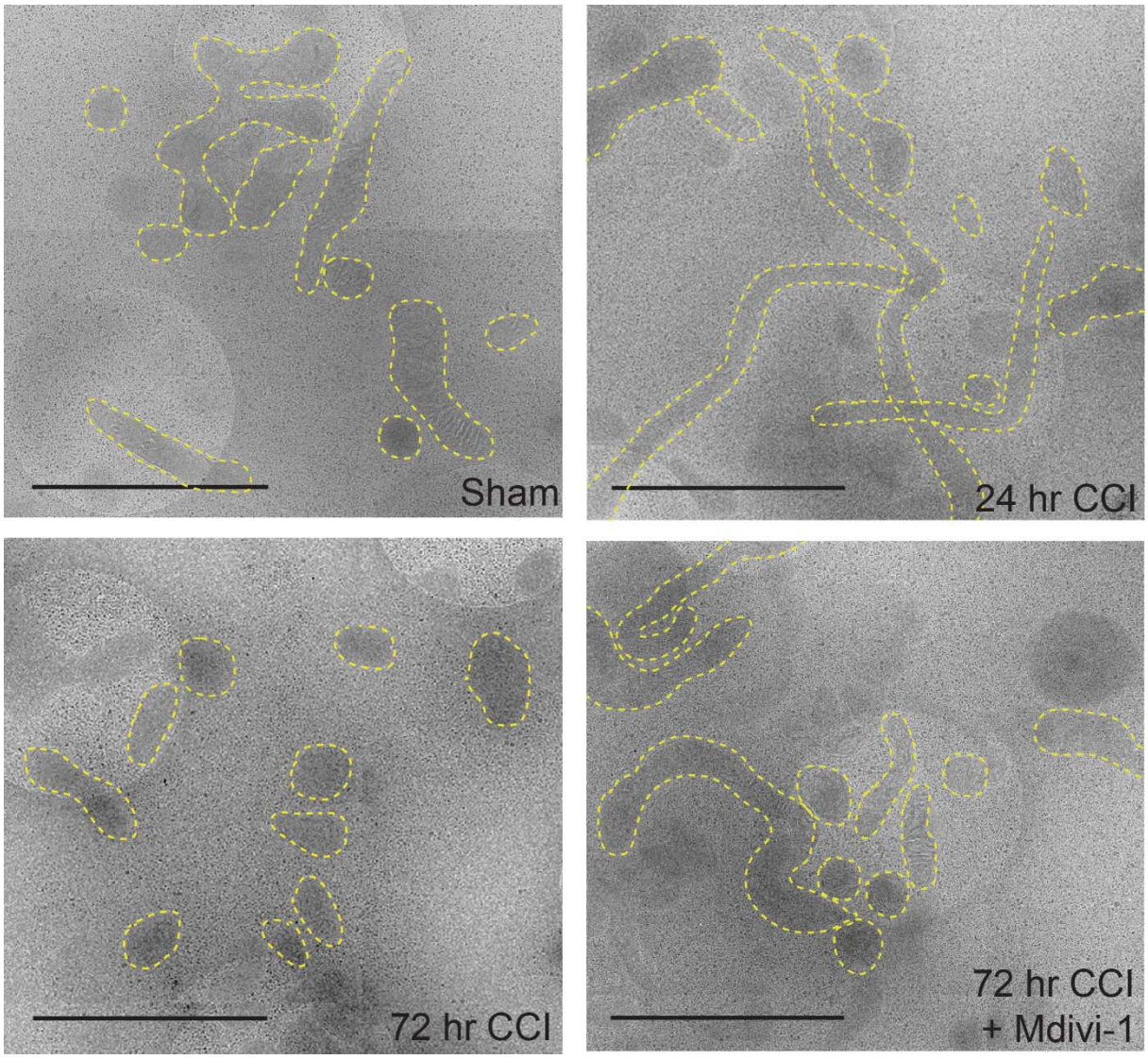

Figure 3.5 Representative cryo-electron micrographs of sham and CCI injured hippocampal mitochondria. Isolated mitochondrial fractions from the hippocampus were applied to freshly glow-discharged Quantifoil copper grids and rapidly frozen in ethane cooled to liquid N_2 temperature. Images were collected at low magnification (4700x) to capture mitochondrial populations from each group and montage maps were generated for measure lengths of the long and short axes of each mitochondria. Mitochondria are outlined in yellow for representative purposes.

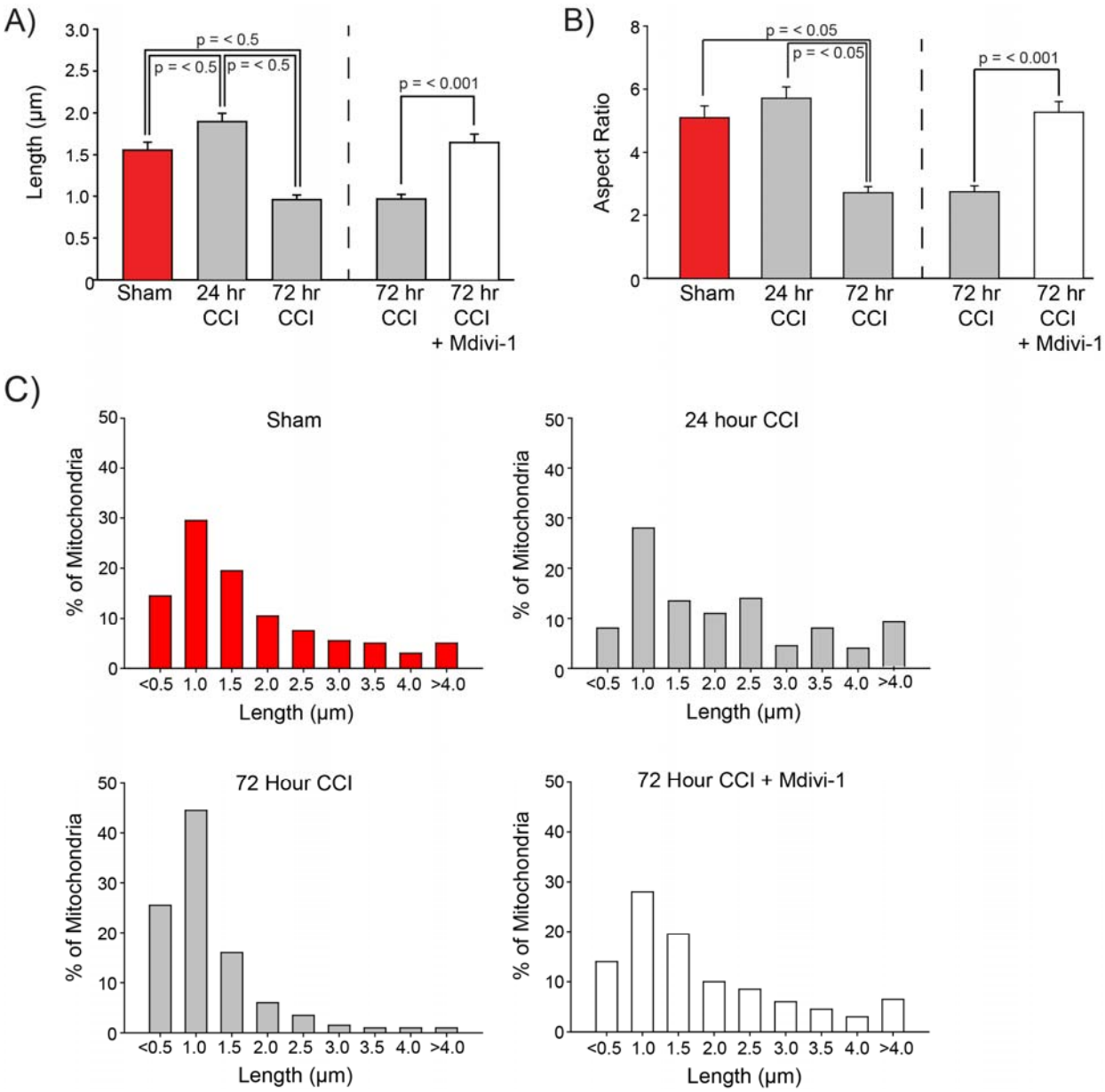

Figure 3.6 CCI injury causes a delayed decrease in mitochondrial size. Lengths were measured along the long and short axes of isolated hippocampal mitochondria (n = 200) from sham and CCI injured (24 hours and 72 hours post-injury) animals (n = 1 sample/group, n = 2 hippocampi/sample). A) Average mitochondrial lengths per group. B) Average aspect ratios (long axis/short axis) of isolated mitochondria per group. Please note that in both figures A and B, results from the 72 hour CCI group are presented twice to represent the statistical comparison to assess mitochondrial length over time after injury (left panel) and the effect of Mdivi-1 treatment at 72 hours post-CCI (right panel). C) Distribution of mitochondrial population lengths within each group. Lengths were binned in 0.5 µm increments and the percent of mitochondria within each bin is shown. X values indicate the higher bin value for each bin (i.e., 1.0 = 0.5 – 1.0 µm). Data are presented as the mean +/- standard error of the mean. *p < 0.05.

3.6 Decreases in Mitochondrial Size after Experimental TBI are Drp1-mediated

Mdivi-1 is a pharmacological inhibitor of Drp1 that has demonstrated rapid elongation and interconnectivity of mitochondrial networks in yeast and mammalian cells.(Cassidy-stone et al., 2008) Given that the maximum increase of Drp1 in mitochondrial fractions was observed at 72 hours post-injury, and there was also a significant decrease in mitochondrial size at this time point, the next question was to determine whether the changes in mitochondrial size after injury were mediated by Drp1. Therefore, a group of injured animals were administered Mdivi-1, a pharmacological inhibitor of Drp1, after CCI injury. Animals were injured using the CCI device (as in previous experiments) and were administered 3 mg/kg Mdivi-1 (i.p.) starting at 30 minutes post-injury, again at 24 and 48 hours post-injury, with a final dosage two hours prior to dissection (n = 2). Mitochondria were isolated from the ipsilateral hippocampus from Mdivi-1 treated animals 72 hours post-injury and then cryo-preserved for cryo-EM imaging and morphological analysis (n = 1 sample, n = 2 hippocampi/sample). **Figure 3.5** (bottom-right) shows a representative cryo-electron micrograph of isolated mitochondria from Mdivi-1 treated, CCI injured hippocampi. Quantification of mitochondrial lengths revealed that Mdivi-1 administration significantly increased lengths at 72 hours post-injury ($U = 13{,}097$, $p = < 0.001$; **Fig. 3.6A**) compared to untreated, CCI injured animals at the same time-point. Aspect ratio quantification also revealed a consistent, significant increase ($U = 12{,}123$, $p = < 0.001$; **Fig. 3.6B**), compared to untreated, injured animals, supporting elongation of mitochondria with Mdivi-1 administration. **Figure 3.6C** (last panel) shows number of mitochondria within the Mdivi-1 treated, CCI injured population sorted into 0.5 μm bins to examine distributions of mitochondrial lengths. The distribution of the lengths within the Mdivi-1 treated, injured population was similar to that seen in sham controls, with 61.5% of mitochondria less than 1.5 μm in length. Therefore, the decrease in mitochondrial size at 72 hours after CCI injury is mediated by Drp1. It is also important to note that in previous studies using negative stain TEM, significant injury-induced changes in size were consistently

observed at 72 hours post-injury, therefore there does not appear to be a difference of experimental outcomes when analyzed within the same EM preparation.

3.7 Decreases in Mitochondrial Size Persist for at least Eight Days after CCI Injury

Previous studies have observed mitochondrial dysfunction to persist for at least one week post-injury after experimental TBI.(Watson et al., 2014) To determine whether the decrease in mitochondrial size persists longer than the 72 hours post CCI injury, mitochondria were isolated from CCI injured hippocampi at eight days post-injury (n = 3 samples, n = 2 hippocampi/sample) and analyzed for changes in morphology. Hippocampal mitochondria were also isolated from a group of non-injured (Naïve) animals separately for comparison (n = 3 samples, n = 2 hippocampi/sample). In these studies, isolated mitochondria were applied and fixed onto carbon-coated copper grids for negative stain TEM. **Figure 3.7** shows a representative electron micrograph of isolated hippocampal mitochondrial from uninjured, naïve animals and CCI injured animals, eight days post-injury. Analysis of hippocampal mitochondrial lengths (n = ~200 mitochondria/sample) revealed a significant decrease in mitochondrial size at eight days post-injury compared to hippocampal mitochondria from naïve animals (**Fig. 3.8A**). Analysis of the distribution of mitochondrial lengths revealed a shift within the CCI injured population compared to Naïve in the less than 0.5 μm population (**Fig. 3.8B**), with an increase from 29.7% in naïve to 47% eight days post-injury. It is important to note that no observable differences of mitochondrial lengths were found between sham-injured and uninjured, naïve animals, confirming them as a proper control (data not shown).

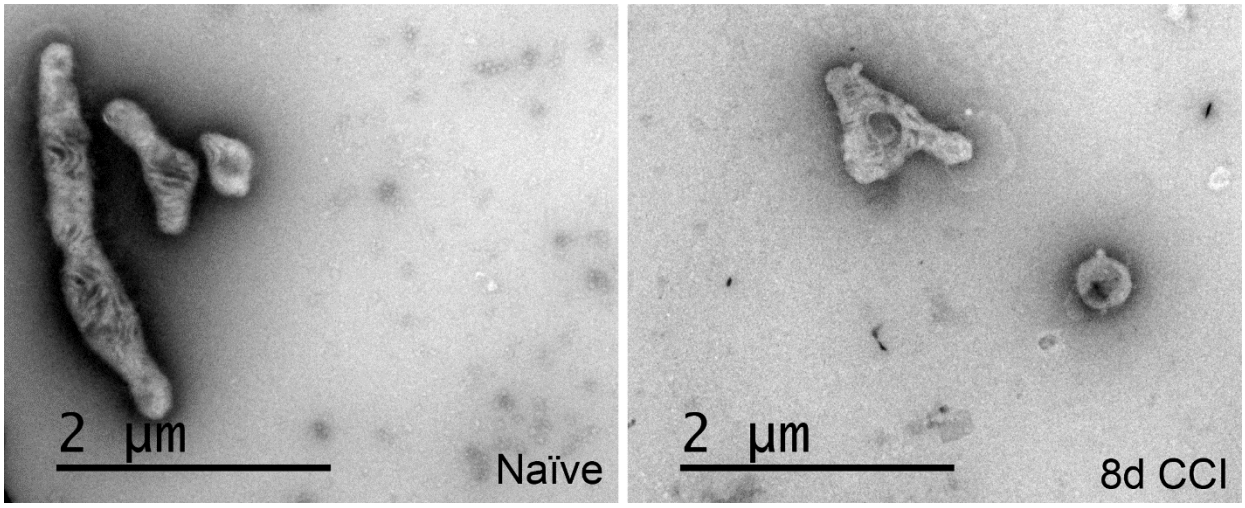

Figure 3.7 Representative negatively stained isolated hippocampal mitochondria from naïve and injured animals, 8 days post-injury. Isolated mitochondria were applied directly to freshly-glow discharged carbon-coated copper grids and fixed with 4% paraformaldehyde. Grids were then negatively stained with methylamine vanadate and TEM images were collected at 15k to capture mitochondrial populations. Length of the long axis was measured for each mitochondria.

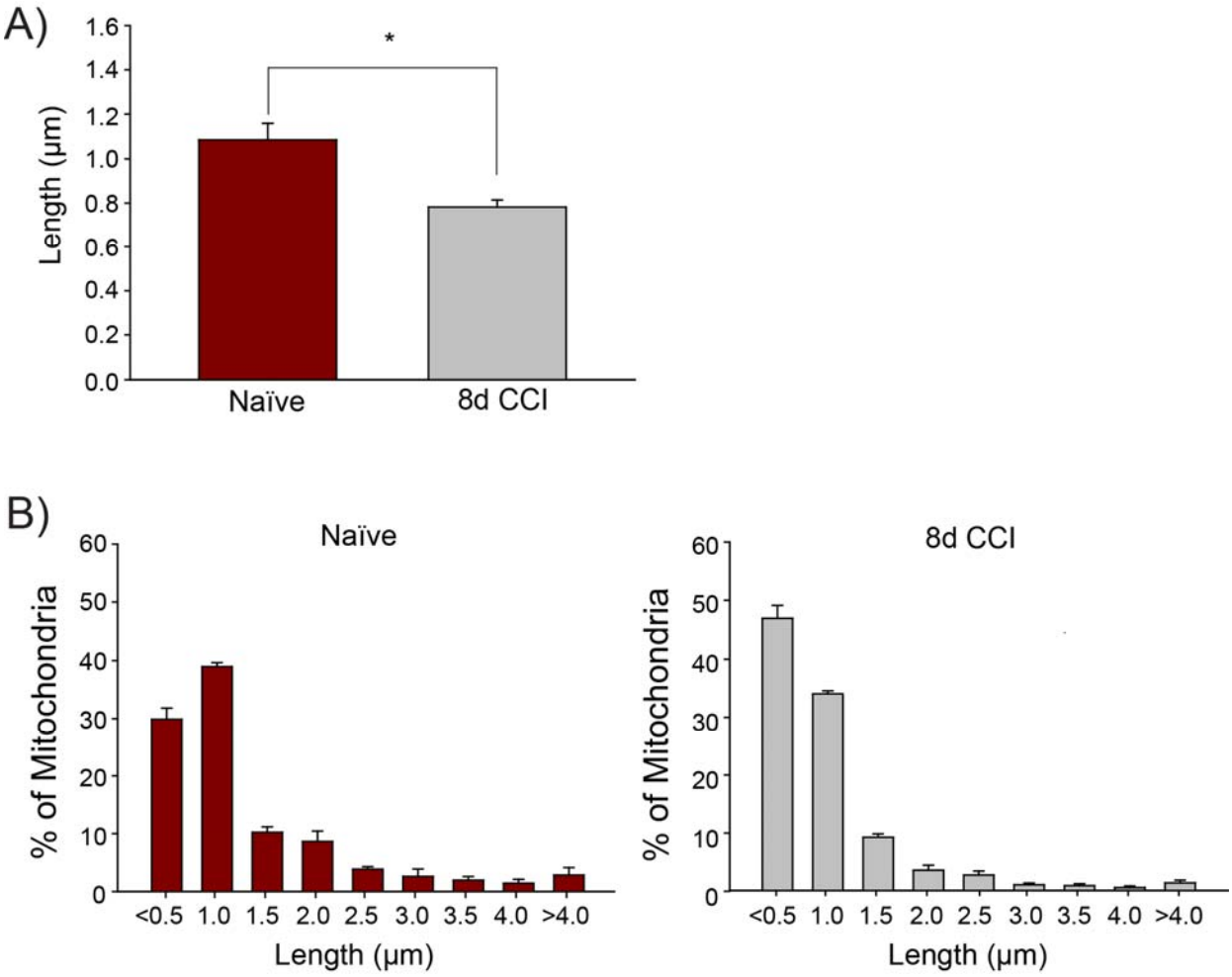

Figure 3.8 Injury induced decrease in mitochondrial size persists for at least 8 days post-injury. Lengths were measured along the long axes of isolated hippocampal mitochondria (n = ~200) from Naïve and 8 day CCI injured animals (n = 3 sample/group, n = 2 hippocampi/sample). A) Average mitochondrial lengths per group. B) Distribution of mitochondrial population lengths within each group. Lengths were binned in 0.5 µm increments and the percent of mitochondria within each bin is shown. X values indicate the higher bin value for each bin (i.e., 1.0 = 0.5 – 1.0 µm). Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

3.8 Cytochrome *c* release after TBI is Drp1-mediated

Cytochrome c is a key player in the activation of caspases in the intrinsic pathway of apoptosis.(Martinou and Youle, 2011) Redistribution of cytochrome *c* into the cytosol has been observed to be an early, time-dependent event post-injury, with significant changes at 6 hours post-injury and maximal redistribution at 24 hours post-injury.(Sullivan et al., 2002) Drp1-mediated mitochondrial fission is critical for cytochrome c release during apoptosis is a key characteristic of pathological fission events. (Martinou and Youle, 2011) Therefore, cytochrome *c* levels were detected in the mitochondrial fractions from sham and injured (vehicle and Mdivi-1 treated) hippocampi at 72 hours post-injury via western blotting to determine if cytochrome c release after TBI is mediated by Drp1. **Figure 3.9A** shows representative Wes images with immunoreactive bands at corresponding molecular weights of cytochrome *c* (~14 kDa). CCI injury caused a reduction of mitochondrial cytochrome c immunoreactivity at 72 hours post-injury in vehicle treated hippocampal mitochondrial fractions compared to sham-injured controls (**Fig. 3.9BB**). Interestingly, Mdivi-1 treated mitochondrial fractions revealed a significant increase in detected mitochondrial cytochrome *c* in comparison to vehicle treated controls and at levels comparable to sham fractions ($t(4) = -3.441$, $p = 0.026$). These findings indicate cytochrome c release after TBI is Drp1-mediated.

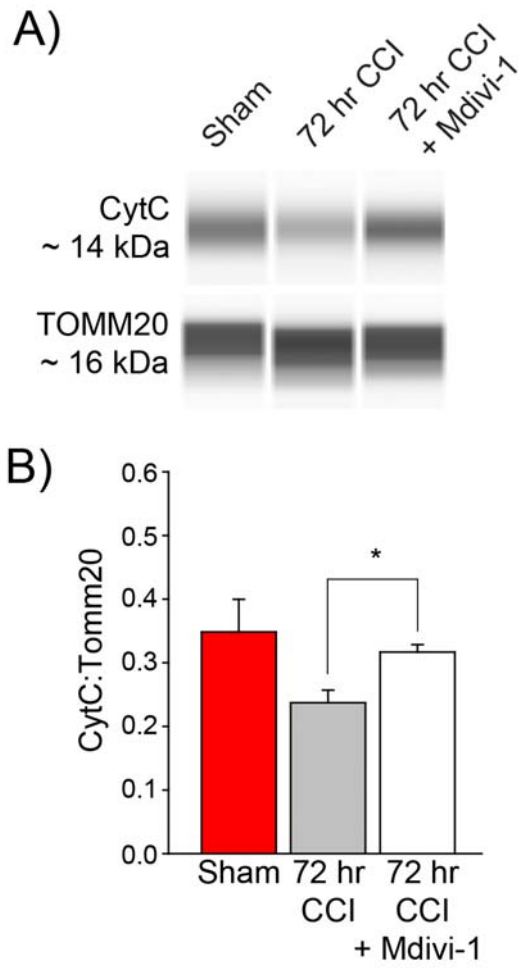

Figure 3.9 Mdivi-1 reduces cytochrome *c* release from mitochondria after injury. A) Representative Wes® results of cytochrome c immunoreactivity in isolated mitochondrial fractions from the ipsilateral hippocampi of sham (n = 3 samples/group, n = 2 hippocampi/sample) and CCI injured animals (untreated and Mdivi-1 treated) (n = 3 samples/group, n = 2 hippocampi/sample). B) Average Drp1 optical densities normalized to TOMM20 optical densities. Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

3.9 Summary

The current studies investigated mitochondrial fission after experimental TBI by examining alterations of Drp1 and mitochondrial morphology in the hippocampus using protein analysis, immunogold labeling, and electron microscopy. Results from these experiments reveal three key findings: 1) experimental TBI increases Drp1 association with mitochondria on the outer membrane, 2) TBI causes acute changes in mitochondrial morphology, eventually resulting in Drp1-mediated mitochondrial fission, and 3) cytochrome c release from mitochondria is Drp1-mediated after experimental TBI. Taken together, results from these studies demonstrate excessive Drp-1mediated fission occurs as a pathological characteristic after TBI. Therefore, strategies aimed at inhibiting Drp1 and mitochondrial fission may have potential for mitochondrial-targeted therapeutic approaches to improve outcome after injury.

Chapter 4. Mitochondrial Division Inhibitor-1 Improves Hippocampal Neuronal Survival and Cognitive Function after Experimental TBI

Clinical and experimental research has shown that structural and functional damage of mitochondria is an early event after TBI that contributes to the exacerbation and progression of secondary damage.(Cheng et al., 2012; Gajavelli et al., 2014) Mitochondrial dysfunction, including decreased respiration and ATP production, as well as oxidative damage in cortical and hippocampal mitochondria occurs acutely within 12 hours post-injury and can last up to 14 days in experimental models of TBI. (Gajavelli et al., 2014; Gilmer et al., 2009; Hill et al., 2016; Singh et al., 2006; Vink et al., 1990) Moreover, mitochondrial damage can result in the release of pro-apoptotic factors, such as cytochrome C, that activate cell death pathways and initiate apoptosis after injury.(Brustovetsky et al., 2002; Sullivan et al., 2002) As mitochondrial dysfunction occurs acutely, and is thought to contribute to early cell death and thus later behavioral deficits, mitochondrial damage may be a pivotal determinant of cell survival and long-term recovery.(Gajavelli et al., 2014) Therefore, mitochondrial-targeted therapeutic approaches to reduce mitochondrial dysfunction may offer neuroprotection and improved cognitive outcome after injury.

Mitochondria form a highly interconnected reticulum within a cell, constantly fusing together and dividing apart to properly respond to metabolic demands and maintain health of the network.(Labbé et al., 2014) Mitochondrial fusion and fission are thus tightly balanced processed that are intimately coupled to mitochondrial function and oxidative phosphorylation.(Mishra and Chan, 2016; Westermann, 2012) Imbalances of fusion and fission, such as excessive fission without sufficient fusion, can therefore be detrimental to cellular function and survival as mitochondria are no longer able to properly respond to the metabolic needs of the cell. Excessive fission, in particular, has been associated with mitochondrial dysfunction in many neurodegenerative models, including reduced respiration, oxidative stress, and release of pro-

apoptotic factors.(Burté et al., 2014; Chen et al., 2005; Itoh et al., 2013; Manczak and Reddy, 2012; Song et al., 2011; Wang et al., 2012) Recent evidence has shown that inhibiting mitochondrial fission machinery can restore equilibrium, improve mitochondrial function, and reduce apoptosis, indicating that these processes may have potential in therapeutic approaches. (Cui et al., 2010; Grohm et al., 2012; Qian et al., 2014; Rappold et al., 2014; Wu et al., 2017; Zhao et al., 2014) As mitochondrial dysfunction is central to TBI neuropathology, approaches that target the balance of fusion and fission may rescue mitochondrial function, and thus increase cell survival and improve recovery after injury.

Mitochondrial division inhibitor-1 (Mdivi-1) is a pharmacological inhibitor of Drp1, the primary protein regulator of mitochondrial fission.(Cassidy-stone et al., 2008; Tanaka and Youle, 2008) Mdivi-1 is proposed to reduce the affinity of GTP binding to Drp1 and therefore allosterically inhibit GTP-dependent Drp1 assembly and oligomerization, the first critical step in mitochondrial fission. Inhibition of Drp1 oligomerization thus also reduces subsequent GTPase activity and membrane scission downstream, creating an elongated mitochondrial network with unopposed fusion in normal conditions (**Fig. 4.1**). Mdivi-1 has been shown to attenuate apoptosis and mitochondrial dysfunction during cellular stress by reducing mitochondrial fission, and delaying cytochrome c release and apoptosis in various cell culture models, as well as in mammals. (Cassidy-stone et al., 2008; Cui et al., 2010; Grohm et al., 2012; Qian et al., 2014; Rappold et al., 2014; Wu et al., 2017; Zhao et al., 2014) Therefore, I hypothesized that Mdivi-1 treatment after injury would improve behavioral and histopathological outcome and reduce mitochondrial dysfunction after injury. To determine whether Mdivi-1 treatment would improve outcome after injury, I assessed the effect of post-injury Mdivi-1 treatment on hippocampal-dependent learning and memory, neuronal death, and mitochondrial function after injury.

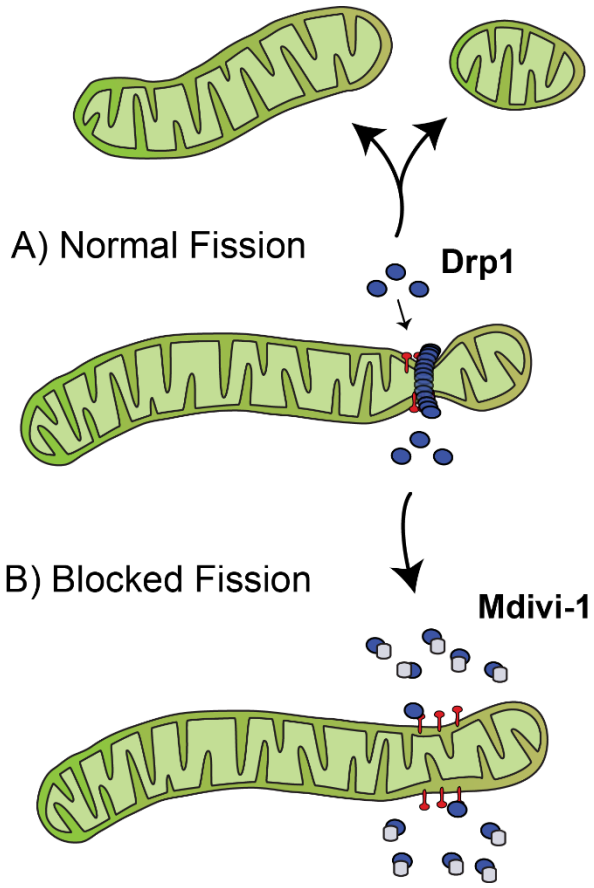

Figure 4.1 Inhibition of mitochondrial fission by Mitochondrial Division Inhibitor 1 (Mdivi-1). The proposed mechanism of action for Mdivi-1 inhibition of Drp1 is that Mdivi-1 has a high affinity for the "taut and unassembled state" of Drp1 dimers, and binds to Drp1 at an allosteric site that stabilizes this conformation. This unassembled state has a low affinity for GTP binding, and therefore Mdivi-1 reduces GTP binding affinity, GTP-mediated assembly and oligomerization of Drp1, GTPase activity, and membrane division.

4.1 Mdivi-1 Treatment Does Not Reduce Mitochondrial Dysfunction in the Hippocampus

Damage and dysfunction of mitochondria is a key characteristic of TBI pathology and is thought to be at the center of secondary injury after TBI and has been associated with the propagation of secondary cascades that cause prolonged dysfunction after injury.(Cheng et al., 2012; Gajavelli et al., 2014) Injury induced mitochondrial dysfunction is characterized by decreases in respiration, oxidative phosphorylation, and mitochondrial permeability transition with eventual release of cytochrome C and apoptosis induction. (Gajavelli et al., 2014; Gilmer et al., 2009; Hill et al., 2016; Lifshitz et al., 2004; Singh et al., 2006; Sullivan et al., 2002; Vink et al., 1990) Specifically, deficits in mitochondrial function are thought to precede cell death and cognitive deficits, with reduced respiration and oxidative phosphorylation observed in the as early as 3 hours, peaking at 72 hours, and persisting as long as 14 days post-injury.(Hill et al., 2016; Lifshitz et al., 2003; Singh et al., 2006; Watson et al., 2014; Xiong et al., 1997) To determine whether Mdivi-1 treatment reduces mitochondrial dysfunction after experimental TBI, respiration (O_2 consumption) of isolated hippocampal mitochondria from Vehicle-treated and Mdivi-1 treated animals was measured 72 hours after CCI injury.

Animals were treated with either vehicle (DMSO:Saline) or Mdivi-1 (3 mg/kg) starting 30 minutes post-injury, with two more injections at 24 and 48 hours post-injury (same paradigm as behavioral assessments). Mitochondria were isolated from sham and injured hippocampal tissues at 72 hours post-injury using the same procedures described in Chapter Two and Three. Mitochondrial function was assessed using the Seahorse XFe96 analyzer and determined via measurement of oxygen consumption rates (pmol/min) of isolated organelles in a classical mitochondrial respiratory control assay. **Figure 4.2** shows the sequence of poison addition and time course of oxygen measurements within each condition from the experiment. Several measures of mitochondrial function can be analyzed based on changes in oxygen consumptions rates in each poison condition, including respiratory States as described by Chance and Williams (1956), and respiratory control.

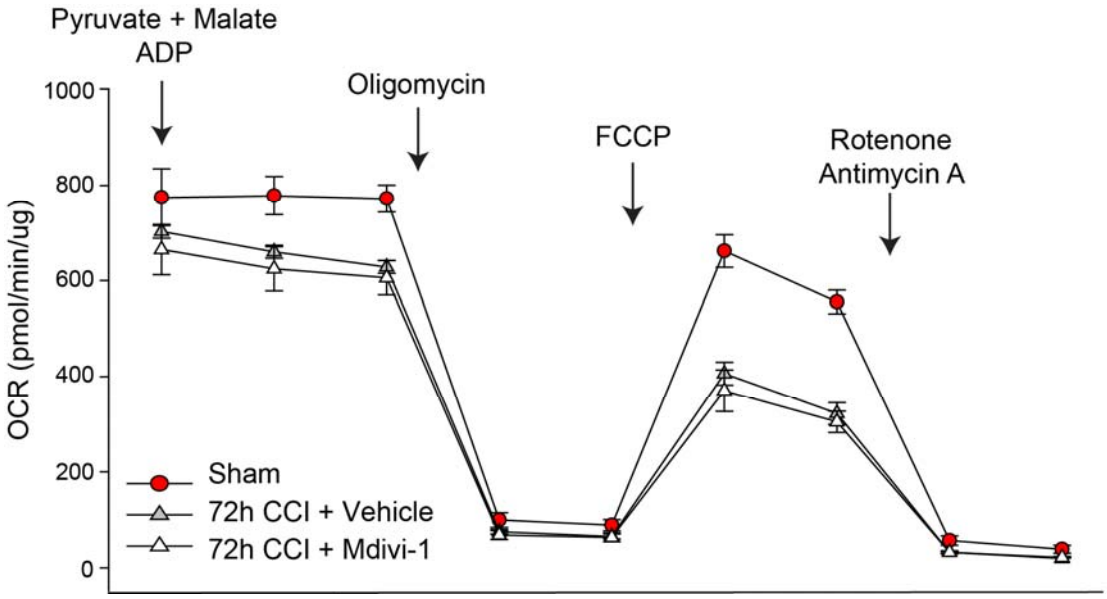

Figure 4.2 Time-course of oxygen consumption rates from sham and injured isolated mitochondria in a classical mitochondrial coupling assay. Mitochondria were isolated from sham (n = 2 samples, n = 2 hippocampi/sample), Vehicle-treated, CCI injured (n = 3 samples, n = 2 hippocampi/sample), and Mdivi-1 treated, CCI injured (n = 3 samples, n = 2 hippocampi/sample) 72 hours post-injury. Each point of measure is an average of replicates (n = 10/condition) across time in the measuring period for each sample in the assay. Respiration was assessed in freshly isolated mitochondria, starting in a state of "normal" respiration with TCA substrates (pyruvate and Malate) and ADP present. Respiration was then measured with subsequent addition of mitochondrial poisons, including Oligomycin (1 μM), FCCP (4 μM), and a combination of Rotenone (2 μM) and Antimycin A (4 μM).

State III-ADP is representative of a “basal” respiration state defined as respiration with substrates (pyruvate and malate) and ADP present. A significant difference in State III-ADP respiration of isolated hippocampal mitochondria was detected between groups ($F(2,5) = 7.542$, $p = 0.031$; **Fig. 4.3 – Top, Right Panel**). Compared to Sham controls, StateIII-ADP was non-significantly reduced in the CCI Injured, Vehicle treated condition ($p = 0.079$), and significantly reduced in the CCI injured, Mdivi-1 treated condition ($p = 0.041$). However, isolated mitochondria from injured, Mdivi-1 treated hippocampi showed no difference in State III-ADP oxygen consumption rates compared to vehicle-treated controls, indicating that Mdivi-1 treatment does not reduce mitochondrial dysfunction after injury ($p = 1.00$).

State III-uncoupled can be defined as respiration in the presence of substrates, ADP, and an uncoupler (FCCP). State III-uncoupled is representative of uninhibited electron flow through the electron transport chain and oxygen consumption in isolated mitochondria. A consistent significant reduction in State III-uncoupled respiration was also observed between groups ($F(2,5) = 14.972$, $p = 0.008$)**; Fig. 4.3 – Top, Right Panel**). Compared to Sham controls, StateIII-Uncoupled was significantly reduced in the CCI Injured, Vehicle treated condition ($p = 0.020$), and in the CCI injured, Mdivi-1 treated condition ($p = 0.010$). However, no significant differences were observed in uncoupled respiration with Mdivi-1 treatment after injury, compared to Vehicle treated controls ($p = 1.00$). It is important to note that the observed ~40% reduction of oxygen consumption rates between the initial State III-ADP condition and the FCCP, uncoupler-stimulated condition in both injured mitochondrial samples. There is some evidence that “injured” mitochondria can experience “rundown”, in which respiratory capacities slowly decline across time when incubated at 37°C and constant respiration in these assays. Additionally, the decrease of uncoupler-stimulated respiration could also reflect a potential defect in substrate utilization, which would be challenged upon maximal electron flow through the electron transport chain. Further experimental analysis is required to determine whether these factors contribute to the

observed results. Nonetheless, these data do reflect respiratory dysfunction in isolated hippocampal mitochondria after CCI injury compared to sham controls.

State IV-oligomycin (StateIVo) represents a state of respiration in the presence of substrates, ADP, and oligomycin, an ATP synthase inhibitor, and reflects oxygen consumption without ATP synthesis. Oxygen consumption without ATP synthesis, therefore can be interpreted as potential proton leakage from the electron transport chain that would allow for electron flow without dissipation of the electrochemical gradient provided by ATP synthase. No differences were found with either CCI injury or treatment in State IVo respiration (**Fig. 3 – Bottom, Left Panel**), indicating that CCI injury does not result in significant leakiness of the electron transport chain in isolated hippocampal mitochondria.

Finally, a ratio can be calculated from oxygen rates in the StateIII-ADP and StateIVo conditions that reflect “respiratory coupling”, or the coupling of respiration and phosphorylation (**Fig. 4.3 – Bottom, Right Panel**). Interestingly, there were no significant differences observed between groups in the respiratory control ratio (RCR), indicating that mitochondrial respiration was, in fact, coupled to ATP production in isolated mitochondria regardless of injury or treatment. Overall, results from these studies show that mitochondria isolated from the injured hippocampus 72 hours after injury are dysfunctional, and Mdivi-1 treatment after injury does not preserve mitochondrial function acutely after injury.

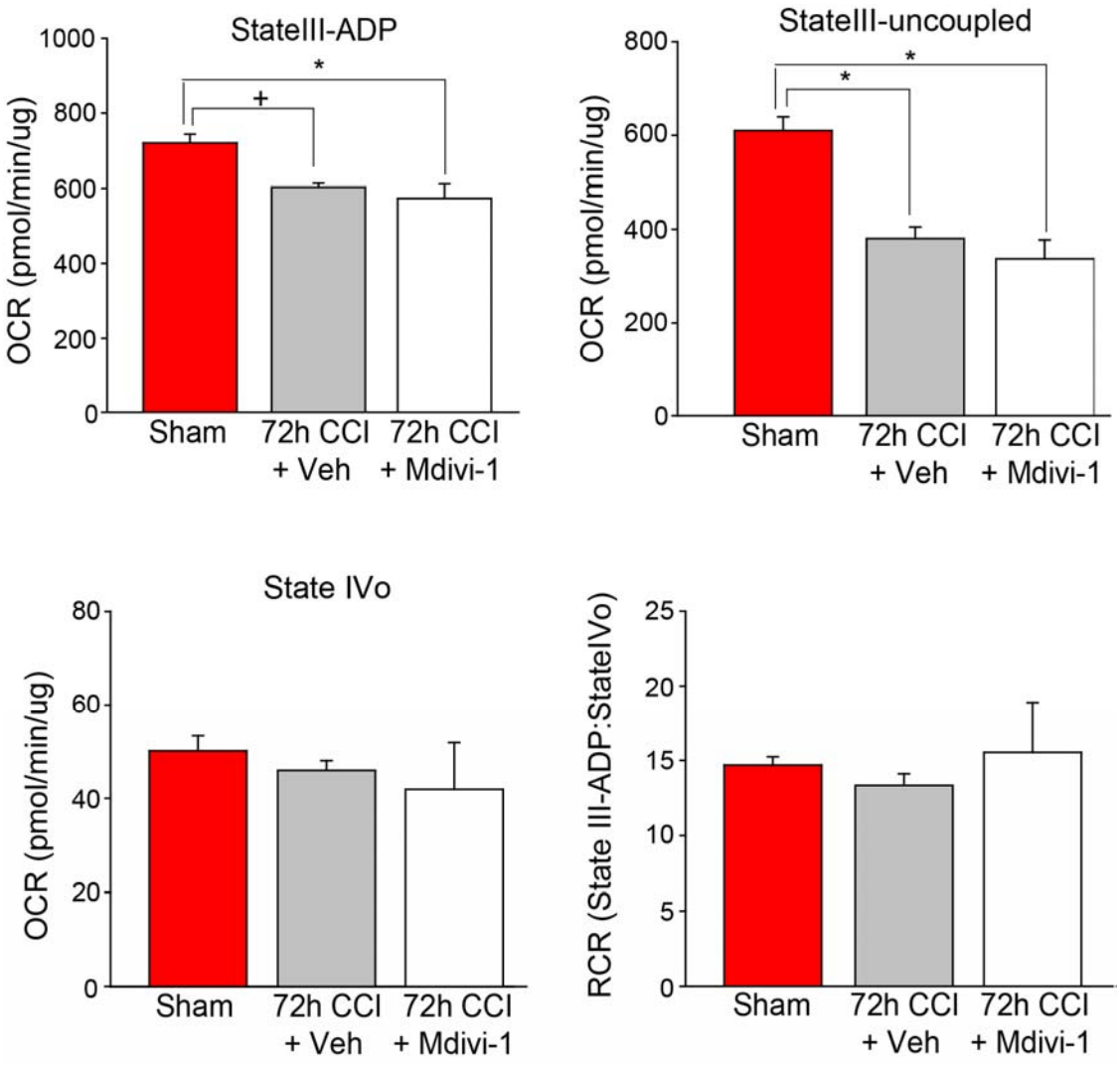

Figure 4.3 Mdivi-1 does not preserve mitochondrial function in the hippocampus after injury. Calculations and analysis of respiratory states in isolated mitochondrial during the classic coupling assay. State III-ADP (top-right panel) represents phosphorylating respiration in a coupled state with TCA substrates and ADP present. Values were taken from the third measurement within this condition. State III-uncoupled (top-right) is respiration in the presence of an uncoupler (FCCP) and represents oxygen consumption without the limitation of the electrochemical gradient. State IV-oligomycin (bottom-left) is respiration in the presence of the ATP synthase inhibitor, oligomycin, and represents the limitations of the electrochemical gradient in a hyperpolarized state. Respiratory Control Ratio (RCR; bottom-right panel) compares values during State III-ADP and State IV-oligomycin and represents coupling of respiration and phosphorylation of ADP. Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

4.2 Mdivi-1 Treatment Improves Neuronal Survival after Injury

The hippocampus is a particularly vulnerable region that experiences regional, and cell-type specific pathologies after both clinical and experimental TBI.(Bigler et al., 1997; Chen et al., 2003b; Marquez de la Plata et al., 2011; Osier et al., 2014) Specifically, death of newborn neurons in the dentate gyrus, as well as mature pyramidal neurons in the CA1 and CA3 regions are characteristic in the CCI model.(Baldwin et al., 1997; Gao et al., 2008; Hall et al., 2005; Scheff and Price, 2005) To determine whether Mdivi-1 treatment reduces hippocampal neuronal loss after injury, histopathology in hippocampal tissues from CCI injured, vehicle treated and CCI injured, Mdivi-1 treated animals were assessed. Tissues were stained for newborn neuronal markers (DCX), mature neuronal markers (NeuN), and dendritic markers (Map2) using immunohistochemical and immunofluorescent approaches. CCI injured animals received either vehicle (DMSO:Saline) or 3 mg/kg Mdivi-1 (n = 5/group) at 30 minutes post-injury, with a second treatment at eight hours post-injury, prior to sacrifice at 24 hours post-injury. A group of sham animals were also simultaneously prepared for comparison (n = 5).

Doublecortin (DCX) is a microtubule-associated protein that is expressed between differentiation and migration stages in embryonic and adult neurogenesis, and is a well-established marker for immature neurons.(Von Bohlen Und Halbach, 2007; Ming and Song, 2011) Loss of newborn, DCX-expressing neurons in the dentate gyrus of the hippocampus has been observed as early as four hours post-injury.(Gao et al., 2008) Therefore, to determine if Mdivi-1 treatment after CCI injury reduced the loss of newborn neurons, hippocampal tissues from sham and CCI injured (Vehicle treated and Mdivi-1 treated) 24 hours after injury were stained with DCX-specific primary antibodies and positive cell populations in the dentate gyrus were quantified. **Figure 4.4A** shows representative photomicrographs of DCX-positive cells in the subgranular zone of the ipsilateral hippocampus from sham and CCI injured (vehicle treated and Mdivi-1 treated) animals. Consistent with previous studies, CCI injury caused a marked reduction of DCX-positive neurons in the ipsilateral hippocampus 24 hours after injury compared

to sham animals (t(8) = 4.817, p = 0.001; **Fig. 4.4B**). Interestingly, Mdivi-1 treatment significantly attenuated the loss of the DCX-positive cell population in the ipsilateral dentate gyrus after injury (t(8) = -2.381, p = 0.044), indicating preservation of newly born neurons after CCI injury. No significant differences were observed on the contralateral side between all groups (**Fig. 4.4B**). Therefore, Mdivi-1 treatment increases cell survival of newly born neurons in the hippocampus after injury.

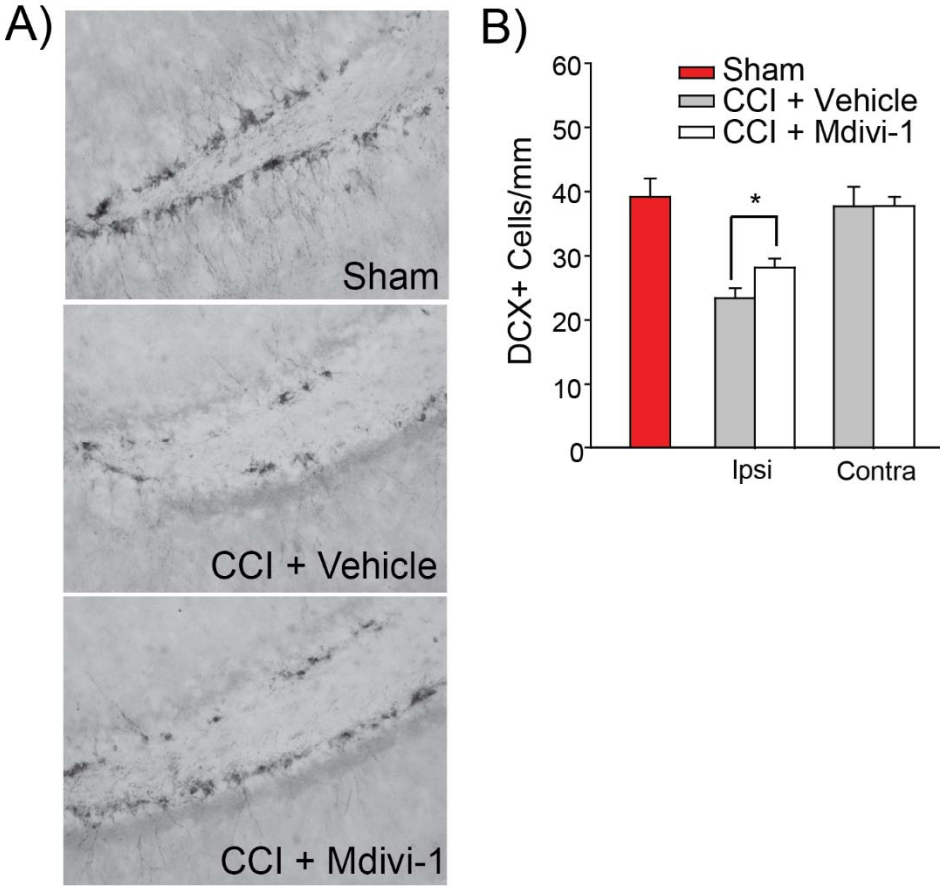

Figure 4.4 Mdivi-1 reduces immature neuronal loss in the dentate gyrus of the hippocampus after CCI injury. (A) Representative photomicrographs of Doublecortin (DCX) positive staining in the dentate gyrus of the ipsilateral hippocampus in a sham and CCI injured animals (vehicle-treated and Mdivi-1 treated) 24 hours post-injury. (B) Quantification of DCX+ cells in the ipsilateral and contralateral dentate gyrus for each group. DCX-positive cell counts for both hippocampi were averaged, as there is no ipsilateral or contralateral factor to sham injury. Data are presented as the mean +/- standard error of the mean. *p < 0.05.

Loss of mature neuronal populations in the CA1 and CA3 regions of the hippocampus are also characteristic of the CCI injury model.(Baldwin et al., 1997; Colicos et al., 1996; Osier et al., 2014; Scheff and Price, 2005) Pyramidal somata in the CA3 region are particularly vulnerable, with rapid cell loss observed as early as one hour post-injury, and maximal loss by 24 hours.(Baldwin et al., 1997) Cell loss in the CA1 region is slightly delayed and less pronounced, with significant observations of death found as early as 24 hours post-injury, peaking at 48 hours post-injury.(Hall et al., 2005) NeuN is a soluble nuclear protein that is expressed in most mature neuronal cell types in the brain and is a common marker for postmitotic neuronal nuclei.(Von Bohlen Und Halbach, 2007) NeuN-positive staining in the hippocampus can be seen in **Figure 4.5A**, in which the cell layers of the hippocampus are clearly defined, including CA1 and CA3. Loss of NeuN-positive cells can be seen within the CA1 and CA3 layers in both CCI injured vehicle-treated and Mdivi-1 treated hippocampi compared to sham animals (**Fig. 4.5B**). However, there does appear to be subtle preservation of NeuN-positive staining observed in both these areas with Mdivi-1 treatment, suggesting attenuation of mature neuronal loss at 24 hours post-injury. However, without quantification of the NeuN-positive cell populations within the CA1 and CA3 areas, it cannot be concluded that the observed differences with Mdivi-1 treatment is in fact significant attenuation of cell loss after injury.

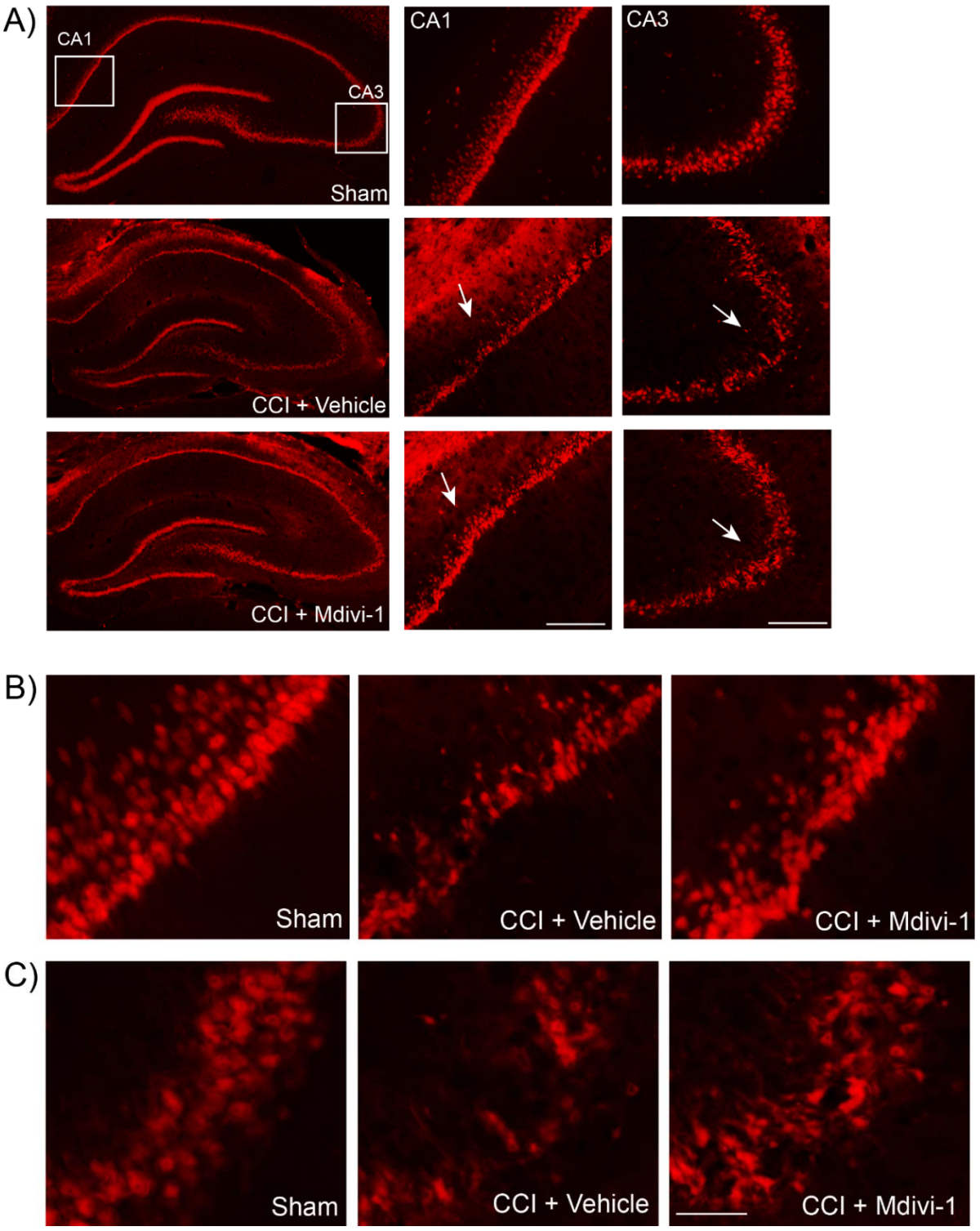

Figure 4.5 NeuN-positive cell populations in the hippocampi of sham, vehicle treated, CCI-injured, and Mdivi-1 treated, CCI-injured animals. A) Representative low-magnification photomicrographs (2.5x) of NeuN staining in the entire ipsilateral hippocampus (left panel) and CA1 and CA3 (right panels; 10x magnification) of sham and CCI injured animals (vehicle-treated and Mdivi-1 treated) 24 hours post-injury. B) High magnification of the CA1 area in the hippocampus. C) High magnification (20x) of the CA3 area in the hippocampus. Loss of NeuN immunoreactivity can be seen in both CCI injured conditions (Vehicle-treated and Mdivi-1 treated). Scale bar = 200 μm.

In addition to loss of pyramidal somota in the CA1 and CA3 neuronal layers, these areas also experience selective dendritic loss primarily within 48 hours after injury.(Hall et al., 2005; Scheff and Price, 2005) In particular, dendritic loss in CA1 is most robust due to death of pyramidal neurons in CA3 and deafferentation of their projections through the Schaffer Collateral pathway. (Baldwin et al., 1997; Colicos et al., 1996; Scheff and Price, 2005) Microtubule-associated protein 2 (Map2) is a cytoskeletal protein primarily expressed in the soma and dendrites of neurons and is common marker to assess dendritic loss within the hippocampus. **Figure 4.6** shows representative photomicrographs of Map2-positive staining in the CA1 and CA3 regions of hippocampal tissues from sham and injured animals (vehicle-treated and Mdivi-1 treated) 24 hours post-injury. Subtle loss of Map2-positive staining can be seen in both CCI injured groups in the CA1 and CA3 regions compared to sham animals (**Fig. 4.6A** and **4.6B**, respectively). Mdivi-1 treatment did appear to modestly preserve Map2-positive immunoreactivity, primarily in the CA1 region, however dendritic loss due to injury was minor and variable within the CCI injured, Vehicle-treated group, making interpretation difficult. It is also possible that 24 hours may be too early of a time-point to observe significant Map2 loss, as maximum dendritic loss is typically observed by 48 hours post-injury, providing a potential explanation to the minor loss and variability observed in this study.(Hall et al., 2005; Scheff and Price, 2005) Nonetheless, without quantification of Map2 immunoreactivity, it cannot be concluded that the observed preservation with Mdivi-1 treatment is in fact significant attenuation of dendritic loss after injury.

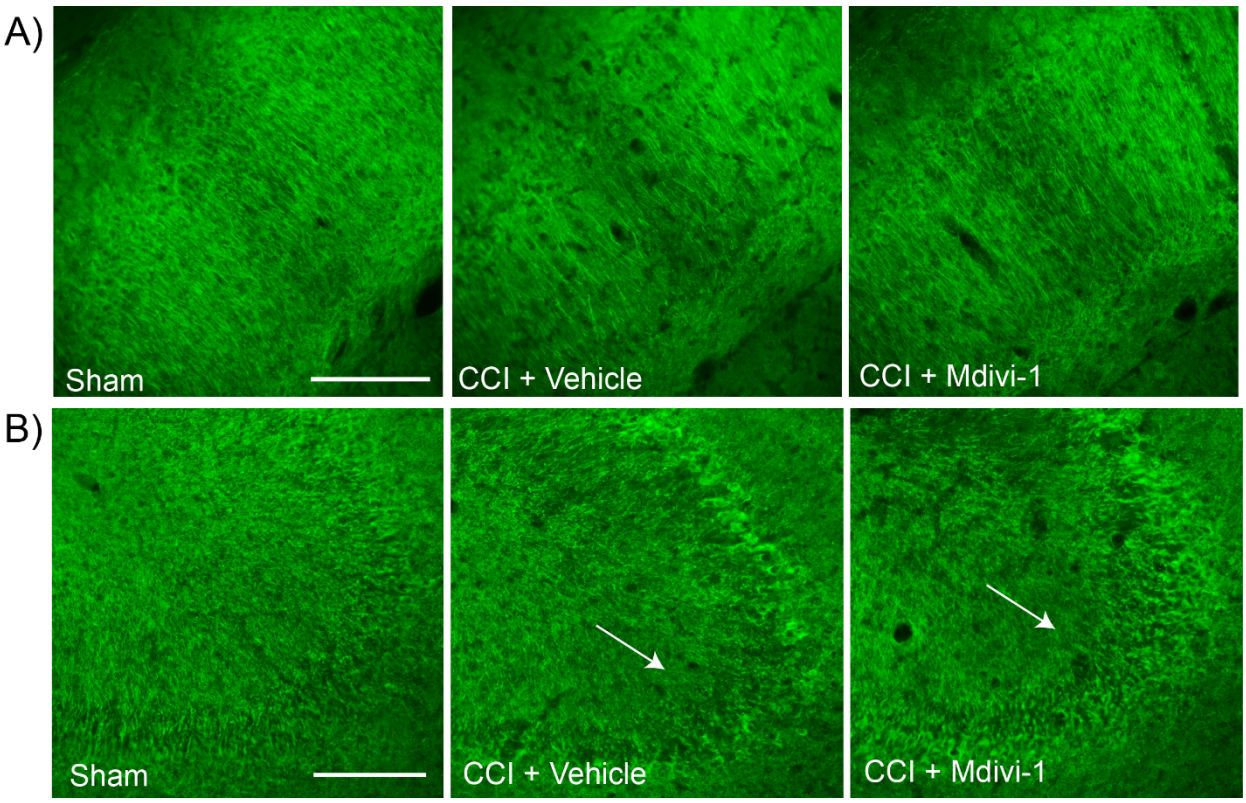

Figure 4.6 Mdivi-1 may reduce dendritic loss in hippocampal areas CA1 and CA3 after CCI injury. Representative high-magnification (photomicrographs of Map2 staining in area (A) CA1 (20x) and (B) CA3 (10x). Loss of Map2 immunoreactivity can be seen in both CCI injured conditions (Vehicle-treated and Mdivi-1 treated). However, Mdivi-1 treated, CCI injured animals appear to have modest attenuation of Map2 loss within these areas. Scale bar = 200 μm.

4.3 Mdivi-1 Treatment Does Not Reduce Astrocytic Inflammation after CCI Injury

Inflammation is typical response of tissue damage and a key characteristic of secondary injury in neuropathology after experimental and clinical TBI.(Corps et al., 2015) The role of the inflammatory response after injury has been widely debated, and is now well accepted that neuroinflammation exhibits both harmful and beneficial effects.(Burda and Sofroniew, 2014; Karve et al., 2016; Woodcock and Morganti-Kossmann, 2013) Astrocytes, in particular, become reactive after injury, in which they proliferate and migrate towards damaged areas, display a hypertrophic morphology with extended processes, and alter gene expression.(Burda and Sofroniew, 2014; Karve et al., 2016; Myer et al., 2006) Reactive astrogliosis has been found to occur as early as 24 hours post-injury and last for up to 60 days in experimental models of TBI and is typically detected by the upregulation of glial fibrillary protein (GFAP).(Susarla et al., 2014; Villapol et al., 2014) Although it is difficult to determine whether reactive astrogliosis is protective or deleterious, it is still widely used as an indicator for severity of damage.(Burda and Sofroniew, 2014; Karve et al., 2016; Myer et al., 2006) Therefore, hippocampal tissues from the same sham and injured animals (vehicle-treated and Mdivi-1 treated) at 24 hours post-injury were immunostained for GFAP to detect reactive astrogliosis in the hippocampus after injury. **Figure 4.7** shows representative photomicrographs of GFAP-positive staining in the hippocampus from sham and injured (vehicle-treated and Mdivi-1 treated) animals. Increased immunoreactivity of GFAP is observed in both the vehicle-treated and Mdivi-1 treated hippocampal tissues after CCI injury compared to sham animals. No observable differences in GFAP expression or distribution were found with Mdivi-1 treatment. Therefore, Mdivi-1 treatment does not appear to alter inflammatory responses of astrocytes 24 hours after experimental TBI.

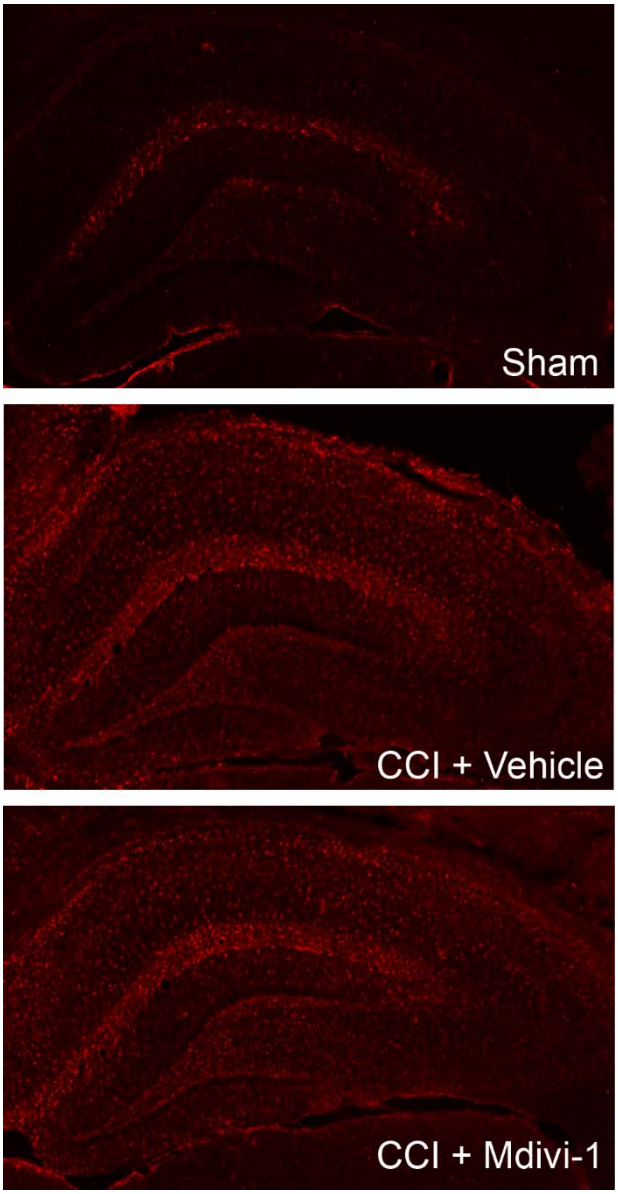

Figure 4.7 Mdivi-1 may does not reduce astrocytic inflammation in the hippocampus after CCI injury. Representative micrographs of GFAP immunoreactivity in the ipsilateral hippocampus in sham and injured (Vehicle-treated and Mdivi-1 treated) animals.

4.4 Mdivi-1 Treatment Reduces Deficits in Hippocampal-Dependent Recognition and Contextual Fear Memory after CCI injury

The hippocampus is a primary area of damage in the controlled cortical impact (CCI) model of experimental TBI.(Baldwin et al., 1997; Colicos et al., 1996; Dixon et al., 2016; Edward Dixon et al., 1991) Hippocampal-dependent cognitive deficits are thus a common characteristic of CCI injury, and represent a functional indicator to the severity of hippocampal pathology after injury.(DASH et al., 1995; Hamm, 1992; Osier et al., 2014) To determine whether Mdivi-1 treatment improves cognitive outcome after injury, CCI injured animals were administered Mdivi-1 treatment and assessed on three hippocampal-dependent tasks, the Novel Object Recognition (NOR) task, a Contextual Fear Conditioning task, and the Morris Water Maze. CCI injured animals were randomly assigned to receive either vehicle treatment (50% DMSO:Saline) or Mdivi-1 (3 mg/kg) systemically by intraperitoneal injection. This dosage was chosen due to previous reports of neuroprotection in other injury and disease models.(Grohm et al., 2012; Rappold et al., 2014) Animals received treatment starting at 30 minutes post-injury, and again at 24 hours and 48 hours post-injury and did not receive any further treatment. **Figure 4.8** illustrates a timeline of treatment and behavioral assessment after injury. Mdivi-1 treatment did not intersect with cognitive assessments, as animals did not receive any additional treatments after the final dosage at 48 hours post-injury. Motor performance was first assessed starting 24 hours post-injury and subsequently at 48 and 72 hours post-injury. CCI injury caused significant deficits in both the Foot Fault test (Left Foot: $F_{(2,21)} = 5.857$, $p = 0.009$; **Fig. 4.9A**) and the Beam Balance test ($F_{(2,22)} = 5.835$, $p = 0.009$; **Fig. 4.9B**). Mdivi-1 treatment did not significantly affect motor performance in either of the tasks across all days of testing (Vehicle vs Mdivi-1: Foot Fault (Left Foot): $p = 0.396$; Beam Balance: $p = 0.334$). Therefore Mdivi-1 treatment did not improve motor deficits acutely after injury.

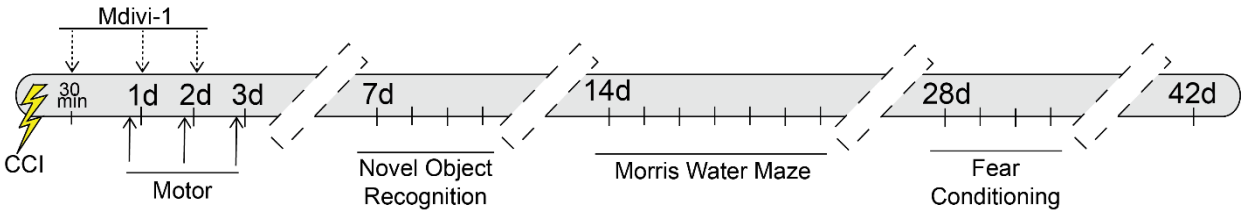

Figure 4.8 Timeline of injury, treatment, and behavioral testing. Animals were randomly assigned to either receive sham injury (n = 5) or controlled cortical impact (CCI) injury (n = 20). CCI injured animals were then randomly assigned to receive vehicle treatment (n = 10) or Mdivi-1 treatment (n = 10). Animals were injured and received intraperatoneal injections of either vehicle (DMSO:Saline) or Mdivi-1 (3 mg/kg) starting at 30 minutes post-injury, and subsequently at 24 and 48 hours post-injury. Motor performance was tested for the three days, starting 24 hours post-injury. Novel Object Recognition was performed from days 7 to 9 post-injury and Contextual. Morris Water Maze was tested for seven days, starting 14 days post-injury. Fear Conditioning was performed days 28 and 29 post-injury.

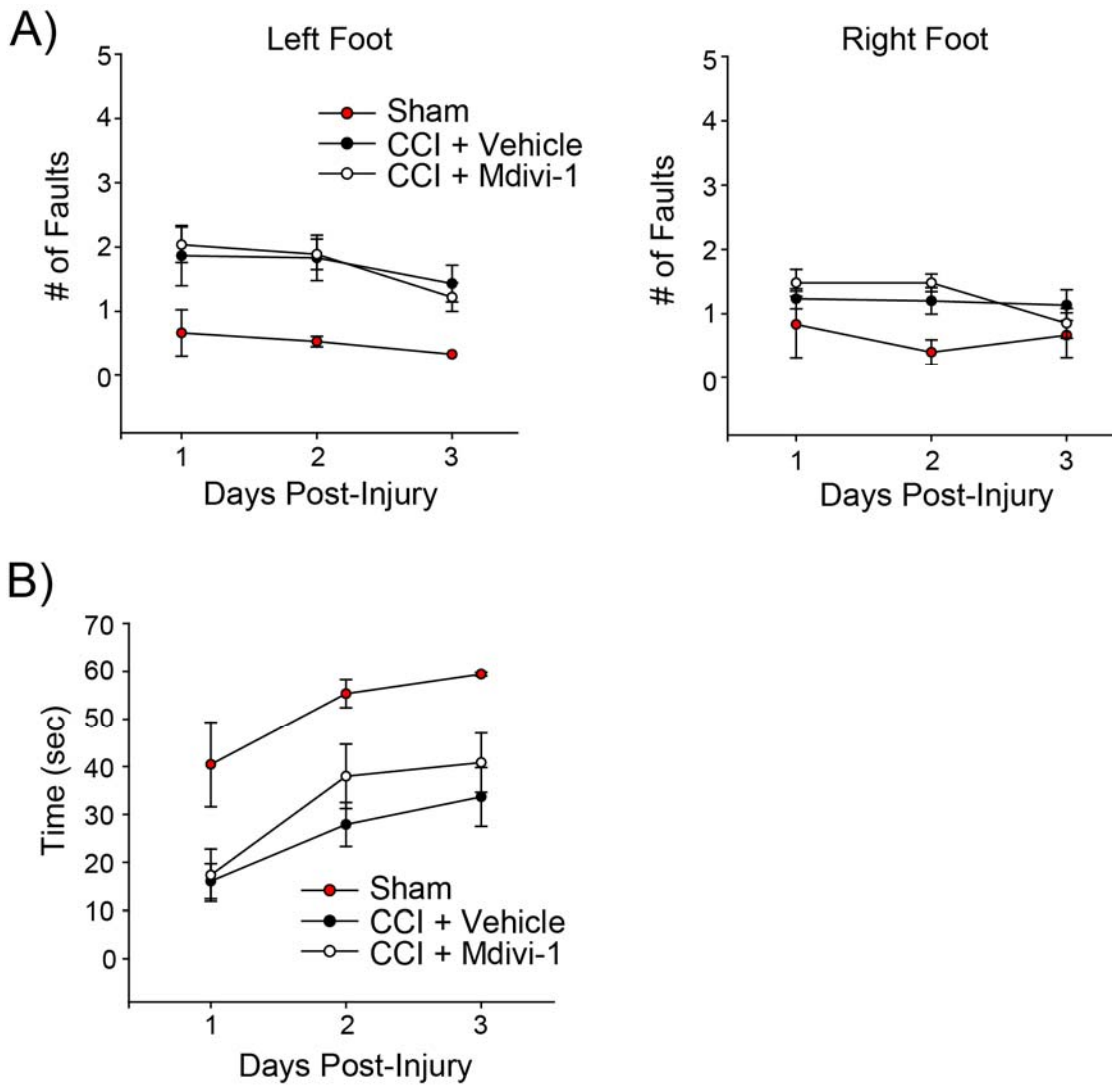

Figure 4.9 Mdivi-1 treatment does not improve motor performance after CCI injury. Motor and vestibulomotor performance in sham (n = 5) and CCI injured (Vehicle-treated or Mdivi-1 treated; n = 10/group) animals on days 1-3 post-injury. A) Average foot faults per left foot (left panel) and right foot (right panel) on the Foot Fault motor performance task. One sham and one CCI injured, Mdivi-1 treated animal were removed due to lack of activity during the Foot Fault task. B) Average time spent on the beam in the Balance task. Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

The Novel Object Recognition (NOR) task is a simple behavioral assessment that relies on the innate exploratory behaviors of rodents and can be used as a measure for object recognition and memory.(Antunes and Biala, 2012; Ennaceur and Delacour, 1988) Although the regional dependence of the NOR task has been controversial, the hippocampus and the perirhinal cortex are thought to play major roles in the acquisition, consolidation, and storage of the nonspatial, object recognition memory.(Broadbent et al., 2010; Cohen and Stackman, 2015) Moreover, deficits in recognition memory are commonly detected on the NOR task after experimental TBI, and performance on the task is thought to model hippocampal function and recovery after injury.(Dash et al., 2015; Osier et al., 2014; Rozas et al., 2015) Therefore, the NOR task was used to assess recognition memory to determine whether Mdivi-1 treatment improves cognitive function after CCI injury.

A three-day paradigm of the NOR task was administered starting seven days post-injury. Animals were first habituated to the testing environment on day one, then were allowed to familiarize themselves with two identical objects on the second day. Long-term memory was tested 24 hours later by replacing one of the familiar objects with a novel object and the amount of time spent exploring each object was recorded and analyzed. Sham animals (n = 5) spent significantly more time exploring the novel object (**Fig. 4.10B**; $t(8) = 6.81$, $p = < 0.001$), indicating intact recognition memory. In contrast, CCI injured animals receiving vehicle treatment (n = 7) spent equivalent times exploring both objects, and did not prefer either the familiar and novel object, indicating impaired recognition memory ($t(12) = -0.548$, $p = 0.594$). Interestingly, Mdivi-1 treated, CCI injured (n = 10) animals show a significant preference for the novel object versus the familiar object ($t(18) = 8.844$, $p = < 0.001$), indicating preserved recognition memory after CCI injury. Therefore, results indicate that Mdivi-1 treatment improves recognition memory deficits after CCI injury. It is important to note that three animals from the vehicle treated, injured group were removed from analysis for inactivity during the task.

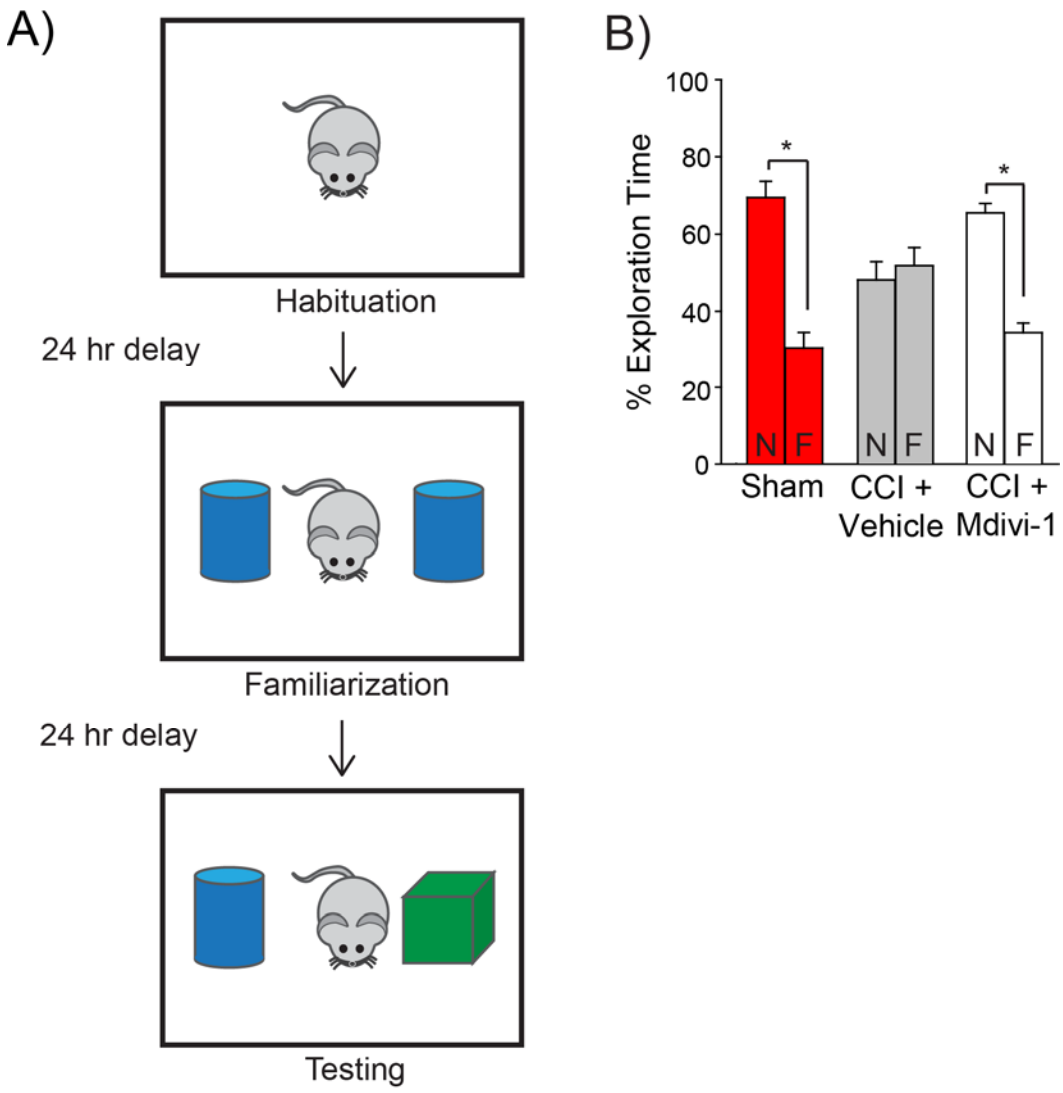

Figure 4.10 Mdivi-1 treatment preserves recognition memory after CCI injury. A) Illustration of the behavioral paradigm for the Novel Object Recognition task. B) Average percent of time exploring both the novel (N) and familiar (F) objects for sham (n = 5), CCI injured, Vehicle treated (n = 7), and CCI injured, Mdivi-1 treated (n =10) animals in the Novel Object Recognition Task. Recognition memory was tested 24 hours after familiarization. Percent exploration time between the novel and familiar objects reflects long-term recognition memory.

The Contextual Fear Conditioning task is an associative learning task that employs a classical conditioning paradigm by pairing of a foot shock (aversive, unconditioned stimulus) with an environment containing particular contextual cues (conditioned stimulus).(Curzon et al., 2014) The freezing response of animals is measured after the association phase and is an indicator of fear memory. Contextual fear memory is primarily dependent on the hippocampus and amygdala, although recruitment of cortical regions, such as the medial prefrontal cortex and the parahippocampal cortex (enthorinal, perirhinal, and postrhinal), are also thought to be important as well.(Maren et al., 2013; Wiltgen, 2006) Specifically, the encoding and storage of the nonspatial, contextual representation of the environment is dependent on intact hippocampal function, with the aversive stimulus pairing requiring recruitment of the amygdala circuitry. Deficits in contextual fear memory are commonly detected after experimental TBI, and freezing responses are thought to represent hippocampal function and recovery after injury.(Osier et al., 2014) Therefore, contextual fear memory after CCI injury and Mdivi-1 treatment was assessed in a One Trial Contextual Fear Conditioning task.

In a separate study, with an identical treatment and behavioral timeline (**Fig. 4.8**), fear conditioning was assessed for Naïve animals (n = 8), vehicle-treated, CCI injured animals (n = 6), and Mdivi-1 treated, CCI injured animals (n = 8). 28 days post-injury, animals were placed in a chamber with visual cues on the walls of the chamber for 2.5 minutes, prior to receiving a mild foot shock. Freezing behavior (defined as the absence of movement excluding movement from breathing) was recorded the entire time the animal was in the chamber. During training, all animals were mobile with minimal freezing behavior in the context prior to delivery of a mild foot shock **(Fig. 4.11B)**. Twenty-four hours later, long-term memory for the training context was tested by placing the animal back into the training environment and recording freezing behavior for three minutes. Sham animals froze approximately 60% of the observation time, whereas CCI injured, vehicle treated animals showed reduced freezing behavior (37%). The CCI injured, Mdivi-1 treated animals froze at a level comparable to shams (65%), and significantly more than the CCI

injured, vehicle treated controls ($t(12) = -2.839$, $p = 0.015$; **Fig. 4.11B**), indicating improved contextual fear memory. Thus, Mdivi-1 treatment preserved contextual fear memory after experimental TBI. This experiment was performed by Dr. Michael Hylin and data were analyzed by Tara D. Fischer. It is important to note that this separate cohort of animals were also assessed for motor performance, Novel Object Recognition, and Morris Water Maze, in which the outcomes were replicated in CCI injured, Mdivi-1 treated animals.

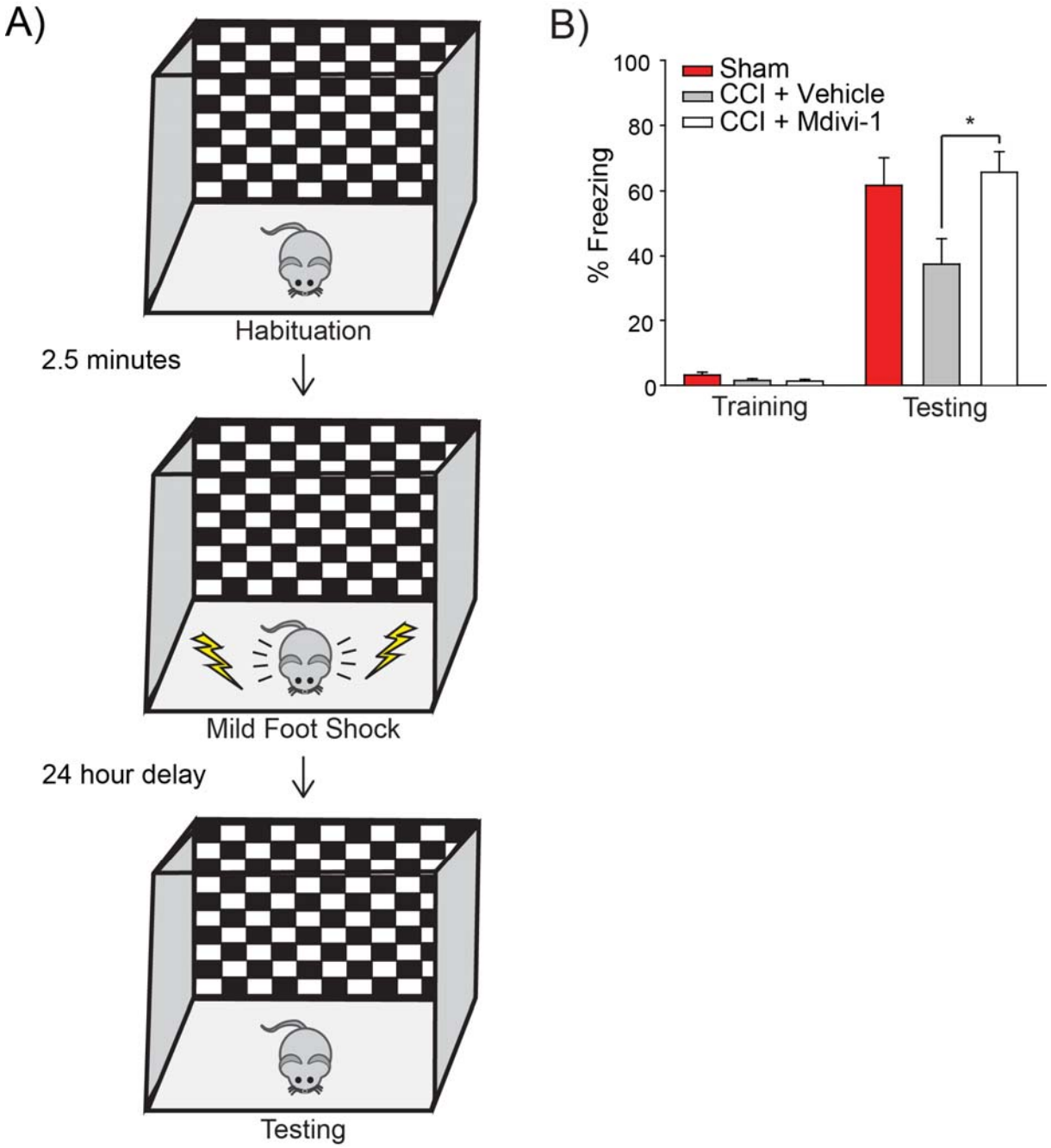

Figure 4.11 Mdivi-1 treatment preserves contextual fear memory after CCI injury. A) Representation of behavioral paradigm for the One Trial Fear Conditioning task. B) Average percent freezing time during training (2.5 minutes prior to shock) and testing for Naïve (n = 8), CCI injured, Vehicle treated (n = 6), and CCI injured, Mdivi-1 treated (n = 8) animals in the Contextual Fear Conditioning task. Fear memory was tested 24 hours post-shock. Percent freezing time in the context reflects long-term fear memory. Data are presented as the mean +/- standard error of the mean. *$p < 0.05$.

The Morris Water Maze (MWM) task is a complex, hippocampal-dependent spatial learning and memory task that is typically regarded as the “gold standard” for assessing hippocampal function. Spatial learning is achieved through multiple training trials per day for several days of the location for a hidden platform in a swimming arena that is complete with external visual cues in the perimeter.(D’Hooge and De Deyn, 2001; Morris, 1984; Vorhees and Williams, 2006) After learning is established, spatial memory for the platform location is assessed by removal of the platform from the swimming arena and measuring the latency between entry into the arena and platform location crossing. The Morris Water Maze requires a combination of behavioral skills, and therefore requires the recruitment and function of several brain regions for successful solving of the task. The hippocampus, specifically, requires large regional intactness and functional connectivity between several hippocampal subregions, and therefore requires a mostly complete hippocampal circuitry for both spatial learning and memory.(Broadbent et al., 2004; Vorhees and Williams, 2006) Deficits in spatial memory are well established as a pathological cognitive outcome in the CCI injury model of TBI.(Hamm, 1992; Osier et al., 2014; Scheff et al., 1997) Therefore, Mdivi-1 treated animals were assessed for spatial memory and MWM performance after injury.

Starting 14 days after injury, sham animals (n = 5), Vehicle treated, CCI injured animals (n = 10), and Mdivi-1 treated, CCI injured animals (n = 10) began training on the MWM task. Animals were trained to find the location of a hidden platform for six days prior to a long-term spatial memory probe. Although sham animals did require less training days to learn the platform location, and consistently exhibited less latency to platform location in the subsequent training trials, no significant differences were found during the training period between the groups in a repeated measured one-way ANOVA (**Fig. 4.12A**). By the final training day, all animals exhibited similar latency times to platform location, indicating successful learning of the maze. Twenty-four hours after the final training session, the platform was removed from the swimming arena and animals were assessed for spatial memory of the platform location. Latency to platform location

times were on average ~14s for sham animals, similar to average latency times in the training trial for the previous day, indicating intact spatial memory (**Fig. 12B**). Latency times for CCI injured, Vehicle treated and Mdivi-1 were ~34s and 27s. Although, latency times for CCI injured animals were longer than sham animals, no significant differences were found in a one-way ANOVA ($H(2) = 3.615$, $p = 0.164$). Similar results were found for number of platform crossings during the probe trial, in which CCI injured animals crossed the platform location less than shams, however statistical significance was not achieved in this measure either ($H(2) = 3.348$, $p = 0.187$); **Fig. 12C**). No significant differences were found when comparing Vehicle treated and Mdivi-1 treated, CCI injured animals in either measure. Therefore, Mdivi-1 treatment did not improve spatial memory on the MWM task after experimental TBI.

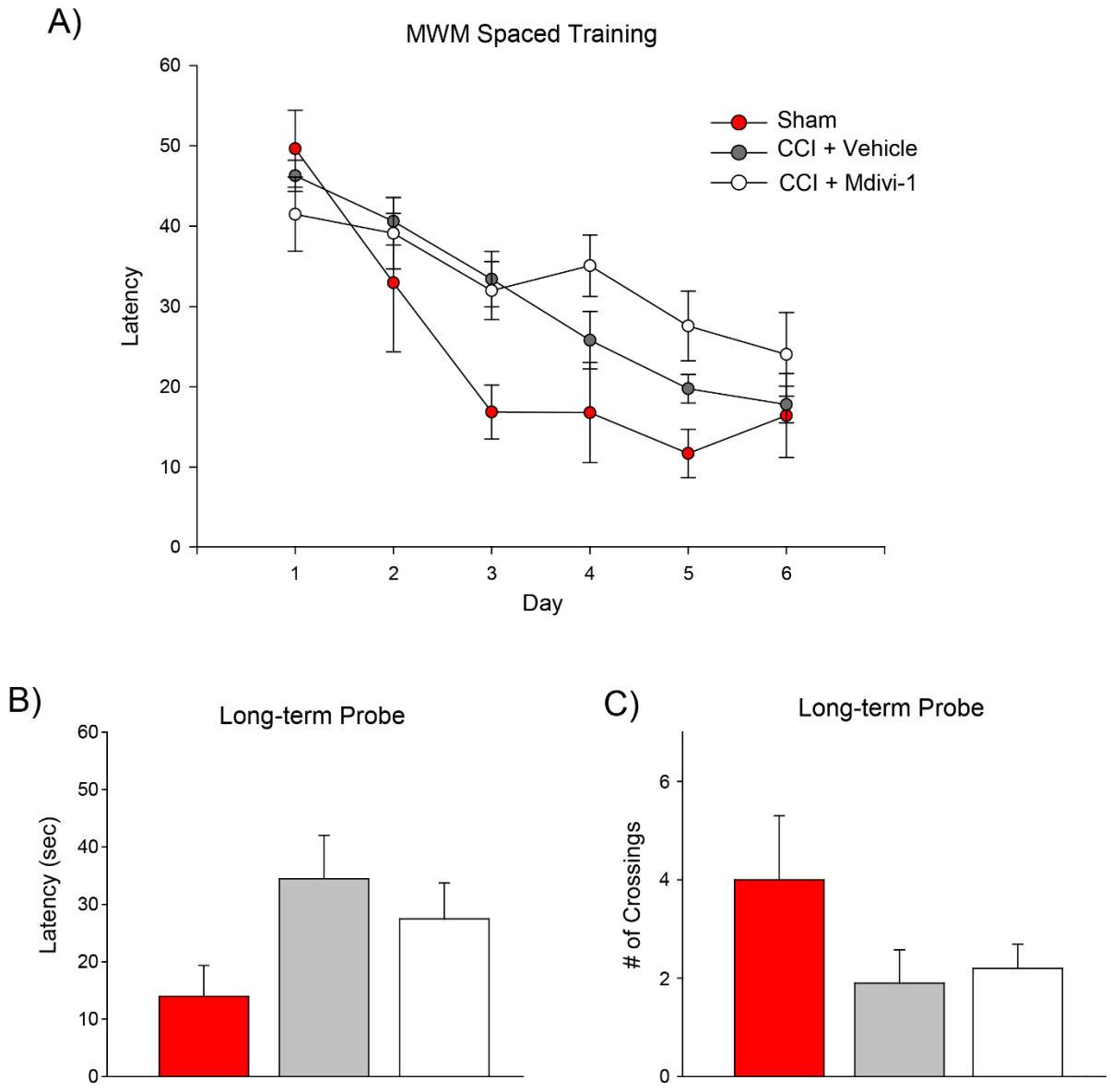

Figure 4.12 Mdivi-1 treatment does not improve performance on the MWM task after experimental TBI. A) Average latency to platform for each training day for sham (n = 5), CCI injured, Vehicle treated (n = 10), and CCI injured, Mdivi-1 treated (n =10) animals in the Morris Water Maze. B) Average latency to platform location for each group in the long-term probe. C) Average platform crossings within the probe trial period for each group.

4.5 Summary

The current studies examined the effect of a pharmacological inhibitor of Drp1, Mdivi-1, on hippocampal outcome in the context of mitochondrial, neuronal, and cognitive pathology after experimental traumatic brain injury. Results from experiments reveal three key findings: 1) Mdivi-1 treatment does not reduce injury-induced hippocampal mitochondrial dysfunction, 2) Mdivi-1 treatment improves newborn neuron survival in the dentate gyrus, and 3) Mdivi-1 treatment improves hippocampal-dependent recognition and contextual fear memory after experimental TBI. Taken together, results from these studies support a role for Drp1 in hippocampal neuropathological outcome after TBI, however without the improvement of mitochondrial function. Therefore, inhibition of Drp1 may provide a potential therapeutic avenue to improve cell survival and cognitive outcome after TBI.

Chapter 5. Discussion

Mitochondria respond dynamically to the metabolic needs of the cell through tightly balanced processes of fusion and fission.(Mishra and Chan, 2016; Westermann, 2012) The delicate balance between fusion and fission plays an integral role in cellular health, and imbalance of these processes can be detrimental to mitochondrial function and cell survival.(Archer, 2013; Detmer and Chan, 2007b) Growing evidence has suggested that excessive mitochondrial fission without sufficient fusion contributes to mitochondrial dysfunction and cell death in neurodegeneration.(Bertholet et al., 2016; Burté et al., 2014; Itoh et al., 2013; Knott et al., 2008) Mitochondrial damage and impairment is a major contributor to secondary injury and neuropathology after traumatic brain injury (TBI); however, whether imbalances in mitochondrial fusion and fission, such as excessive fission, contribute to mitochondrial dysfunction and cell death after injury is not well known. The studies described in this dissertation investigated whether dysregulation of Dynamin-related protein 1 (Drp1) and mitochondrial fission play a role in neuropathology after TBI. Results from these studies revealed six key findings: 1) Experimental TBI increases Drp1 translocation with mitochondria; 2) Experimental TBI causes acute changes in Drp1-mediated mitochondrial morphology that persists post-injury; 3) mitochondrial Cytochrome *c* release after TBI is Drp1-mediated; 4) post-injury treatment with a pharmacological inhibitor of Drp1, Mdivi-1, does not reduce injury-induced mitochondrial dysfunction; 5) Mdivi-1 treatment improves survival of newly born neurons acutely after injury; 6) Mdivi-1 treatment improves hippocampal-dependent recognition and contextual fear memory. Taken together, results from these studies reveal that TBI causes excessive Drp1-mediated mitochondrial fission and that this pathological fission state may play a key role in hippocampal neuronal death and cognitive deficits after TBI. Additionally, inhibition of Drp1 may be a potential therapeutic strategy to improve neuronal recovery and cognitive function after injury.

5.1 Primary Protein Regulators of Mitochondrial Dynamics and Experimental TBI

Dynamin-related protein 1 (Drp1) is a cytosolic GTPase that is actively recruited to the mitochondrial outer membrane (MOM) to mediate fission events through interactions with integral mitochondrial outer membrane (MOM) receptors.(van der Bliek et al., 2013; Smirnova et al., 1998, 2001) Drp1 self-assembly into an oligomeric, ring-like structure around the mitochondrion, constricts and drives membrane division through hydrolysis of bound GTP. Dysregulation of Drp1 has been implicated in inappropriately causing excessive mitochondrial fission that contributes to mitochondrial dysfunction and cell death in neurodegenerative diseases.(Manczak and Reddy, 2012; Manczak et al., 2011; Reddy et al., 2011; Song et al., 2011; Wang et al., 2011) The rate of mitochondrial fission can be altered by Drp1 activity through changes in cellular concentrations of Drp1 (e.g., expression/degradation) or changes in the critical steps that drive membrane division (e.g., translocation or GTPase activity).(Cereghetti et al., 2008; Chang and Blackstone, 2007a, 2010; Mai et al., 2010; Santel and Frank, 2008; Uo et al., 2009) Therefore, to determine whether Drp1 dysregulation occurs after experimental TBI, changes of total Drp1 levels and mitochondrial Drp1 levels were investigated in the hippocampus and cortex within 72 hours after injury. Results revealed that experimental TBI does not alter levels of total Drp1 in either injured brain area, indicating no observed changes in expression or degradation of Drp1 acutely after injury. However, a time-dependent increase of Drp1 was detected in isolated mitochondrial fractions from injured brain areas at 24 and 72 hours post-injury, with significant changes observed in the hippocampus and nonsignificant changes in the cortex (**Chapter 3.1**). Drp1 in the isolated mitochondrial fractions was also found localized on the outer membranes of mitochondrial structures using electron microscopy, confirming association of Drp1 with the mitochondrial membrane in the fraction (**Chapter 3.3**). Together, these data demonstrate an increase in translocation of Drp1 to the mitochondrial outer membrane after experimental TBI. Several *in vitro* and *in vivo* injury models have observed similar increases in translocation of Drp1 to the mitochondrial network, including models of hypoxia/ischaemia, subarachnoid hemorrhage,

brain lesion, and excitotoxicity.(Barsoum et al., 2006; Cavallucci et al., 2014; Gomez-Lazaro et al., 2008; Liu et al., 2012; Owens et al., 2014; Wu et al., 2017; Zhao et al., 2013, 2014) Consistent with the current studies, increases in Drp1 translocation are observed concomitantly with fragmentation of the mitochondrial network. The consistency of observed changes in Drp1 activity observed across several studies using different injury conditions and different model systems implicates modulation of Drp1 as a maintained pathological consequence of cellular insult. Further exploration into the precise upstream mechanisms that drive Drp1 translocation is necessary to determine potential changes in the cellular environment that regulate Drp1 and mitochondrial fission after insult or injury.

As Drp1 is primarily cytosolic (97%), its activity is sensitive to modifications by environmental signaling to communicate changes in the metabolic status of the cell and to regulate the rate of fission.(Chang and Blackstone, 2010; Smirnova et al., 1998, 2001) Communication from the cellular environment is essential for proper dynamic responses of the mitochondrial network, but also exposes Drp1 to inappropriate regulation by environmental disturbances, such as after TBI.(Detmer and Chan, 2007b; Otera et al., 2013; Santel and Frank, 2008) Several post-translational modifications (PTMs) are known to mediate the critical steps in Drp1 activity, such as translocation, and alter the rate of mitochondrial fission in response to environmental signaling.(Cereghetti et al., 2008; Chang and Blackstone, 2007a, 2010; Cho et al., 2009; Harder et al., 2004; Karbowski et al., 2007; Santel and Frank, 2008) One of the most well characterized PTMs, is the phosphorylation/ dephosphorylation of the conserved serine residue 637 (humans; 656 in rats) located in the GTPase effector domain.(Cereghetti et al., 2008; Chang and Blackstone, 2007a; Cribbs and Strack, 2007) Phosphorylation by cyclic-AMP (cAMP)-dependent protein kinase A (PKA) inhibits Drp1 GTPase activity by blocking intra-molecular interactions necessary for GTP hydrolysis, thereby reducing mitochondrial fission and permitting mitochondrial elongation due to unopposed fusion.(Chang and Blackstone, 2007a; Cribbs and Strack, 2007) Conversely, dephosphorylation by the calcium/calmodulin serine-threonine

phosphatase, Calcineurin (CaN; also known as protein phosphatase-2B or PP2B), increases mitochondrial fission by specifically enhancing Drp1 translocation to the MOM, resulting in fragmentation of the mitochondrial network and increases sensitivity of cells to apoptotic stimuli.(Cereghetti et al., 2008; Cribbs and Strack, 2007) Phosphorylation at Drp1^{Ser637} has also been directly associated with adaptation of mitochondrial function during cellular starvation and autophagy, in which mitochondria are spared from degradation, and mitochondrial elongation is complemented by cristae formation and increased ATP synthesis.(Gomes et al., 2011) Interestingly, experimental TBI is known to cause changes in both cAMP-PKA signaling and calcium-induced activation of calcineurin that have been linked to neuropathological outcomes and cognitive dysfunction after injury.(Atkins et al., 2007; Bales et al., 2010a, 2010b; Kobori et al., 2015; Okonkwo and Povlishock, 1999; Walker and Tesco, 2013) For example, in a fluid percussion injury model of TBI, cAMP levels were found to be acutely depressed, and the activity of its substrate PKA was also reduced specifically in the hippocampus after injury.(Atkins et al., 2007) Similar results were found in the medial prefrontal cortex after controlled cortical impact injury and was directly contributed to working memory deficits.(Kobori et al., 2015) Regulation of this pathway is well characterized in relation to synaptic plasticity, and deficits in learning and memory, particularly through the cAMP-PKA-CREB pathway.(Kandel, 2012) Additionally, although there appears to be differential regional and isoform-specific regulation reported in the literature, calcineurin activity and expression is also observed to be altered after experimental injury.(Bales et al., 2010a, 2010b) Furthermore, pharmacological approaches targeting these pathways, such as phosphodiesterase inhibitors (Rolipram – PDE4 inhibitor) that increase PKA activity, and calcineurin inhibitors, such as Cyclosporin A and FK506, demonstrate neuroprotection and improved outcome in experimental injury models.(Atkins et al., 2007; Reeves et al., 2007; Sullivan et al., 1999; Titus et al., 2015) Therefore there is some evidence in the literature of injury-induced environmental signaling changes that could be implicated in dysregulation of Drp1 at serine 637, a site that specifically mediates Drp1 translocation to mitochondria. However, whether Drp1-mediated mitochondrial fission is affected in these

pharmacological approaches, or whether excessive mitochondrial fission is mediated through downstream modification of Drp1 by cAMP-PKA or calcinuerin after experimental injury is unknown. It would be reasonable to hypothesize that regulation of Drp1-Ser637 due to injury-induced changes in cAMP-PKA signaling and/or calcineurin activity contributes to Drp1-mediated pathological fission events after TBI (**Fig. 5.1**). Further studies are necessary to determine the precise mechanisms involved in Drp1 dysregulation after injury. Elucidating the connection between injury-induced changes in the cellular environment and Drp1 regulation will be critical in developing specific, targeted therapeutic approaches after TBI.

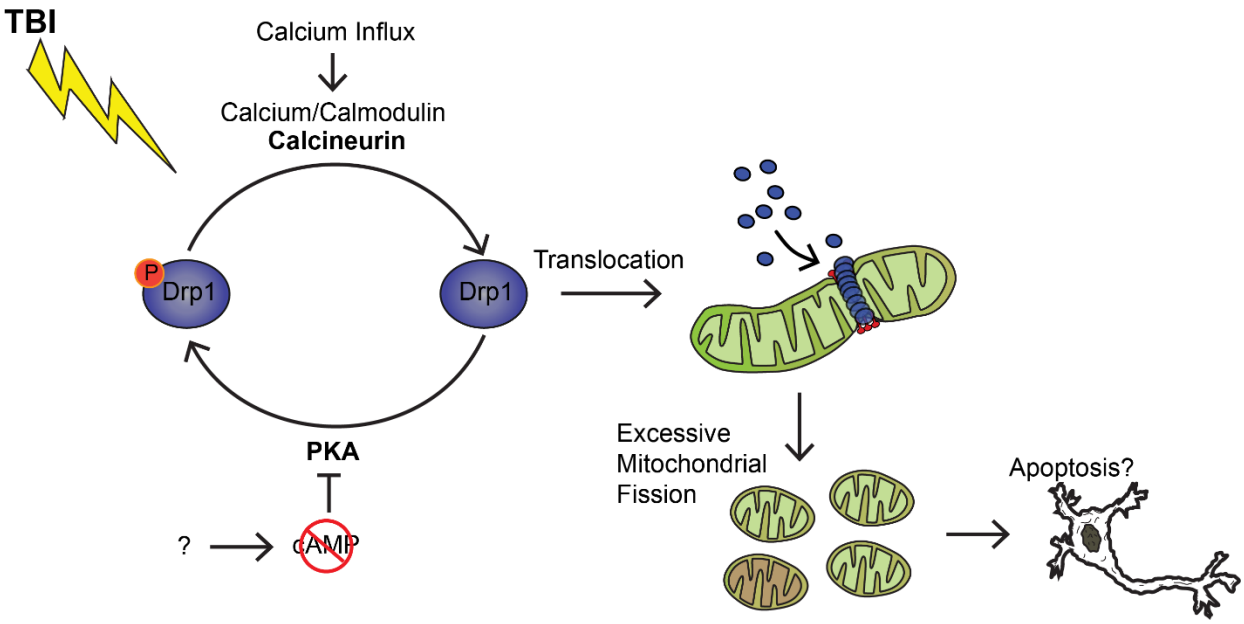

Figure 5.1 Hypothesis of injury-induced upstream mechanisms of Drp1 regulation

It is important to discuss the findings that a pharmacological inhibitor of Drp1, Mdivi-1, did not reduce or inhibit Drp1 association with mitochondria after injury (**Chapter 3.2**). To examine these results, the proposed mechanism of action of Mdivi-1 and mechanisms of Drp1 recruitment to the mitochondrial outer membrane must be considered. Cassidy-stone et al. (2008) identified Mdivi-1 as an inhibitor of mitochondrial-specific division through the selective inhibition of the primary protein regulator of yeast and mammalian mitochondrial fission, DNM1 and Drp1, respectively.(Cassidy-stone et al., 2008) The proposed mechanisms of Drp1 inhibition by Mdivi-1 is that Mdivi-1 binds to an allosteric site on Drp1 and stabilizes a conformation that has a low affinity for GTP binding. Due to the inability of Drp1 to bind GTP, the downstream formation of GTP-dependent oligomeric spiral structures, and the GTP hydrolysis required for membrane division is blocked. Thus, Drp1-mediated fission is blocked and Drp1 remains in an unassembled, likely dimeric, conformation. Recently, two new receptors, MiD49 and MiD51, have been identified on the mitochondrial outer membrane to bind Drp1 and mediate mitochondrial fission.(Palmer et al., 2011; Zhao et al., 2011) However, whether binding of Drp1 to these specific receptors exhibit pro-fission or anti-fission regulation has been unclear due to contradictory results in overexpression and knockdown studies.(Palmer et al., 2011; Zhao et al., 2011) Specifically, multiple groups have demonstrated that knockdown of MiD49 and MiD51, both together and individually, result in reduced mitochondrial recruitment of Drp1 and mitochondrial elongation.(Liu and Chan, 2015; Losón et al., 2013; Palmer et al., 2011; Zhao et al., 2011) Expectedly, overexpression studies from the same reports increase Drp1 recruitment; however, unexpectedly, mitochondrial elongation is observed, indicating inhibition of fission. Only until recently have potential mechanisms been elucidated in two elegant studies, explaining the perplexing regulation of Drp1-mediated fission through MiD49/51 receptors.(Liu and Chan, 2015; Losón et al., 2013) First, Loson *et al.* (2013) observed that although overexpression of MiD49/51 increased Drp1 recruitment to the mitochondrial outer membrane, inhibitory phosphorylation of Drp1 at serine site 637 was also increased. The removal of phosphorylation via site-directed mutagenesis allowed for normal mitochondrial fission to proceed. These results reveal the

potential for differential regulation of Drp1-mediated fission via MiD49/51 depending on the modification of Drp1, and potentially the physiological state of the cell. A later study by the same group further elucidated the precise mechanisms involved by identifying that each of the known receptors for Drp1 exhibits preferential recruitment and affinity depending on the conformational state of Drp1.(Liu and Chan, 2015) In the cytosol, Drp1 can be found in many conformational states, including dimer, tetramers, and higher-order assemblies.(Zhu et al., 2004) Lui et al. (2015) observed that MiD49/51 receptors preferentially bind dimeric, inactive forms of Drp1, unlike the other primary receptor, MFF, which only has a high affinity for active, tetramer and higher-order assemblies of Drp1. Taken together, the proposed model is that while MFF preferentially binds a subpopulation of fission-competent Drp1, MiD49/51 binds inactive forms of Drp1 that require an additional stimulus to initiate oligomerization and fission events. This stimulus may be dependent on modifications of Drp1 and the physiological state of the cell. In the context of the current studies, the recruitment of different subpopulations of Drp1 to the mitochondrial outer membrane from the cytosol call into question whether Mdivi-1 would inhibit mitochondrial recruitment of Drp1, *per se*. As the mechanism of Mdivi-1 blocks self-assembly of Drp1, it is likely that this also results in increased Drp1 dimers in the cytosol, thus it is possible that the inactive forms of Drp1 could be found associated with the mitochondrial outer membrane via recruitment by the MiD49/51 receptors. These reports clearly demonstrate the complexity in the mechanisms involved in Drp1-mediated mitochondrial fission, as well as the potential for environmental regulation of Drp1 and mitochondrial fission, most of which have only recently been elucidated. This further supports the necessity to uncover the precise mechanisms involved in the regulation of Drp1 and mitochondrial fission after injury to determine whether these events contribute to pathological outcome and have therapeutic potential after TBI.

Pathological fission events are also known to be associated with disruptions in fusion proteins.(Archer, 2013; Detmer and Chan, 2007b) Mitochondrial inner membrane fusion is regulated by Opa1 (Optic Atrophy 1), a large GTPase that is both integral and associated with

the inner mitochondrial membrane, depending on the isoform, while outer membrane fusion is mediated by integral Mitofusins (Mfn1 and Mfn2).(van der Bliek et al., 2013) Most notably, loss-of-function mutations of Opa1 and Mfn2 are directly associated with two neurodegenerative diseases, Autosomal Dominant Optic Atrophy and Charcot-Marie Tooth subtype 2A peripheral neuropathy (respectively).(Delettre et al., 2000; Züchner et al., 2004) Therefore, specific loss of fusion GTPases and mitochondrial fusion deficiencies result in cell death and degenerative disease due to unopposed fission. Additionally, it has been observed that loss of fusion proteins co-occur with excessive mitochondrial fission during cellular insult and apoptosis, (Arnoult et al., 2005a; Jahani-Asl et al., 2011; Sanderson et al., 2015) Specifically, *in vitro* models have demonstrated cleavage of Opa1 from the inner membrane and release into the cytosol corresponding with stress-induced pathological fission events prior to apoptosis.(Arnoult et al., 2005a; Sanderson et al., 2015) Mfn1 and Mfn2 are also both substrates of Parkin, and are targeted for proteasomal degradation via ubiquitination during mitophagy. (Gegg et al., 2010; Glauser et al., 2011; Leboucher et al., 2012) Thus, the loss of Opa1, Mfn1/2, or both, consequently commit the mitochondrial network to a pathological fission state due to cessation of fusion and unopposed fission. In basal state, this regulation is critical and is a protective mechanism to ensure damaged mitochondria do not re-fuse back into the network. However, a few studies have shown that rescue of fusion by the overexpression of Opa1 or the stabilization of Mfns restores mitochondrial function and increases cell survival, therefore fusion proteins may also be inappropriately regulated by injury-induced environmental changes.(Jahani-Asl et al., 2011; Leboucher et al., 2012) To determine whether fusion proteins are altered after experimental TBI, Opa1 and Mfn1 levels were detected in mitochondrial fractions from injured hippocampi (**Chapter 3.4**). Non-significant reductions were detected in both Opa1 and Mfn1 at 24 hours and 72 hours after injury. Therefore, mitochondrial fusion proteins in the injured hippocampus do not appear to be altered acutely after CCI injury. One technical caveat should be considered when determining whether Opa1 is specifically affected after experimental TBI. Fusion-competent mitochondria require both long forms and short forms of Opa1 to inner

membrane fusion. In states of stress-induced mitochondrial inner membrane depolarization, activation of the *m*-AAA protease, OMA1, completely cleaves the long forms of Opa1 into s-Opa1, which can be released in outer membrane permeabilization during apoptosis.(MacVicar and Langer, 2016) Therefore, to appropriately determine whether there is an injury-induced change in Opa1 using gel electrophoresis, the short form of Opa1 should be probed for specifically, as well as release from the mitochondrial compartment. This can be achieved by running the protein on a polyacrylamide gel with higher percentage gradient than the gels used for these studies (12-14%) to yield better separation and resolution for Opa1 detection. Therefore, it is possible that separating the detection of the long and short form of Opa1 and probing for changes in the levels of s-Opa1, specifically, would have revealed changes in mitochondrial Opa1 levels post-injury that would support excessive fission events. The elegant molecular machinery involved in mitochondrial fission and fusion mechanisms, as well as the complex metabolic and environmental regulations that mediate these delicately balanced processes is fascinating. More precise and specific investigation into these mechanisms is essential to elucidate injury-induced changes in fission and fusion proteins, and their contribution in neuropathological outcomes after TBI.

5.2 Experimental TBI and Mitochondrial Morphology

Mitochondrial populations in neurons, typically exist in morphologically heterogeneous, connected networks that are constantly elongating, dividing, and being transported along axons.(Bertholet et al., 2016; Saxton and Hollenbeck, 2012) A canonical characteristic of pathological mitochondrial fission is an observably fragmented mitochondrial network, with morphologically smaller and rounder mitochondria.(Knott et al., 2008; Reddy et al., 2011) To determine whether experimental TBI causes excessive mitochondrial fission, morphology (length and aspect ratios) of isolated hippocampal mitochondria was measured after injury (**Chapter 3.5**). Results from the current studies reveal that mitochondria isolated from injured hippocampi

display time-dependent morphological changes after injury. At 24 hours post-injury, mitochondrial lengths and aspect ratios (length of long axis: length of short axis) are significantly increased, indicating mitochondrial elongation. However, at 72 hours post-injury lengths of injured hippocampal mitochondria are significantly reduced, and aspect ratios are nearing values of 1 (a perfect circle), indicating smaller and morphologically more round mitochondria after injury. Thus, isolated hippocampal mitochondria display morphological characteristics consistent with pathologically excessive fission events 72 hours after experimental TBI, the same time-point to which maximal Drp1 in the mitochondrial fraction was observed. Additionally, data shows that these morphological features of isolated mitochondria persist for at least eight days post-injury (**Chapter 3.7**). These results demonstrate that experimental TBI causes acute changes in mitochondrial morphology, eventually resulting in a prolonged decrease in mitochondrial size after injury.

The observation of mitochondrial elongation at 24 hours post-injury, prior to a major reduction in mitochondrial size at 72 hours after injury is interesting and may indicate the metabolic state of hippocampal mitochondria. Increased mitochondrial fusion and elongation in certain metabolic states has been directly associated with increases in mitochondrial respiration and oxidative phosphorylation.(Gomes et al., 2011; Mishra and Chan, 2016; Mishra et al., 2014; Rossignol et al., 2004) Interestingly, a few studies have observed a biphasic response in mitochondrial dysfunction, with an initial decline and recovery within 24 hours after injury, and a secondary decline that peaks at 72 hours and remains reduced. [4,13] The acute mitochondrial elongation reported in these studies may thus be reflective of increased mitochondrial function in the hippocampus at 24 hours after experimental TBI. Consistently, decreases in mitochondrial size and states of excessive fission are characterized by reduced mitochondrial respiration and oxidative phosphorylation. Thus, the change in mitochondrial morphology at 72 hours post-injury correspond with the further observation of decreased respiration at the same time-point in these studies. Further studies are required to determine whether changes in mitochondrial morphology

correspond to injury-induced alterations in mitochondrial function and whether specific mechanisms of mitochondrial fission and fusion are involved in these states after injury. These observations could reveal a key time-point of mitochondrial injury and dysfunction after injury, and thus may provide a potential time window to target metabolic and mitochondrial dysfunction for treatment after TBI.

As the decreases in mitochondrial size at 72 hours post-injury were consistent with significant increases in Drp1 association with mitochondria at the same time point, it was essential to determine whether the changes in mitochondrial morphology were mediated by Drp1. Pharmacological inhibition of Drp1 with Mitochondrial Division Inhibitor 1 (Mdivi-1) reduces mitochondrial fission via allosteric modulation of GTP-dependent assembly and oligomerization necessary for membrane division. Therefore, Mdivi-1 was administered to animals after injury and hippocampal mitochondria lengths were measured at 72 hours-post injury, the same time point that mitochondrial size was observed to be significantly reduced (**Chapter 3.6**). Mdivi-1 administration significantly increased mitochondrial lengths at 72 hours after injury, indicating Drp1 as a key mediator of injury-induced decreases in mitochondrial size. Overall, results from these studies reveal that experimental TBI both increases Drp1 translocation to mitochondria after injury and causes acute changes in mitochondrial morphology that eventually result in a prolonged increased of Drp1-mediated fission.

It is important to note that morphological measurements of mitochondria isolated from the hippocampus present a few limitations that need to be addressed. Firstly, although all samples were prepared simultaneously and consistently, thereby allowing for direct comparisons between sham and injured conditions, it is not possible to rule out the potential for the isolation procedure to alter mitochondrial morphology. Thus, mitochondrial morphologies in the hippocampus may display different results when observed *in vivo*. Secondly, mitochondria isolated from injured hippocampi originate from all cell types in the hippocampus, and thus do not present any regional or cell-type specificity. It is possible that excessive mitochondrial fission could be a graded

response based on injury severity, and therefore might manifest differently depending on distance from the locus of injury or in areas that experience more pathophysiological damage after injury. Additionally, different cell types might exhibit vulnerability due to dysregulation of mitochondrial fission and fusion compared to other cell types. The preferential improvement in survival of the newly born neuron population in the dentate gyrus of the hippocampus with Mdivi-1 treatment observed in these studies may be reflective of such vulnerability. Therefore, potential differential in responses of mitochondrial fission in distinct cell types, or specific changes in different regions of the hippocampus, may mask or skew specific mitochondrial morphological changes due to injury in a homogeneous isolated mitochondrial preparation. To potentially examine *in vivo* mitochondrial morphological changes in different regions and cell types in the hippocampus after injury, we examined a PhAM^{floxed} (photo-activatable mitochondria) mouse that conditionally expresses a mitochondrial-targeted green fluorescent protein (mito-Dendra2) in combination with a neuronal and astrocytic-specific promoter (CaMKIIa-Cre and GFAP-Cre) via intrahippocampal adenoviral infusion prior to injury. Preliminary analysis of mitochondrial morphologies in the injured hippocampus 72 hours after injury displayed consistent reductions in mitochondrial size detected by random sampling (~15,000 mitochondria/condition) in the hippocampus and automated morphometric analysis (n = 1 animal/group; **Fig. 5.2**).(Pham et al., 2012) These experiments support the premise for pathological mitochondrial fission in the hippocampus after TBI *in vivo*. Further study is necessary to identify potential differential regional and cell-type specific changes in mitochondrial dynamics after experimental TBI, and determine possible vulnerability of specific populations to mitochondrial dysfunction and cell death after injury.

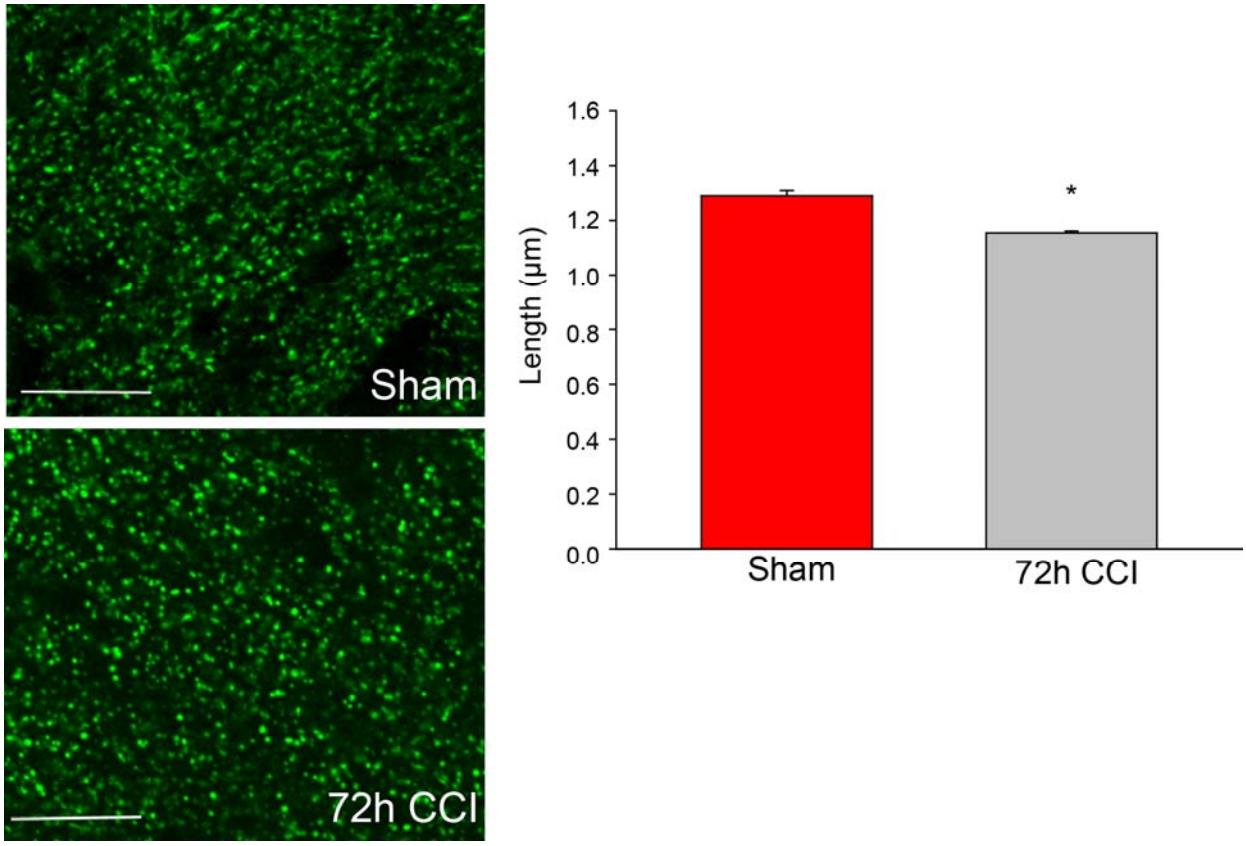

Figure 5.2 Mitochondrial morphology in the hippocampi of sham and injured MitoDendra2-CaMKIIa-Cre mouse. PhAM^{floxed} (photo-activatable mitochondria) mouse that conditionally expresses a mitochondrial-targeted green fluorescent protein (mito-Dendra2) in combination with a neuronal and astrocytic-specific promoter (CaMKIIa-Cre and GFAP-Cre) via intrahippocampal adenoviral infusion prior to injury. Preliminary analysis of mitochondrial morphologies in the injured hippocampus 72 hours after injury displayed consistent reductions in mitochondrial size detected by random sampling (~15,000 mitochondria/ condition) in the hippocampus and automated morphometric analysis (n = 1 animal/group). P = <0.05. Scale bar = 20 um).

5.3 Cytochrome *c* Release after Experimental TBI is Drp1-mediated

Drp1 mediates many cellular processes, including control of apoptosis through the intrinsic pathway.(Frank et al., 2001; Lee et al., 2004) The formation of Bax/Bak foci on the mitochondrial outer membrane forms a pore (mitochondrial outer membrane permeabilization – MOMP) that allows for Cytochrome *c* and other inter membrane proteins to be released into the cytosol and thus activate caspases that initiate apoptosis. It is well known that Drp1 is involved in Bax/Bak mediated apoptosis, and is specifically involved in the release of Cytochrome *c*.(Dewson and Kluck, 2009; Karbowski et al., 2004; Lee et al., 2004; Suen et al., 2008) Cytochrome *c* release and caspase activation are known to occur in a time-dependent manner early after TBI.(Cheng et al., 2012; Raghupathi et al., 2000; Sullivan et al., 2002) Interestingly, Drp1-dependent apoptosis has also been linked to aberrant calcium signaling, which is also an early pathological event resulting in excitotoxic apoptosis after TBI.(Szabadkai et al., 2004; Weber, 2012) Therefore, mitochondrial Cytochrome *c* levels were examined with Mdivi-1 treatment 72 hours after injury (**Chapter 3.7**). Results show that Cytochrome *c* levels were reduced in untreated CCI injured hippocampal mitochondria at 72 hours post-injury. Mdivi-1 treatment significantly increased mitochondrial Cytochrome *c* in the injured hippocampus, indicating an inhibition of release after injury. Thus, Cytochrome *c* release from mitochondria after experimental TBI Drp1-mediated. These results, in tandem with the increase of Drp1 translocation may implicate Drp1-mediated mitochondrial fission and Cytochrome *c* release as a potential mechanisms of cell death via induction of the apoptotic pathway after injury. Additionally, Cytochrome *c* is an essential component of electron transport chain function and oxidative phosphorylation. Therefore, drp1-mediated Cytochrome *c* release may also contribute to mitochondrial dysfunction after TBI along with apoptotic cell death. Further research is necessary to specifically elucidate the precise mechanisms involved in Drp1-mediated Cytochrome *c* release after TBI and to determine whether inhibiting Drp1 might directly block cell death via this pathway after injury.

5.4 Mdivi-1 Treatment and Mitochondrial Function after TBI

Mitochondrial dysfunction is at the center of secondary damage and contributes to the propagation of pathological biochemical cascades after TBI.(Cheng et al., 2012; Gajavelli et al., 2014) As mitochondria are metabolic hubs of many critical cellular processes, mitochondrial impairment is particularly detrimental to function and cell survival.(Detmer and Chan, 2007b) Several neuropathological processes after TBI can be associated with disruptions in essential mitochondrial functions including, inflammation, excitotoxicity, oxidative damage, and apoptosis.(Gajavelli et al., 2014; Lifshitz et al., 2004) Specifically, injury-induced mitochondrial dysfunctions involve dysregulation of calcium handling, generation of reactive oxygen species, induction of apoptosis, and most importantly, disruptions in oxidative phosphorylation. (Gajavelli et al., 2014; Hill et al., 2016; Lifshitz et al., 2004; Okonkwo and Povlishock, 1999; Pandya et al., 2013; Robertson, 2004; Sullivan et al., 1999) It has become increasingly evident in recent research that mitochondrial fission and fusion are tightly coupled to metabolic adaptation in changing cellular environments.(Mishra and Chan, 2016; Westermann, 2012) Consistently, imbalances of fission or fusion favored in either direction, have been associated with mitochondrial damage and dysfunction, including mtDNA damage, reduced respiration and oxidative phosphorylation, and release of pro-apoptotic factors and cell death. (Burté et al., 2014; Chen et al., 2005; Itoh et al., 2013; Manczak and Reddy, 2012; Song et al., 2011; Wang et al., 2012) Approaches to restore the balance of fusion and fission in these conditions have proven beneficial to mitochondrial function and cell survival (Cui et al., 2010; Grohm et al., 2012; Qian et al., 2014; Rappold et al., 2014; Wu et al., 2017; Zhao et al., 2014) Therefore, mitochondrial respiration was examined after experimental TBI to determine whether Mdivi-1 treatment reduces injury-induced mitochondrial dysfunction (**Chapter 4.1**). Consistent with other reports of mitochondrial dysfunction in the hippocampus, results from these studies showed that respiration of isolated hippocampal mitochondrial was decreased and that respiration was also uncoupled from oxidative phosphorylation at 72 hours after injury.(Singh et al., 2006) However, Mdivi-1 treatment did not improve mitochondrial respiration or respiratory coupling at this time-point after

injury. Therefore, Mdivi-1 treatment or inhibition of Drp1 acutely after injury does not reduce mitochondrial dysfunction after injury.

It is important to note that the observation that acute Mdivi-1 treatment does not reduce mitochondrial dysfunction after injury, does not answer the question of whether dysregulation of Drp1 is a mechanism involved in injury-induced mitochondrial dysfunction. Mdivi-1 treatment was administered during the critical time window of mitochondrial dysfunction and cell death after injury, therefore it is possible that temporary reductions in mitochondrial dysfunction could have occurred during treatment while the drug was present.(Singh et al., 2006) Reductions of mitochondrial dysfunction during treatment might have been beneficial or protective for ongoing cellular processes during this time and resulted in later improvements in outcome measures after the injury. Determining the consequences of excessive fission after injury is essential to determine whether Drp1 and inhibiting mitochondrial fission is a potential therapeutic approach to improve outcome after injury.

Many mitochondrial defects have been identified to contribute to mitochondrial dysfunction after injury, however the precise causal mechanisms underlying mitochondrial dysfunction after TBI have not yet been resolved.(Lifshitz et al., 2004) When considering the rate limiting reactions in mitochondrial oxidative phosphorylation (see Ch. 2.2 for review), several factors can be implicated in mitochondrial respiratory dysfunction after TBI, including 1) metabolite availability, 2) enzymatic control, 3) structural defects. Firstly, alterations in metabolite availability can slow mitochondrial respiration without significant damage to other critical components required for oxidative phosphorylation. Specifically, the availability of pyruvate and NADH, are key upstream regulators of respiration, as a major respiratory substrate that feeds into the TCA cycle, and a major product that feeds into the respiratory chain, respectively. Pyruvate availability, in particular, can be altered by either upstream regulation in the glycolytic pathway or deficits of transport into the mitochondrial matrix via the mitochondrial pyruvate carrier. Microdialysis studies have observed increases in extracellular lactate, as well as

increases of the lactate/pyruvate ratio in TBI patients and have been highly correlated with poor outcome.(Carpenter et al., 2015; Jalloh et al., 2015a) Studies have suggested that the increase in lactate may be due to increases in glycolysis and the preferential conversion of pyruvate to lactate via lactate dehydrogenase due to mitochondrial dysfunction and increases of extramitochondrial pyruvate availability. Production of lactate through the Pentose-Phosphate pathway has also been implicated, as shunting of Glucose-6-Phosphate from the glycolytic pathway has also been observed. However, the contribution of each to increases of extracellular lactate concentrations in the brain after injury has not been fully resolved. Additionally, it is possible that the inability of pyruvate to enter the matrix due to defects in the Mitochondrial Pyruvate Carrier (MPC) could result in reduced respiration without mitochondrial damage.(Divakaruni et al., 2013) However, given that the MPC was only recently been discovered at the molecular level, its activity and potential contribution to disease has not been well characterized. Furthermore, availability of mitochondrial NADH levels can be altered by defects in the enzymatic reactions of the TCA cycle, and thus also slow mitochondrial respiratory activity. Some enzymes of the TCA cycle are prone to oxidative damage due to exposed Fe/S centers required for enzymatic activity, such as aconitase.(Yan et al., 1997) Therefore, enzymatic activity is also vulnerable due to close proximity to reactive oxygen species in mitochondria. Most notably, the enzymatic activity of Complex I is also prone to oxidative damage and can significantly reduce mitochondrial respiration directly through defects in the electron transport chain.(Hirst, 2013) In the present study, reduced mitochondrial respiration acutely after experimental TBI were determined with the use of pyruvate and malate as respiratory substrates, and therefore implicate defects in complex I in mitochondrial dysfunction after injury. However, the contribution of other factors that could influence respiratory activity post-injury, as discussed above, cannot be ignored in these studies. Lastly, physical damage to mitochondrial ultrastructure can reduce mitochondrial oxidative phosphorylation by disrupting the ability of cristae formation, respiratory complex assembly, and ATP dimerization required for oxidative phosphorylation. Significant ultrastructural damage has been observed in isolated mitochondria

from injured brain tissues, characterized by fragmented cristae and mitochondrial swelling.(Lifshitz et al., 2003) This may be a consequence of direct mechanical forces from the initial impact, or the disruption of key protein regulators of inner membrane structure. The potential for ultrastructural damage is of particular interest to the current studies and mitochondrial dynamics, as Opa1 has been found to regulate cristae morphology and formation. Additionally, structural complexes have been recently identified to play a role in stabilization of cristae structure, such as the MICOS complex. Disruption of these complexes could result in cristae destabilization and contribute to mitochondrial dysfunction after injury; however, whether these mechanisms are disrupted after injury are unknown. Several additional factors not discussed here could also contribute to deficits in mitochondrial function after TBI. Further investigation is required to elucidate the precise mechanisms involved in injury-induced mitochondrial dysfunction and will be critical in developing mitochondrial-targeted therapeutic approaches that improve pathological outcome after TBI.

5.5 Mdivi-1 Treatment and Hippocampal Neuroprotection after TBI

Specific cell death and dysfunction in various cell types within the hippocampus are key contributors to neuropathological and cognitive outcomes in both experimental and clinical TBI.(Baldwin et al., 1997; Colicos et al., 1996; Hall et al., 2005; Zhou et al., 2012) Several mechanisms contribute to cell death and damage in the hippocampus including, inflammation, excitoxicity, mitochondrial dysfunction, and apoptosis.(Arundine and Tymianski, 2004; Chen et al., 2003b; Corps et al., 2015; Mazzeo et al., 2009; Raghupathi et al., 2000; Sullivan et al., 2002) In experimental models, neuronal death occurs acutely within the first 24 hours after injury, and can last from days to weeks post-injury due to propagation of secondary cascades and delayed cell death.(Chen et al., 2003b; Hall et al., 2005; Osier et al., 2014) Specific neuronal populations and hippocampal regions also appear to be particularly vulnerable after CCI injury, such as newborn neurons in the dentate gyrus and mature pyramidal neurons in the CA1 and CA3

regions.(Baldwin et al., 1997; Gao et al., 2008; Hall et al., 2005; Scheff and Price, 2005) Consequently, denervation in the axonal pathways between these areas and dendritic loss also occurs, causing profound functional impairment in the hippocampal circuitry.(Atkins, 2011; Marquez de la Plata et al., 2011; Scheff and Price, 2005) To determine whether Mdivi-1 treatment after experimental injury improved neuropathological outcome in the hippocampus, Mdivi-1 treated, injured hippocampal tissues were immunostained and examined for somal and dendritic loss in vulnerable neuronal populations at 24 hours post-injury (**Chapter 4.2**). Results from immunostaining for newly born neurons in the dentate gyrus of the hippocampus and quantification of the cell populations revealed a significant increase in cell survival with Mdivi-1 treatment after injury. Additionally, immunostaining for mature neurons and observation of populations in the CA1 and CA3 regions appeared to be slightly preserved with Mdivi-1 treatment after injury as well. In support of preservation of mature pyramidal soma with Mdivi-1 treatment, immunostaining for dendrites also appeared to show some preservation of dendritic integrity in the CA1 and CA3 areas. However, quantification was not obtained in either these populations, and therefore whether the observed protection is significant is not known. No differences were found in astrocytic responses after experimental TBI with Mdivi-1 treatment (**Chapter 4.3**). These results demonstrate that inhibiting Drp1 with Mdivi-1 treatment is protective of newly born neurons, and may be protective of some mature neuronal population as well after experimental TBI. In addition to hippocampal neuroprotection, these results are consistent with one other study that also observed improved cell survival in cortical neurons after mild experimental TBI and Mdivi-1 treatment.(Wu et al., 2016) Improved cell survival and functional benefits can also be found with pharmacological and genetic approaches targeting Drp1 in other brain injury and neurodegenerative models, such as ischaemia, Parkinson's and Huntington's, supporting the premise for potential therapeutic approaches targeting mitochondrial fission in neurodegeneration.(Cui et al., 2010; Grohm et al., 2012; Qi et al., 2013; Rappold et al., 2014; Wu et al., 2017; Zhao et al., 2014)

The selective death of different neuronal cell types and in different subregions of the hippocampus, and the potential differential preservation of selective populations with Mdivi-1 treatment pose several interesting questions concerning the underlying mechanisms involved in the vulnerability of these populations after TBI. Firstly, it is unknown whether subpopulations of cells in the hippocampus experience differences in disruptions in mitochondrial dynamics after injury. Secondly, it is unknown whether different cell types in the hippocampus may depend differently on mitochondrial fission and fusion for normal functioning, and therefore might be specifically more vulnerable to disruptions in these mechanisms after injury. Cell types in different brain regions have demonstrated varying dependence on functional mitochondrial dynamics revealed in knock out models of critical fission and fusion protein regulators.(Delettre et al., 2000; Manczak et al., 2012; Oettinghaus et al., 2016; Wakabayashi et al., 2009; Züchner et al., 2004) For example, models that produce pathological mitochondrial fission, such as Opa1 heterozygous knockout mice and Mfn2 inducible knock out mice, both demonstrate specific degeneration in the optic nerve and of cerebellar purkinje neurons in the cerebellum, respectively.(Burté et al., 2014; Davies et al., 2007; Detmer and Chan, 2007a) These particular cell populations are known to be highly metabolically demanding, and thus balanced mitochondrial dynamics might be critical for maintaining energy requirements for normal cellular functioning. In the studies reported in this dissertation, the observation that newly born neurons in the dentate gyrus, are selectively protected with Mdivi-1 treatment, suggests that disruption of mitochondrial fusion and fission plays a major role in the death of these cells after injury. Thus, newborn neuron function may be particularly dependent on the balance between these processes, contributing to their susceptibility after injury. Interestingly, a recent study employing inducible knock out models of various mitochondrial fission and fusion proteins, including, Drp1, Opa1, and Mfn1/2, demonstrated that mitochondrial dynamics is critical for neural stem cell identity, self-renewal, and fate during development. (Khacho et al., 2016) The same study also observed specific depletion of the adult neural stem cell population in an Mfn1/2 inducible knockout mouse, supporting the requirement of functional mitochondrial fission and fusion in

adult hippocampal neurogenesis. Studies of mitochondrial dynamics, in other proliferating cell types, such as oocytes and hematopoietic stem cells have also revealed very interesting regulation of fission and fusion that are critical for different steps of proliferation.(Chen and Chan, 2017; Khacho and Slack, 2017) Further study is necessary to identify potential differential regional and cell-type specific changes in mitochondrial dynamics after experimental TBI, and to determine possible vulnerability of specific populations to mitochondrial dysfunction and cell death after injury.

5.6 Mdivi-1 Treatment and Hippocampal-Dependent Cognitive Outcome after TBI

Both clinical and experimental evidence demonstrates lasting deficits in cognitive function and memory impairment after a TBI.(Ashman et al., 2006; Osier et al., 2014) The hippocampus, a structure critical for learning and memory, is particularly vulnerable to structural and functional damage after TBI.(Bigler et al., 1997; Chen et al., 2003b; Marquez de la Plata et al., 2011) The Controlled Cortical Impact (CCI) is a focal, moderate-severe injury model that targets the parietal cortex directly above the hippocampus in rodents, and thus results in profound hippocampal damage and dysfunction.(Chen et al., 2003b; Edward Dixon et al., 1991; Osier et al., 2014) After CCI injury, rodents exhibit reproducible deficits in many hippocampal-dependent behavioral tasks, including spatial navigation tasks, such as Morris Water Maze and Barnes Maze, and recognition/episodic tasks, such as Fear Conditioning and Object Recognition.(Hamm, 1992; Osier et al., 2014; Scheff et al., 1997; Xiong et al., 2013) The reproducible nature of deficits in these tasks using the CCI model, thus offers a functional indication of hippocampal damage after TBI and can be used as a measure to assess effects of pharmacological treatment on hippocampal-dependent cognitive outcome after injury. In the current studies, injured animals were administered Mdivi-1 treatment and assessed on three hippocampal-dependent tasks, the Novel Object Recognition (NOR) task, a One-Trial Contextual Fear Conditioning task, and the Morris Water Maze task (**Chapter 4.1**). Results from these studies revealed that Mdivi-1

treatment reduced impairments of recognition and contextual fear memory after experimental TBI; however, Mdivi-1 treatment did not reduce deficits in spatial memory. One study investigating Mdivi-1 treatment in mice after experimental TBI using a weight drop injury model, did observe improvements on the Morris Water Maze task after injury.(Wu et al., 2016) However, the weight drop injury model is a less severe, mild injury model, compared to the CCI model used in the current studies, and thus results in less hippocampal damage and cognitive impairment.(Christiane and Sirén, 2010; Xiong et al., 2013) It is possible that Mdivi-1 treatment may benefit milder functional deficits in the hippocampus that would result in cognitive improvements reflected in assessment of hippocampal-dependent spatial memory compared to a more severe injury. Nonetheless, inhibiting Drp1 with Mdivi-1 treatment did improve hippocampal-dependent cognitive performance in two hippocampal-dependent memory tasks after experimental TBI.

Variability in the results of Mdivi-1 treatment on cognitive outcome between different behavioral tasks pose an interesting question to why improvements would be revealed in selective hippocampal-dependent memory tasks. Morris Water Maze (MWM) remains the gold standard to detection of sensitive hippocampal function, so what underlying neurobiology would explain improvements in other hippocampal-dependent tasks and not MWM? Although a difficult question to answer, to compose a potential explanation, the behavioral complexity of these tasks, the hippocampal recruitment required for each task, as well as the regional and cell-type specificity of hippocampal injury after experimental TBI must be considered.

Firstly, both the Novel Object Recognition (NOR) task and the Contextual Fear Conditioning (FC) task are relatively simple learning paradigms. The NOR task entails mere exposure of an animal to objects, relying on the innate exploratory behaviors of rodents for familiarization and learning of the objects, and does not require rule learning or reinforcement.(Antunes and Biala, 2012) The Contextual FC task employs classical conditioning of an aversive stimulus to a particular contextual environment, and relies on incidental “gestalt”

learning of the contextual features.(Maren et al., 2013) The Morris Water Maze, on the other hand, is a technically challenging task, as it requires the engagement of multiple behavioral abilities and sophisticated information processing for successful task completion.(D'Hooge and De Deyn, 2001; Vorhees and Williams, 2014) These requirements encompass visuospatial acquisition, self-localization and allocentric navigation, strategy and path integration, route replay, and nonspatial navigational skills.(Redish and Touretzky, 1998; Vorhees and Williams, 2014, 2006) Furthermore, all of these learning components must converge for effective encoding, consolidation, storage, and retrieval of spatial memory.(Bird and Burgess, 2008) Therefore, the learning paradigm in the MWM is complex and requires the engagement and interaction of multiple behaviors, where as simple tasks such as the NOR and the Contextual FC tasks do not require as much behavioral "skill" *per se*.

Inherent to task complexity, learning and memory in each of these tasks also requires particular circuitry for successful task completion, specifically in the hippocampus.(Bird and Burgess, 2008; Maren et al., 2013; Redish and Touretzky, 1998; Squire et al., 2007) Although each of these tasks are hippocampal-dependent, all have demonstrated differential reliance on hippocampal intactness and function.(Broadbent et al., 2004, 2010; Cohen et al., 2013; Maren et al., 2013; Squire et al., 2007; Zelikowsky et al., 2012) Spatial learning and memory in the MWM task, in particular, demands almost complete integrity of the dorsal hippocampus.(Bird and Burgess, 2008; Broadbent et al., 2004) Conversely, recognition and contextual fear memory have both demonstrated less dependence on complete hippocampal integrity and can be maintained in conditions of considerable hippocampal damage.(Broadbent et al., 2004; Zelikowsky et al., 2012) Thus, learning and memory performance on these tasks can be graded depending on the damage and functional integrity in the hippocampus, and the effect of treatment on hippocampal damage can therefore also be graded. Thus, subtle improvements from treatment might manifest in some tasks compared to others. For example, in a simple, yet comprehensive lesion study, Broadbent *et al.* (2004) demonstrated that deficits of spatial memory on a water maze task were

observed when 30-50% of total dorsal hippocampal volume was lesioned, whereas deficits in recognition memory did not manifest until 75-100% of total dorsal hippocampal volume was lesioned.(Broadbent et al., 2004) Thus, greater hippocampal integrity is required for spatial learning and memory compared to object recognition memory. After CCI injury, there is major damage to the hippocampus and, consistent with the current studies, deficits in spatial memory are observed in the MWM task.(Hamm, 1992; Scheff et al., 1997) Therefore, to observe improvements in spatial memory on the MWM task with treatment after injury, a significant inhibition or repair of hippocampal would be required. On the other hand, improvements in recognition memory in the NOR might manifest with subtler effects of treatment on hippocampal damage. It is important to note, that hippocampal involvement in recognition memory has been widely debated in the literature.(Antunes and Biala, 2012; Broadbent et al., 2010) However, an accumulation of recent evidence has demonstrated the hippocampus is in fact important for consolidation of nonspatial, recognition memory.(Antunes and Biala, 2012; Broadbent et al., 2004; Cohen and Stackman, 2015; Cohen et al., 2013; Squire et al., 2007)

The differences between hippocampal dependence for spatial memory and contextual fear memory are less clear, as contextual fear memory does contain both nonspatial and spatial memory characteristics and thus also requires considerable hippocampal intactness.(Maren et al., 2013) However, experimental evidence has demonstrated that specific hippocampal regions and cell types that are important for contextual fear memory, may be dispensable for spatial learning and memory on the MWM task in certain experimental conditions. (Deng et al., 2010; Denny et al., 2012; Drew et al., 2010; Saxe et al., 2006) Specifically, neurogenesis and newly born neurons in the dentate gyrus of the hippocampus are especially critical for contextual fear memory, as genetic or irradiation-induced ablation of neurogenesis completely abolish the ability for animals to complete the task.(Deng et al., 2010; Denny et al., 2012; Drew et al., 2010; Saxe et al., 2006) Conversely, although studies have yielded mixed results, in certain conditions of the MWM task spatial memory is preserved with ablation of neurogenesis. (Deng et al., 2010; Garthe

and Kempermann, 2013; Saxe et al., 2006) In the context of injury, these hippocampal cell populations are known to be selectively vulnerable to cell death after experimental TBI.(Gao et al., 2008; Zhou et al., 2012) Results from the current studies observed that Mdivi-1 treatment significantly increased the survival of newly born neurons after injury (**Chapter 4.2**). Therefore, it is possible that the selective increase of survival in this particular cell population that is distinctly critical for contextual fear memory could result in improvement of fear memory on this task. Furthermore, neurogenesis and newly born hippocampal neurons have also been observed to be critical for object recognition memory.(Deng et al., 2010; Jessberger et al., 2009; Suárez-Pereira and Carrión, 2015) Thus providing further evidence in support of improved hippocampal-dependent cognitive function with Mdivi-1 treatment after experimental injury observed on the NOR and Contextual FC tasks, and not the MWM task.

The hippocampal circuitry and manifestations of hippocampal-dependent behavior are incredibly complex and been widely debated in the literature. It is important to note that investigations of hippocampal contributions in learning and memory on each of these tasks have yielded highly variable results and appear to be dependent on the injury model, severity of injury, and specific task parameters in each experiment. Therefore, the employment of different hippocampal-dependent tasks and the careful interpretation of results should be considered to properly use these tasks as a functional measure for hippocampal damage after TBI.

5.7 Mdivi-1, inhibiting Drp1, and blocking mitochondrial fission as potential therapeutic strategies after TBI

Mitochondrial Division Inhibitor 1 (Mdivi-1) has been characterized as a specific inhibitor of Drp1 and mitochondrial fission in yeast (Dnm1), by allosterically blocking the self-assembly of Drp1 and stabilizing a conformation that reduces the affinity for GTP binding.(Cassidy-stone et al., 2008) Thus, GTPase activity is reduced and mitochondrial division is inhibited. The studies

described in this dissertation demonstrate that treatment with Mdivi-1 after experimental TBI significantly improves cognitive performance, increases survival of a vulnerable cell population in the hippocampus, inhibits injury-induced mitochondrial Cytochrome *c* release, and results in the elongation of mitochondria. These results thus implicate pharmacological inhibition of Drp1 and mitochondrial fission, and Mdivi-1, as potential therapeutic strategies after TBI. However, the underlying mechanisms that contribute to improved neuropathological outcome must be critically examined to specifically determine which mechanisms are involved in the observed improvements after experimental TBI.

Firstly, it is important to discuss the potential therapeutic implications for these results when considering the distinction between Drp1-mediated mechanisms and mitochondrial fission. Results from the current studies demonstrated that Mdivi-1 treatment causes both mitochondrial elongation and inhibition of Cytochrome *c* release after experimental injury. Although Drp1 is the primary protein regulatory of mitochondrial fission and blocking Drp1 activity will result in reduced mitochondrial fission, Drp1 is also critically involved in regulatory mechanisms other than mitochondrial fission, such as apoptosis. Therefore, with the approach of inhibiting Drp1, it is difficult to determine whether the improvements observed in these studies with Mdivi-1 treatment after TBI are due to the inhibition of Drp1, specifically, or the inhibition of mitochondrial fission. Given the role of Drp1 in apoptotic signaling, and the potential benefits of restoring balance of mitochondrial fission and fusion, it is likely that both mechanisms together contributed to the improvements in neuronal survival and cognitive dysfunction observed in these studies. Nonetheless, excessive mitochondrial fission was observed after experimental TBI, therefore to determine whether targeting mitochondrial fission and fusion, and/or their regulatory mechanisms, offers therapeutic potential, it is essential to elucidate the precise mechanisms involved in the injury-induced disruption of balance between fission and fusion. Recent research has shown evidence for the potential of manipulating fusion proteins to restore the balance of fusion and fission during pathological fission states.(Detmer and Chan, 2007a; Jahani-Asl et al.,

2011; Sanderson et al., 2015; Varanita et al., 2015) In these studies, overexpression of Opa1 and Mfn1 both promote fusion, reduce mitochondrial dysfunction, and increase cell survival in cell culture models of injury and disease. However, whether targeting the fusion proteins to restore the balance of fusion and fission after injury improves outcome is unknown. Currently, no known pharmacological agents are available that directly target the fusion proteins or promote fusion through other mechanisms without also affecting the fission pathways. Development of such approaches will be critical for future studies to determine whether the promotion of fusion or the inhibition of fission improves outcome after TBI.

Secondly, for the potential of Mdivi-1 specifically as a therapeutic approach it is important to discuss a recent study that reported findings that challenge the specificity of Mdivi-1 as an inhibitor of Drp1.(Bordt et al., 2017) In these studies, several cells lines were used to measure the effect of Mdivi-1 on mitochondrial morphology, including primary cortical neurons, and the cell line used in the origin paper identifying Mdivi-1 as a specific chemical inhibitor of mitochondrial fission (COS cells). The same concentrations and incubation times as the original findings were used, as well as increased concentrations and incubations; however, no effect was observed on mitochondrial morphology in any of the cell lines. Additionally, the GTPase activity of recombinant human Drp1 protein was also assessed in the presence of Mdivi-1, in which Mdivi-1 only weakly inhibited GTPase activity at much higher concentrations than previously reported to inhibit mitochondrial division in the original publication. Most notably, these studies found that complex-I dependent respiration was significantly impaired with Mdivi-1 treatment in intact cells and in isolated mitochondria, and that this inhibition is reversible with washout of the drug. Reduced complex I-driven respiration was found to be independent of Drp1, as respiratory defects were observed with Drp1 KO in embryonic fibroblasts. Furthermore, the reduced mitochondrial respiration was rescued with ectopic expression of yeast NADH dehydrogenase enzyme, a protein that is used as a functional substitute to complex I, indicating that complex I is a direct substrate of Mdivi-1 and the source of reduced respiration. It is important to note that

the observed effects of Mdivi-1 treatment in this study were less pronounced in neurons compared to the other cell lines investigated, indicating the potential for cell-specific responses of Mdivi-1 treatment. Additionally, these studies also showed that during complex II driven respiration (using succinate as substrate), Mdivi-1 dose-dependently inhibited reactive oxygen production in isolated mitochondria from rat brain. During complex II-driven respiration, complex I is known to produce massive ROS due to reverse electron transport (RET).(Hirst, 2013; Murphy, 2009) RET-ROS has been characterized a pathological feature of ischaemia-reperfusion injuries due to the accumulation of succinate in the absence of oxygen.(Chouchani et al., 2014) Bordt et al. (2017) thus proposes that benefits reported with Mdivi-1 treatment in disease and injury models, may be attributed to the ability of Mdivi-1 to inhibit RET-ROS and oxidative damage. In theory this could be possible, but the occurrence of RET-ROS is a relatively recent finding and has not yet been characterized in many other pathological conditions unrelated to ischaemia-reperfusion.(Chouchani et al., 2014; Scialo et al., 2016)

The original identification of Mdivi-1 as a specific inhibitor of the yeast homolog, Dnm1, and mitochondrial fission by Cassidy-stone et al. (2008) was an elegant and thorough study.(Cassidy-stone et al., 2008) However, the evidence reported for the specific mechanisms of action for Mdivi-1 inhibition of Drp1 were only well-characterized in the Dnm1 yeast homolog, and the mammalian inhibition of Drp1 was inferred from the observation of mitochondrial elongation and localization of Drp1 in apoptotic conditions with Mdivi-1 treatment. Thus, considering the recent reports from Bordt et al. (2017), whether Mdivi-1 directly inhibits Drp1 in mammalian cells remains unclear. Nonetheless, in the studies described in this dissertation, Mdivi-1 treatment did in fact result in elongation of mitochondria during a state of excessive fission, and also reduced cell death and cognitive dysfunction after experimental TBI. Taken together, although Mdivi-1 has demonstrated benefits in several tissues, spanning multiple injury and disease models, the precise mechanism of action in mammalian systems requires further investigation to discern the direct mechanisms underlying the observed benefits.

5.8 Concluding Remarks and Future Directions

Traumatic brain injury (TBI) is a multifaceted disease that initiates complex pathological processes resulting in neuronal death, regional dysfunction, and lasting cognitive deficits. Metabolic and mitochondrial dysfunction has been identified as a primary indicator of poor patient outcome after injury, therefore mitochondrial-targeted therapeutic approaches may be key in improving recovery after TBI. Growing evidence in the literature indicates that imbalances of mitochondrial fission and fusion are detrimental to cell survival and a common characteristic in many neurodegenerative diseases. The findings reported in this dissertation are of the first to elucidate that Drp1-mediated disruptions in mitochondrial fission occur after experimental TBI and contribute to neuropathological outcome (**Fig. 5.3**). Specifically, these results indicate that TBI increases Drp1 association with mitochondria in the hippocampus after injury, and causes acute changes in mitochondrial morphology, eventually resulting in a prolonged increase in Drp1-mediated fission. Additionally, it was found that Drp1 also mediates injury-induced Cytochrome c release from mitochondria. Furthermore, Mdivi-1, a pharmacological inhibitor of Drp1, increased the survival of newly born neurons after injury, and reduced hippocampal-dependent cognitive deficits. However, Mdivi-1 treatment did not reduce mitochondrial dysfunction after injury. Overall, these results indicate a potential role for Drp1 and excessive mitochondrial fission in hippocampal neuronal death and cognitive dysfunction after TBI, and thus mechanisms of Drp1-mediated fission may offer a mitochondrial-targeted therapeutic approach to improve outcome after brain injury.

The studies presented in this dissertation have made considerable advances in the current working knowledge underlying the dysregulation of mitochondrial fission and neuropathological outcome after TBI. However, numerous questions remain unanswered. Further research is essential to more thoroughly elucidate the precise mechanisms involved in the dysregulation of Drp1 and mitochondrial fission after injury to qualitatively determine and refine therapeutic approaches that target these mechanisms. A few hypotheses have been

discussed in great detail above, but several questions stem directly from this work and should be acknowledged.

Firstly, what environmental changes after injury and upstream mechanisms drive Drp1 to the outer mitochondrial membrane to induce excessive fission events? And What role do mechanisms of mitochondrial fusion play in injury-induced pathological fission?

Secondly, are mitochondrial morphological changes indicative of the metabolic state of cells after injury? Additionally, are there ultrastructural changes of the inner mitochondrial membrane that may influence mitochondrial function and adaptation of metabolic response after injury?

Thirdly, is there cell type or regional specificity in disruptions of mitochondrial fission after injury? And, are certain cells inherently more vulnerable to imbalances of fusion and fission?

Fourthly, does inhibition of Drp1 block apoptosis after TBI? What are the separate contributions of Drp1 dysregulation and/or mitochondrial fission in neuropathology after TBI?

Lastly, what are the primary mechanisms involved in the dysfunction of mitochondrial respiration and oxidative phosphorylation after injury? Many features of mitochondrial function can and should be considered, such as structural changes, metabolic substrate concentrations, enzymatic defects, respiratory chain defects, disruption of mitochondrial transporters, and many, many more.

Well, looks like there is a lot of work to do. Let’s get to it!

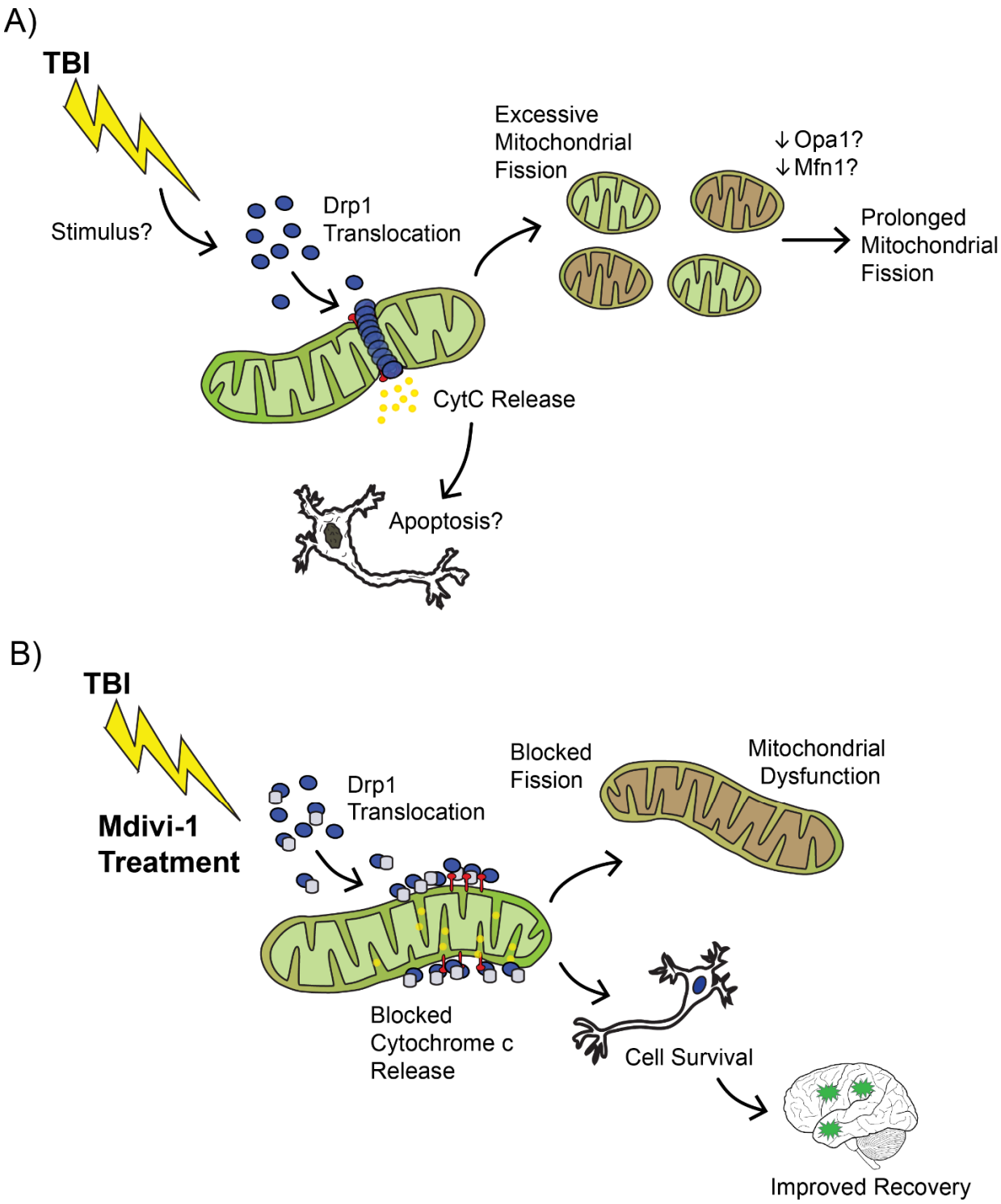

Figure. 5.3 Revised hypothesis model.

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