Characterizing Stomatin-like Protein 2 and its Role in Neuron Survival

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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CHARACTERIZING STOMATIN-LIKE PROTEIN 2 
AND ITS ROLE IN NEURON SURVIVAL
(Thesis format: Monograph)

by

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
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The School of Graduate and Postdoctoral Studies
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Abstract

Stomatin-like Protein 2 (SLP-2) has been identified as a stress-inducible transcript and has been shown to interact with and stabilize mitochondrial proteins. Since mitochondria are critical for neuronal function, we hypothesized that SLP-2 regulates neuron survival in response to stressful stimuli. A conditional SLP-2 knockout mouse (deletion) and the SN56 cell line (upregulation) were employed to study the role of SLP-2 in mitochondrial dynamics and neuron survival. SLP-2 deficient primary cortical neurons displayed significantly decreased levels of various mitochondrial respiratory chain proteins, indicating SLP-2 contributes to maintenance of mitochondrial membrane integrity. SLP-2 was up-regulated in response to oxidative stress and DNA damage, but was not required for stress-induced mitochondrial biogenesis. The level of SLP-2 expression had a significantly different effect on neuron survival, depending on the target of the insult. The results of this study improve our understanding of the role of SLP-2 in mitochondrial stress responses, bioenergetics, and survival in neurons.

Keywords

Stomatin-like protein 2, neuron, mitochondria, respiratory chain, mitochondrial membrane
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List of Abbreviations, Symbols, Nomenclature

3-NP - 3-nitropropionic acid
AD - Alzheimer’s disease
ADP - adenosine diphophate
AIP - apoptosis-inducing factor
ALS - amyotrophic lateral sclerosis
ANOVA - analysis of variance
ATP - adenosine triphosphate
BCA - bicinechonic acid
Bcl-2 - B cell lymphoma 2
BF-1 - brain factor 1
bp - base pair
CaCl₂ - calcium chloride
C57/BL6 - C57 black 6
CCCP - carbonyl cyanide m-chlorophenyl hydrazone
CD95 - cluster of differentiation 95
cDNA - complementary DNA
CMV - cytomegalovirus
CMXRos - chloromethyl-X-rosamine
CNS - Central nervous system
CO₂ - carbon dioxide
CoQ - coenzyme Q
CPT - camptothecin
Cre - Cre recombinase
CT - cycle threshold
cytc - cytochrome c
ddH₂O - double distilled water
DMEM - Dulbecco’s Modified Eagle Medium
DNA - deoxyribonucleic acid
DOA - dominant optic atrophy
Drp1 - dynamin-related protein 1
E - embryonic day
EDTA - ethylenediaminetetraacetic acid
EGFP - enhanced GFP
ER - endoplasmic reticulum
ESCC - esophageal squamous cell carcinoma
ETC - electron transport chain
Fis1 - mitochondrial fission protein 1
Foxg - forkhead box G1
GAM - goat anti-mouse
GAR - goat anti-rabbit
GFP - green fluorescent protein
H$_2$O$_2$ - hydrogen peroxide
HBS - HEPES buffered saline
HBSS - Hanks’ balanced salt solution
HD - Huntington’s disease
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO* - hydroxyl radical
HRP - horseradish peroxidase
IgG - immunoglobulin G
IMS - intermembrane space
lox - LoxP sequence
MEFs - mouse embryonic fibroblast
Mfn1 - mitofusin 1
Mfn2 - mitofusin 2
MgSO$_4$ - magnesium sulfate
MIM - mitochondrial inner membrane
MMP - mitochondrial membrane permeabilization
mNCE - mitochondrial sodium calcium exchanger
MPP+ - 1-methyl-4-phenylperidinium
MPTP - 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine
mRNA - messenger RNA
mtDNA - mitochondrial DNA
Na$_2$HPO$_4$ - disodium phosphate
NBM - neurobasal media
NDUFS3 - NADH dehydrogenase (ubiquinone) iron-sulfur protein 3
NMDA - N-methyl-D-aspartic acid
NRF-1 - nuclear respiratory factor 1
NRF-2 - nuclear respiratory factor 2
O$_2$ - oxygen
O$_2^-$ - superoxide anion
OMM - outer mitochondrial membrane
OPA1 - optic atrophy 1
OXPHOS - oxidative phosphorylation
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PD - Parkinson’s disease
pEGFP - plasmid containing EGFP DNA
PFA - paraformaldehyde
PGC1-$\alpha$ - peroxidome proliferator-activated receptor $\gamma$ coactivator 1$\alpha$
pSLP-2GFP - plasmid containing fused SLP-2-GFP DNA
PTPC - permeability transition pore complex
RC - respiratory chain
RIPA - radio immunoprecipitation assay
RIRR - ROS-induced ROS release
RNA - ribonucleic acid
RNAi - RNA interference
ROS - reactive oxygen species
RT-PCR - reverse transcription polymerase chain reaction
SDS - sodium dodecyl sulfate
SE - standard error
SIMH - stress-induced mitochondrial hyperfusion
SLP-1 - stomatin-like protein 1
SLP-2 - stomatin-like protein 2
SLP-2<sup>lox/lox</sup> - *Lox*P sequences flanking SLP-2 on both alleles
SLP-2<sup>lox/lox/Cre</sup> - *Lox*P sequences flanking SLP-2 on both alleles, expressing Cre
SLP-2<sup>lox/wt</sup> - *Lox*P sequences flanking SLP-2 on one allele
SLP-3 - stomatin-like protein 3
SOD - superoxide dismutase
SPFH - stomatin prohibitin flotilin Hflk
tBid - truncated Bid
TCA - tricarboxylic acid
TCR - T-cell receptor
Tfam - mitochondrial transcription factor A
TNF - tumor necrosis
TOM20 - translocase of outer mitochondrial membrane 20 protein
TOMM20 - translocase of outer mitochondrial membrane 20 gene
Tris - trisaminomethane
UV - ultraviolet
wt - wild-type
Chapter 1: Introduction

1.1 Mitochondria and Neuronal Activity

The central nervous system (CNS) uses approximately twenty percent of the oxygen consumed at rest (Gutowicz, 2011; Kann & Kovacs, 2007). Neurons are highly metabolic, differentiated cells that require large amounts of adenosine triphosphate (ATP), in order to carry out their various functions. As a result, they rely heavily on proper mitochondrial function for a number of fundamental neuronal processes such as energy production, synaptogenesis, calcium buffering, axonal and dendritic transport, and synaptic transmission (Knott et al., 2008; Kann & Kovacs, 2006).

Although it has a large oxidative capacity, the brain has limited ability to withstand oxidative stress. The majority of ATP in neurons is generated by the mitochondria through oxidative phosphorylation (OXPHOS). As a result, neurons have a tremendous dependence on mitochondrial OXPHOS and a particular vulnerability to mitochondrial dysfunction. Furthermore, neurons are functionally and morphologically heterogeneous between cell type and brain region. As a result, there is a difference in metabolic requirement between neuronal cell type, as well as the axonal and presynaptic regions of each individual neuron, which necessitates differential regulation of energy production (Kann & Kovacs, 2006; Calabrese et al., 2001).

Neuronal activity is associated with transient elevations in calcium, a second messenger important for neural plasticity that can also, in excessive amounts, lead to cell excitotoxicity. Mitochondria play an important role in buffering cellular calcium levels, especially at the level of the synapse, in order to maintain proper neuronal function. Additionally, synaptic transmission requires maintenance of plasma membrane potential, release and reuptake of neurotransmitters, and in the case of high frequency or prolonged firing, build up of a reserve pool of neurotransmitter-containing vesicles as well as
localized protein translation. All of these processes are extremely energy demanding and require an adequate number of mitochondria at the synapse (Kann & Kovacs, 2006; Billups & Forsythe, 2002; Vos et al., 2010).

Due to their ability to undergo fission and fusion, migrate through cells, and coordinate regulated turnover, mitochondria are able to adapt to changes in cellular requirements depending on physiological cues or environmental stimuli (Chen & Chan, 2006, 2009). Cooperation of mitochondrial fission and fusion with mitochondrial transport is important, especially in neurons with long axons, to ensure that the proper number of mitochondria arrive at the synapses. Furthermore, proper mitochondrial dynamics are critical in neurons as they allow mitochondria to interact and communicate with each other. This in turn facilitates mitochondrial movement and distribution, over long distances, to synapses and areas of high metabolic activity, where they are needed the most. Synaptogenesis, which includes dendritic spine morphogenesis, requires localization of mitochondria into dendritic sites of development and plasticity. Mitochondrial proteins Drp1 (dynamin-related protein 1) and OPA1 (optic atrophy 1), which are involved in fission and fusion respectively, are essential in preserving the level of mitochondrial content necessary for spine formation, further illustrating the importance of proper mitochondrial dynamics and movement (Li, 2004; Lu, 2009; Chen & Chan, 2006, 2009).

Figure 1 provides a schematic describing the mechanisms by which mitochondrial dysfunction can result in improper mitochondrial organization within neurons, and thereby contribute to neuronal dysfunction. The importance of the interaction between mitochondrial dynamics, energy metabolism and neuronal function is supported by the level of cellular dysfunction that ensues when mitochondrial dynamics are disrupted. Mitochondrial defects appear to have an indisputable role in neuronal deficiency, given that oxidative stress, alterations in apoptosis and impairment of electron transport chain (ETC) complexes have all been shown to contribute to neurodegenerative disease
progression (Lu, 2009; Chen & Chan 2006, 2009; Santos & Cardoso, 2012; Calabrese et al., 2001).

Alzheimer’s disease (AD) is the most common age-related neurodegenerative disorder and is marked by cognitive impairment due to progressive neuronal cell loss due to the accumulation of amyloid-β (Aβ) plaques and neurofibrillary hyperphosphorylated tau protein tangles (NFT) (de Moura et al., 2010). Impaired mitochondrial function in this diseased state triggers Aβ deposition, synaptic dysfunction and degeneration, and generation of NFT. Experimental studies have shown an over-expression of Aβ protein resulting in decreased expression of fission proteins Drp1 and Fis1 and fusion protein OPA1 (Section 1.2.1). The altered expression of these mitochondrial proteins in AD is associated with mitochondrial dysfunction and fragmentation, depolarization of the mitochondrial membrane, and a resultant increase in reactive oxygen species (ROS) and ATP production thereby contributing to a cycle of increased ROS production (Section 1.4.2) (Wang et al., 2008).

Parkinson’s disease (PD), the second most prevalent neurodegenerative disorder following AD, is characterized by extensive and progressive loss of dopaminergic neurons in the substantia nigra pars compacta as well as an accumulation of intraneuronal inclusions, known as Lewy bodies, in the remaining neurons. Several studies have linked PD with mitochondrial function and oxidative stress. The most common alterations detected in patients with PD are complex I deficiency, dysfunction and decreased activity of Complexes I and IV, and an increase in oxidative damage (Sections 1.4.1, 1.4.2) (Moran et al. 2012; Zhu & Chu, 2010; Xie et al., 2010; Henchcliffe & Beal, 2008; Muftuoglu et al., 2004; Palacino et al., 2004; Poole et al., 2008). Complex I inhibitors including 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP), 1-methyl-4-phenylperidinium (MPP+), and rotenone, can elicit PD-like symptoms in both humans (MPTP) and animal models (MPP+, rotenone) (Jones & Miller, 2008; Perier et al., 2005; George et al., 2010; Cicchetti et al., 2005).
Huntington’s disease (HD) is a progressive neurodegenerative disorder that differs from AD and PD in that it is solely caused by a genetic abnormality. Due to an abnormal CAG trinucleotide repeat in the N-terminal region, a conformational change results in the huntingtin protein and leads to a number of neurological defects (Imarisio et al., 2008). Mitochondrial dysfunction, involving reduced ETC activity at complexes II, III and IV (Section 1.4.1), has been strongly linked to HD (Benchoua et al., 2006; Gu et al., 1996). Irreversibly inhibiting complex II with 3-nitropropionic acid (3-NP) results in increased ROS production and damage to mitochondrial but not nuclear DNA in both cellular and animal models, with additional neurodegeneration observed in rats and nonhuman primates (Mandavilli et al., 2005; Brouillet et al., 2005; Acevedo-Torres et al., 2009; Damiano et al., 2010).
Figure 1. Mechanisms of neuronal dysfunction. In healthy neurons (a) mitochondria (green) are actively distributed over long distances from the cell body to dendritic and axonal termini where they are required for the production of ATP and calcium homeostasis. Reduced mitochondrial fusion (b) results in fragmented, dysfunctional mitochondria (red) with structural defects. Subsequently, a lack of fusion results in defective transport capacity and prevents proper distribution of mitochondria to areas distant from the soma. Reduced mitochondrial fission (c) results in mitochondrial lengthening and formation of a highly interconnected network. A portion of these mitochondria are also defective (red), and poorly distributed, due to their aggregation near the soma. Mitochondria that are defective primarily in transport capacity (d) result similarly in unequal distribution of mitochondria to peripheral axons and dendrites. This figure is adapted from Chen and Chan, 2009.
1.2 Mitochondrial Dynamics

Mitochondria, the double membrane bound powerhouses of eukaryotic cells, are highly metabolic organelles that serve a critical bioenergetic role in many cellular functions. In addition, these tubular-shaped structures are central to a number of cell regulatory and signaling processes by way of response to an assortment of physiological and genetic stresses, communications from other organelles, and signaling in cell proliferative and death pathways. As a convergence point for a number of signaling pathways, mitochondria appear to play an important role in receiving, coordinating, and transmitting cellular signals, by altering their own shape and dynamic response. Mitochondrial functions include a number of metabolic pathways such as homeostatic calcium and iron regulation, energy production, apoptosis regulation and generation of reactive oxygen species (ROS) (Da Cruz et al., 2003, Youle & Biekk, 2012; Chen & Chan, 2006; Knott et al., 2008; Santos & Cardoso, 2012; Moran et al., 2012).

The extremely protein-rich mitochondrial inner membrane (MIM) encloses the matrix, and is organized into folds, or cristae, that house the membrane-bound complexes of the electron transport chain, the ATP Synthase (Complex V) and additional electron transport proteins such as cytochrome c (cyt c). The outer mitochondrial membrane (OMM) is a smooth phospholipid bilayer that encapsulates the inner membrane and intermembrane space. The OMM is responsible for coordinating mitochondrial function with extramitochondrial signaling, thereby regulating mitochondrial dynamics. Finally, the mitochondrial matrix contains the various enzymes that are involved in many of the metabolic functions of the organelle (Youle et al., 2012; Krauss et al., 2001; Calabrese et al., 2001; Benard & Karbowski, 2010).

Through differences in permeability between the outer and inner membranes, such that the MIM is much less permeable to smaller molecules and ions than the OMM is, mitochondria are able to achieve compartmentalization, which is important for many of their wide-ranging cellular roles. By acting both as a molecular barrier and an insulator for electrical gradients, the inner membrane is able to facilitate the separation of the
mitochondrial matrix and its contents from the cytosol (Youle et al., 2012; Krauss et al., 2001; Calabrese et al., 2001; Benard & Karbowski, 2010).

1.2.1 Fission and Fusion

Due to their dynamic nature, mitochondria continuously fuse and divide in order to form interconnected networks or small individual units within a cell. These opposing processes of fission and fusion are frequent in healthy cells and are adjusted relative to cellular needs in order to maintain organelle homeostasis, normal cell function and cell survival (Figure 2).

Fission and fusion dynamics control mitochondrial morphology and function in response to cell physiology, developmental state, and a variety of environmental factors. Proper mitochondrial dynamics are essential for the organelle to be able to carry out its many metabolic and regulative roles in a number of fundamental eukaryotic processes. Additionally, mitochondrial dynamics allow for communication between mitochondria, without which the mitochondrial population would become functionally separated and impaired (Suen et al., 2008; Lee et al., 2004; Parone et al., 2008; Chen & Chan, 2006; Benard & Karbowski, 2009; Chan, 2006).

An imbalance between fission and fusion is closely related to alterations in mitochondrial function and ultrastructure, loss of mitochondrial DNA (mtDNA), mitochondrial membrane depolarization, and has been linked to several neurodegenerative diseases, apoptosis, cancer and ageing. (Muster, 2010; Knott & Bossy-Wetzel, 2008; Wang et al., 2009; Chen & Chan, 2009; Santos & Cardoso, 2012; Moura et al., 2010; Suen et al., 2008; Sheridan & Martin, 2010).
Figure 2. Schematic overview of mitochondrial fission and fusion cycles. Under normal cell physiological conditions, mitochondria undergo regular maintenance that involves fusion mediated by both Mitofusin 1 and 2 (Mfn1/2) and long- and short-isoforms of OPA-1 (L/S-OPA1) as well as fission mediated by dynamin-related protein 1 (Drp1) and mitochondrial fission protein 1 (Fis1). Under conditions of low stress, mitochondria undergo stress-induced mitochondrial hyperfusion (SIMH), which is mediated by Mfn1 and L-OPA1 (but not Mfn2 or S-OPA1). SIMH confers stress-resistance to cells by promoting mitochondrial biogenesis and increased ATP production. Under high stress conditions, or if low levels of stress are maintained, cells eventually succumb to an apoptotic pathway that involves increased mitochondrial fragmentation and dysfunction. This figure is adapted from van der Bliek, 2009.
The mitochondrial genome is formed by a circular 16,569 base pair DNA that encodes 37 genes. The majority of these genes code for transfer ribonucleic acids (tRNAs) that are required for mitochondrial translational machinery, while the remainder encode ribosomal ribonucleic acids (rRNAs) and various subunits of the mitochondrial respiratory chain. The maintenance and replication of mitochondrial DNA (mtDNA) as well as the majority of proteins required to maintain proper mitochondrial function, are encoded by nuclear DNA and subsequently imported into the mitochondria. Thereby exemplifying the intricate relationship between mitochondrial function and whole-cell physiology (Moura et al., 2010; Chial & Craig, 2008; Lee & Wei, 2007).

Due to the lack of an efficient DNA repair system and the absence of histones and other protective proteins, the mitochondrial genome is much more prone to mutation than the nuclear genome. Synchronized fusion of inner and outer membranes allows for the exchange of lipid membrane and mitochondrial contents (e.g. mtDNA) between individual mitochondria, thereby intermixing mitochondrial contents and decreasing the amount of defective mitochondria within a given population. Mitochondrial fusion is critical for maintenance of the inner mitochondrial membrane potential and is necessary for normal respiratory function in cells (Chen et al., 2003; Wang et al., 2009; Tondera et al., 2009; Chan et al., 2006; Hoppins et al., 2007; Knott et al., 2008). Although it is not required for mitochondrial fusion itself, an organized cytoskeleton is important for distribution, organization and movement of mitochondria so that they may carry out organelle fusion (Chan, 2006; Rappaport et al., 1998; Yaffè, 1999; Karbowski et al., 2001; Knowles et al., 2002; Varadi et al., 2004). Dysfunctional mitochondrial fusion leads to a number of developmental defects in mice and is associated with a number of neurodegenerative diseases in humans (Chan 2006; Wakabayashi et al., 2009; Wang et al., 2009; Knott & Bossy-Wetzel, 2008).

Fission, conversely, allows for selective elimination of damaged or depolarized mitochondria and its contents through mitophagy, thereby also contributing to mitochondrial stability and homogeneity within a cell (Chen et al., 2005; Chen, 2006;
Twig et al., 2008). Additionally, mitochondrial fragmentation allows for generation of small mitochondria in order to facilitate transport to distant cellular regions where there may be a higher energy requirement, and therefore greater necessity for the organelle. Finally, mitochondrial fission is integral for segregation of mitochondria between daughter cells during mitosis (Moran et al., 2012; Detmer & Chan, 2007; Twig et al., 2008) and also closely linked to a number of cell signaling pathways during apoptosis. Excessive mitochondrial fragmentation is commonly seen when mitochondria are dysfunctional (Suen et al., 2008; Tondera et al., 2009; Duvezin-Caubet et al., 2006; Guillery et al., 2008).

Both fission and fusion are regulated by large dynamin-related GTPases that have opposing effects (Suen et al., 2008; Bleazard et al., 1999; Sesaki and Jensen, 1999; Youle and Bliek, 2012). Fusion in mammalian cells depends mainly on mitofusins 1 and 2 (Mfn1, Mfn2), and OPA1. The fusion of the outer mitochondrial membrane is controlled by the mitofusins, which are localized to the outer mitochondrial membrane, while inner membrane fusion depends on OPA1, which is located within the mitochondrial intermembrane space (Griparic et al., 2004; Herlan et al., 2003; Olichon et al., 2002; Satoh et al., 2003; Wong et al., 2000).

Mammalian OPA1 was first identified as the gene mutated in autosomal dominant optic atrophy (DOA), an inherited form of visual loss through degeneration of retinal ganglion cells (Alexander et al., 2000; Delettre et al., 2000). OPA1 is essential for mitochondrial inner membrane fusion, and may also play a role in the control of inner membrane cristae structure. Knockdown of OPA1 has been shown to result in decreased mitochondrial fusion accompanied by increased fragmentation and severe abnormalities of mitochondrial cristae structure (Chen et al., 2005; Cipolat et al., 2004; Griparic et al., 2004). Due to a broad spectrum of splice variants that yield eight mRNA isoforms, as well as potential post-translational modifications, the OPA1 gene is able to produce multiple protein isoforms (Delettre et al., 2001). Both long and short isoforms are
required in combination to support organized and efficient mitochondrial fusion activity (Song et al., 2007).

Fission of the mitochondrial outer membrane requires dynamin-related protein 1 (Drp1) and mitochondrial fission 1 protein (Fis1). The majority of Drp1 is soluble and found in the cytoplasm of mammalian cells, while a smaller pool is also found localized to the mitochondria in punctate foci, a portion of which will later develop into sites where fission will occur (Chan et al., 2006). Fis1 is an integral outer mitochondrial membrane protein and has been shown to functionally interact with Drp1 as an adaptor protein for its recruitment from the cytosol to fission cites in order to carry out mitochondrial fragmentation (Lu, 2009; Heymann et al., 2009; Liesa et al., 2009; James et al., 2003; Yoon et al., 2003).

Mitochondrial fission and fusion are essential for development in mice as well as neuronal survival and function in both mice and humans. Mutations in Drp1 have been associated with alterations in its distribution and resultant assembly into cytosolic aggregates, while depletion of Drp1 has been linked to immense impairments in mitochondrial bioenergetics, apoptosis resistance, impaired proliferation, and disrupted mitochondrial transport along microtubules (Lee et al., 2004; Benard et al., 2007; Varadi et al., 2004). In yeast, impaired Fis1 function similarly results in defective fission and formation of a ‘net-like’ mitochondrial morphology (Suzuki et al., 2005; Karren et al., 2005). On the other hand, cells lacking integral fusion proteins Mfn1/2 or OPA1 show severe cellular defects including diminished growth and decreased levels of oxygen consumption through a decline in activity of all five respiratory complexes (Olichon et al., 2003; Arnoult et al., 2005; Chen et al., 2005; Lee et al., 2004). Mice lacking these fusion proteins die at an early embryonic age (Chen et al., 2003; Davies et al., 2007; Tondera et al., 2009). In humans, point mutations in Mfn2 and OPA1 lead to severe neurodegenerative diseases such as Charcot-Marie-Tooth type 2A and DOA, respectively (Alexander et al., 2000; Delettre et al., 2000; Zuchner et al., 2004), thereby further
illustrating the critical importance of mitochondrial dynamics in neuronal cell function, homeostasis, and survival.

When cells are exposed to selective stresses, including DNA damaging agents or agents that inhibit cytosolic protein synthesis, mitochondria undergo a process known as stress-induced mitochondrial hyperfusion (SIMH). SIMH involves a subsequent increase in mitochondrial biogenesis and ATP production, and presents cells with an adaptive, pro-survival response to stress. This is supported by the fact that elongated mitochondria tend to display higher efficiency in ATP production which may aid in their ability to recover from certain stressful stimuli (Tondera et al., 2009; Rambold et al., 2011). SIMH has been shown to function independently of Mfn2 as well as the prohibitins, an evolutionarily conserved family of membrane proteins shown to be involved in cell proliferation, cristae morphogenesis and mitochondrial functional integrity (Merkwirth et al., 2008). On the other hand, SIMH requires Mfn1, the long isoform of OPA1 (L-OPA1), and stomatin-like protein 2 (SLP-2), our protein of interest (Tondera et al., 2009). SIMH is disrupted when cells are treated with chemical inhibitors of oxidative phosphorylation, such as CCCP (carbonyl cyanide m-chlorophenyl hydrazone) or oligomycin, which are known to disrupt the mitochondrial membrane potential (Tondera et al., 2009), thereby further indicating the importance of mitochondrial bioenergetics in this adaptive mechanism.

1.3 Stomatin-like Protein 2

SLP-2, also known as STOML2 or RPB72, is a widely expressed mitochondrial protein found in a number of different tissues and species. SLP-2 is encoded by an approximately 1.5 kilobase mRNA transcribed from a 3250bp gene located on chromosome 9p13. The amino acid sequence for SLP-2 predicts a 38,537 kDa protein that shares 20% similarity with stomatin (Da Cruz et al., 2010; Cui et al., 2007; Kirchof et al., 2008). Stomatin, in humans, is a 31kDa erythrocyte membrane protein of unknown
function that is suggested to function in the control of ion channel permeability, mechanoreception, and lipid domain organization (Cui et al., 2007). Mice with a homozygous null mutation in the murine orthologue of the stomatin gene appear to be phenotypically normal (Zhu et al., 1999).

SLP-2 is a member of the highly conserved stomatin family of proteins, whose homologues are widely expressed in different species, and include stomatin, SLP-1, SLP-2 and SLP-3 (Dong Chang et al., 2009; Seidel & Prohaska, 1998; Goldstein et al., 2003; Wang & Morrow, 2000). Similarly to SLP-2, the precise function of other stomatin family proteins is currently unknown. These proteins do, however, share a common stomatin prohibitin flotilin Hflk (SPFH) domain, a signature sequence that implicates their enrichment in lipid rafts, as well as their involvement in a number of cellular functions. These functions include protein turnover and cell signaling, assembly and regulation of ion channels, lipid domains and mechanosensation receptors, and an ability to interact and mediate interactions with cell membranes (Kirchof et al., 2008; Stewart et al., 1992; Wetzel et al., 2006; Cui et al., 2007; Hajek et al., 2007; Tavernakis et al., 1999; Nadimpalli et al., 2000; Da Cruz et al., 2008, 2010).

SLP-2 interacts with the peripheral erythrocyte cytoskeleton and other integral membrane proteins, but, unlike other members of the stomatin family, not directly with the membrane lipid bilayer (Cui et al., 2007). Instead, SLP-2 is proposed to link integral membrane proteins to the peripheral cytoskeleton, and thereby play a role in regulating ion channel conductance and organization of lipid rafts (Cui, 2007; Wang et al., 2000).

SLP-2 has been identified in two major pools throughout cells. This includes primarily an association with the inner mitochondrial membrane, but to a lesser extent also with the plasma membrane (Hajek et al., 2006, 2007; Wang et al., 2000; Kirchof et al., 2008; Da Cruz et al., 2003; Mootha et al., 2003). Unlike the other stomatin family proteins, SLP-2 uniquely lacks a putative amino-terminal hydrophobic transmembrane domain. It does, however, contain six myristoylation/palmitoylation sites and an amino-terminal mitochondrial targeting sequence, which could explain its attachment to mitochondrial
and plasma membranes (Kirchof et al., 2008; Hajek et al., 2006; Owcsarek et al., 2001). The primary sequence of SLP-2 does not indicate involvement of an enzymatic domain or any traditional protein-protein interaction domain, suggesting that SLP-2 may instead form indirect associations through other molecules, and thereby also suggest a role for SLP-2 as a potential scaffolding or assembling protein (Kirchof et al., 2008).

SLP-2 forms hetero-oligomers with Mfn2, a large GTPase on the outer mitochondrial membrane that is essential for mediating mitochondrial fusion (Hajek et al., 2006; Chen et al., 2003; Ishihara, 2004). One group suggests that other proteins may interact with the SLP-2-Mfn2 complex, to form higher molecular weight complexes (Hajek et al., 2006). This further implicates a role for SLP-2 as an important scaffolding protein in the inner mitochondrial membrane that additionally interacts with fusion machinery on the outer mitochondrial membrane.

SLP-2 has been shown to be involved in the regulation of mitochondrial calcium homeostasis. In HeLa cells over-expressing SLP-2, mitochondrial calcium release was delayed, while in HeLa cells depleted of SLP-2 by RNA interference (RNAi), this calcium release was accelerated (Gouriou et al., 2011; Da Cruz et al., 2010; Palty et al., 2012). Additionally, in mouse embryonic fibroblasts (MEFs) SLP-2 is shown to stabilize the long (L-) isoform of OPA1 (Tondera et al., 2009), a protein not only required for mitochondrial fusion but also for the proper formation of mitochondrial cristae junctions (Frezza et al., 2006; Merkwirth et al., 2008; Palmer et al., 2011). Loss of cristae junctions could lead to redistribution of signaling molecules within the inner mitochondrial membrane thereby negatively regulating the activity of the mitochondrial sodium-calcium exchanger (mNCE) (Da Cruz et al., 2010). In HeLa cells depleted of SLP-2 by RNAi, the levels of calcium in the cytosol were elevated, while in SLP-2 over-expressing HeLa cells these cytosolic calcium elevations were decreased, suggesting that these mitochondrial defects are a cause rather than a result of the cytosolic calcium defect (Da Cruz et al., 2010; Gouriou et al., 2011; Palty et al., 2012).
Studies in MEFs have shown that SLP-2 is required to stabilize the fusion mediator protein L-OPA1 during SIMH, such that this pro-survival response of mitochondrial biogenesis does not occur in the absence of SLP-2. Depletion of SLP-2 results in increased proteolytic cleavage of L-OPA1 and inhibited mitochondrial fusion following treatment with SIMH-inducing cell stressors. SLP-2 depletion alone, however, does not fragment mitochondria in non-stressed MEFs, but does lead to mitochondrial fragmentation in response to various DNA damaging agents, including UV irradiation and Actinomycin D. While SLP-2 depleted MEFs are more prone to mitochondrial fragmentation, over-expression of SLP-2 does not appear to alter mitochondrial morphology. The tendency for mitochondria to fragment in SLP-2 depleted cells might further contribute to reduced mitochondrial calcium uptake in these cells, because mitochondrial fragmentation often leads to relocation of mitochondria away from ER calcium release sites, thereby impairing calcium propagation within mitochondria (Szabadkai et al., 2004; Da Cruz et al., 2010).

The overexpression of SLP-2 was first reported in human esophageal squamous cell carcinoma (ESCC), with a significant correlation between high SLP-2 expression and depth of ESCC invasion, and associated with accelerated cell growth in HEC-1B cells (Cui et al., 2007). SLP-2 is highly expressed in several other types of human cancer tissues including lung cancer, endometrium adenocarcinoma, laryngeal squamous cell carcinoma (LSCC), and primary invasive breast cancer, wherein high levels of SLP-2 correlate with distant metastasis, and decreased patient survival (Cao et al., 2007; Dong et al., 2009; Zhang et al., 2006). Furthermore, depletion of SLP-2 in KYSE150 cells (an ESCC cell line) by small interfering RNA (siRNA), resulted in increased sensitivity to chemotherapeutic reagents by altering the cell cycle without any significant change to apoptosis (Wang et al., 2009). Additionally, depleting the level of SLP-2 expression in esophageal cancer cells resulted in reduced cell growth and adhesion, indicating that SLP-2 could act as a novel potential oncogene (Cui et al., 2007; Zhang et al., 2005, 2006; Dong et al., 2009).
Consistent with the emerging role of proteins containing an SPFH domain, SLP-2 has been shown to divide into lipid rafts, and interact with polymerized actin and T-cell receptor signalosome components. Furthermore, SLP-2 has been shown to contribute to human T-cell activation and sustained T-cell receptor signaling, such that changes in expression levels of SLP-2 resulted in changes in T-cell activation and responses (Kirchof et al., 2008; Christie et al., 2011). Although this may suggest that SLP-2 has the potential to act in immunotherapeutics, the role of SLP-2 in neurons and neuronal injury has yet to be examined.

1.4 Mitochondrial Bioenergetics

As mentioned, mitochondria play a large functional role in many cellular processes. Their most important function, however, involves the generation of the majority of cellular energy in the form of ATP. ATP production requires the coordinated action of the mammalian OXPHOS system, which is composed of five multi-protein complexes (Complexes I to V) and two mobile electron carriers, coenzyme Q (CoQ) and cyt c (Hatefi, 1986; Lenaz & Genova 2009; Shoubridge, 2012; Muster et al., 2010).

1.4.1 The Electron Transport Chain

The first four major complexes, which are responsible for the redox portion of this process, comprise the respiratory chain (RC) and are located within the mitochondrial inner membrane. These include NADH-CoQ reductase (Complex I, or NADH dehydrogenase), succinate-CoQ reductase (Complex II, or succinate dehydrogenase), CoQ-cytochrome c reductase (Complex III, or Cytochrome c reductase) and cytochrome c oxidase (Complex IV) (Muster et al., 2010; Lenaz & Genova, 2009; Irwin et al., 2012).

Complex I and II transfer electrons from NADH and FADH$_2$ respectively to coenzyme Q, through a series of cofactors, thereby linking the respiratory chain to the tricarboxylic acid (TCA) cycle. The reduced coenzyme Q freely diffuses along the membrane to
Complex III, which through its ‘Q-cycle’, receives the two electrons from reduced coenzyme Q and transfers them individually, through a series of cofactors, to cytochrome c. Finally, Complex IV accepts electrons from cytochrome c and through a series of cofactors, transfers electrons to oxygen (O$_2$), the final electron acceptor, thereby reducing it to water (H$_2$O) (Vafai et al., 2012; Muster et al., 2010; Balaban et al., 2005).

Complex V of the OXPHOS system is commonly termed the F$_0$F$_1$-ATP synthase as it utilizes the proton motive force, of the proton gradient created by the translocation of electrons across the inner membrane by the RC, to drive the phosphorylation of ADP to ATP (Vafai et al., 2012; Muster et al., 2010; Balaban et al., 2005, Acin-Perez et al., 2008). Figure 3 provides a schematic overview and description of oxidative phosphorylation and the mitochondrial respiratory chain.

Some of the components of OXPHOS proteins are encoded and produced in the mitochondria, however the majority of OXPHOS enzyme complexes are encoded by the nuclear genome. Complexes I and III-V consist of multiple polypeptide subunits that are encoded by both nuclear and mitochondrial DNA, while Complex II is encoded entirely by the nuclear genome (Matsumoto et al., 2012; Irwin et al., 2012; van den Heuvel & Smeitink, 2001). The proper biosynthesis of the OXPHOS complexes is therefore a very intricate and extremely regulated process that requires coordinated action between the two genomes. Any disturbance in OXPHOS complex biosynthesis can result in aggregation of unassembled complexes or other mitochondrial translation products in the inner mitochondrial membrane. This would subsequently lead to a mitochondrial electron leakage and ROS production, decreased ATP production, and release of apoptosis factors ultimately resulting in cell death and potential degeneration of the tissues involved (Moran et al., 2012; DiMauro et al., 2009)

Recent structural and functional evidence has suggested that mitochondrial complexes associate with one another to form supramolecular structures, known as supercomplexes, in order to carry out their roles as “respirasomes” (Acin-Perez et al., 2008). These supercomplexes seem to offer structural and functional benefits to the OXPHOS system
by stabilizing and helping to prevent degradation of RC complexes. This appears to improve substrate channeling and electron transport efficiency, and contribute to reduced levels of mitochondrial electron and proton leakage (Moran et al., 2012; Koopman et al., 2010; Lenaz et al., 2010; Wittig et al., 2009). Due to the structural interdependence among RC complexes however, structural changes affecting one particular complex may have a significant effect on others. As a result, genetic deficiency of a single complex can result in multiple RC enzyme deficiencies (Acin-Perez et al., 2004; Li et al., 2007; Schagger et al., 2001)

Complexes I/III have been shown to form the most abundant and stable supercomplexes, particularly in mouse liver mitochondria were the majority of Complexes I and III are found in supercomplex form. Additionally, associations of Complexes III/IV, I/III/IV, II/III/IV and I/II/III/IV have also been observed, however Complexes II and IV display a larger unassociated fraction than the other respiratory complexes. Some supercomplexes additionally include CoQ and cyt c, and retain the ability to transfer electrons from NADH or Complex II to oxygen, thereby indicating a functional role for these respirasomes (Schagger and Pfeiffer, 2001; Schagger, 2001; Cruciat et al., 2000; Acin-Perez et al., 2008).

These reactions, especially those at Complexes I and III, result in formation of ROS, which have the potential to become toxic by-products. Mutations in Complex II have also been shown to result in ROS production through complex I by way of reverse electron flow (Kann et al., 2007; Moura et al., 2010; Chen et al., 2003; Guzy et al., 2008; Moran et al., 2012; Murphy et al., 2009). Additionally, Complex IV itself does not produce ROS, however, changes to its state of phosphorylation can lead to production of radicals at other points along the respiratory chain (Chen et al., 2003; Kadenbach et al., 2009).
Figure 3. Schematic overview of oxidative phosphorylation and the complexes of the mitochondrial respiratory chain. All five complexes are embedded in the mitochondrial inner membrane (MIM). The transfer of electrons through Complexes I-IV (CI-CIV) is coupled to the production of ATP by complex V (CV). Complexes I and II represent entry points for electrons into the respiratory chain. Complexes I, III, and IV transfer protons to the intermembrane space (IMS) thereby creating an electrochemical gradient, and a net negative charge in the mitochondrial matrix. Complex V utilizes the energy of this gradient, while translocating protons back into the mitochondrial matrix, for the phosphorylation of ADP to produce ATP. CoQ and cyt c function as electron carriers. This figure is adapted from Perier and Vila, 2012.
1.4.2 Reactive Oxygen Species

ROS collectively refers to the molecular oxygen-derived free radicals and precursors formed as a result of incomplete reduction of oxygen. This includes the superoxide anion \((O_2^-)\), hydrogen peroxide \((H_2O_2)\) and the hydroxyl radical \((HO^*)\). ROS can be produced during oxidative phosphorylation when electrons are captured by oxygen along the ETC to produce \(O_2^-\) radicals, and become further converted by manganese superoxide dismutase, to hydrogen peroxide. Hydrogen peroxide can be further broken down within the mitochondria, by antioxidant enzymes such as glutathione peroxidase or peroxiredoxins, to produce water. Additionally, cells contain other nonenzymatic ROS scavengers including vitamin E, vitamin C, ubiquinone and glutathione to protect them from oxidant injury (Uttara et al., 2009; Trachootham et al., 2008; Kushnareva et al., 2002; D’Autreaux and Toledano 2007). ROS have the ability to cause functional damage, such as reduced coupling and enhanced ROS production, thereby posing a threat to the proper function of both mitochondria and the cells they are in, as well as organs and an entire organism if left uncontrolled. Effects of ROS however, are buffered through the enzymatic functions of superoxide dismutase and catalase, as well as glutathione, thioredoxin and thiol redox buffer systems (Kushnareva et al., 2002; D’Autreaux and Toledano 2007).

Although ROS for the most part, are thought to mediate the toxic effects of oxygen due to their chemical reactivity, they can also function as important signaling molecules in healthy cells. This however, remains a subject of debate as signaling is thought to require precision, and ROS tend to have non-specific, indiscriminate reactivity (Imlay et al., 2003; D’Autreaux and Toledano, 2007; Halliwell et al., 1999)

Under certain pathological conditions, diminished availability of antioxidants or increased radical production can result in an imbalance between ROS production and protection, thereby resulting in oxidative stress. Oxidative stress is used to describe the resultant oxidization of proteins, lipids and nucleic acid, and altered cell integrity that
follows the excess presence of ROS (Ott et al., 2007; Cherz-Shouval et al., 2007; Van Houten et al., 2010)

Mitochondrial DNA is not only more susceptible to ROS damage, but particularly prone to mutation due to the absence of protective proteins, such as histones, and a high-efficiency repair system. Normal mitochondrial dynamics are suggested to fulfill a ROS-damage rescue function through their ability not only to intermix defective and functional mtDNA through fusion, but also to sequester damaged components through fission and subsequent autophagic degradation (Muster et al., 2010). Chronic mtDNA damage, however, can lead to a cycle of decreased synthesis of electron transport chain proteins, thereby resulting in increased ROS production, depolarization of mitochondrial membrane potential, and decreased ATP production. These factors can result in further amplification of oxidative stress and injury in a number of diseased states, and ultimately lead to cellular dysfunction and potentially death (Ishii et al., 2006; Van Houten et al., 2006; Ou et al., 2007; Moran et al., 2012; Fernandez-Checa et al., 2010).

Additionally, there exists a tightly coordinated feedback loop between ROS production and mitochondrial dynamics. The initial ROS signaling cascade, modulated by mitochondrial dynamics, can in turn lead to further scaling of ROS signaling and modifications to the level of oxidative stress and damage that can further impact mitochondrial dynamics and cell viability. Mitochondrial ROS production can also act as a redox signal in the regulation of autophagy and apoptosis. Low levels of oxidative damage may induce mitochondrial depolarization which can provide the necessary signals for induction of autophagy and thereby result in autophagic removal of potentially dangerous radical-producing mitochondria. If the mitochondrial damage were to occur prior to excess ROS production, however, this protective pathway may be impaired, resulting in accumulation of ROS-producing organelles and subsequent increase in oxidative stress and damage that could eventually result in apoptosis or necrosis instead (Yen et al., 2008; Scherz-Shouval and Elazar, 2007; Scherz-Shouval and Elazar, 2011).
Mitochondrial response to oxidative stress also seems to be modulated in a dose-dependent manner, such that at higher concentrations or longer exposure to ROS, subsequent increases in the extent of mitochondrial fragmentation and cell death rates are observed (Park et al., 2011; Wei and Lee, 2002; ref. Jahani-Asl et al., 2007; Jendrach et al., 2008). Lower ROS doses, on the other hand, appear to induce mitochondrial hyperfusion associated with decreased expression of fission protein Fis1, and increased expression of fusion proteins Mfn1 and Mfn2 (Yoon et al., 2006).

Altering the number, density or spatial distribution of mitochondria can modulate a process known as ROS-induced ROS release (RIRR). RIRR refers to the subsequent induction of ROS generation in the mitochondria surrounding a smaller population of ROS-producing, damaged, or dysfunctional mitochondria, in a given subcellular area. RIRR in effect contributes to a whole-cell increase, through mitochondrial propagation, in ROS levels, thereby affecting overall cell viability (Park et al., 2011; Zorov et al., 2000).

The peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a potent stimulator and major regulator of mitochondrial biogenesis, gene transcription, and respiration. As a transcriptional co-activator, PGC-1α functions to transduce physiological signals into specific metabolic programs, often through a concomitant increase in mitochondrial activity. This is achieved through activation of nuclear receptor ERRα (estrogen-related receptor alpha) and nuclear respiratory factors NRF-1 and NRF-2 whereby the expression of mitochondrial transcription factor A (Tfam) is regulated (Mootha et al., 2004; Wu et al., 1999; St-Pierre et al., 2006; Wareski et al., 2009). Tfam is essential for maintenance, replication, and transcription of mtDNA. Additionally, PGC-1α activates expression of other nuclear encoded proteins required for mitochondrial function, including subunits of RC complexes (Wareski et al., 2009).

PGC-1α knockout mice display reduced mitochondrial gene expression, decreased metabolic capacity, increased sensitivity to damage caused by oxidative stress (Arany 2005, Leone 2005, St-Pierre 2006) and behavioral abnormalities secondary to
neurodegenerative lesions in the brain (Leone et al., 2005; Lin et al., 2004). Furthermore, this increase in sensitivity to neurodegeneration is associated with increased oxidative damage and apoptotic cell death (St-Pierre et al., 2006).

PGC-1α is itself induced by ROS, but it in turn is also involved in regulating cellular ROS metabolism through activation of genes encoding mitochondrial ROS-detoxifying enzymes, such as mitochondrial superoxide dismutase (SOD) (Kukidome et al., 2006; St-Pierre et al., 2003; Valle et al., 2005, D’Atreaux and Toledano, 2007).

Currently, there is a substantial amount of evidence linking mitochondrial dysfunction and oxidative stress to neurodegenerative diseases such as AD, PD and HD. Respiratory chain dysfunction due to enzymatic or assembly defects, increased ROS production, alterations to the morphology of the mitochondrial network and an impaired equilibrium between protective cell death are recurrent features of neurodegenerative diseases associated with a variety of genetic origins (Enns et al., 2003; Mandemakers et al., 2007; Sas et al., 2007; Godvaze et al., 2009; Jellinger et al., 2009).

1.5 Apoptosis

Apoptosis is an endogenously programed form of cell death that is, under physiological conditions, active during normal embryonic and postembryonic development and tissue homeostasis. This highly regulated process leads to the organized disassembly of a cell and requires active participation of the cell itself (Karbowki et al., 2010; Parone and Martinou, 2006; Parone et al., 2006; Desagher et al., 2000; Haeberlein et al., 2004; Suen et al. 2008; Youle et al., 2005; Nijhawan et al., 2000). Apoptosis is essential in multicellular organisms to eliminate unnecessary, ectopic, damaged or mutated cells, and consequently the disabling of apoptosis is considered a pathogenic event implicated in both oncogenesis and cancer progression. Excessive apoptosis on the other hand, especially in postmitotic cells such as neurons or cardiomyocytes, can also be directly linked with disease (Youle et al., 2005; Haeberlein et al., 2004).
Apoptosis may be initiated by a number of molecular pathways, the most prominent and well characterized known as the extrinsic and intrinsic pathways. The extrinsic pathway of apoptosis, also known as the death-receptor-dependent pathway, is triggered by ligand-induced activation of cell-surface death receptors such as tumor necrosis factor (TNF) receptor-1, and the CD95/Fas receptor. The intrinsic pathway, known as the mitochondrial pathway, is on the other hand, triggered by intracellular events, such as DNA damage, cytokine withdrawal, oxygen radicals or endoplasmic reticulum (ER) stress, and controlled by pro- and anti-apoptotic B cell lymphoma 2 (Bcl-2) family proteins. (Otera et al., 2012; Haeberlein et al., 2004; Kroemer et al., 2007; Henry-Mowatt et al., 2004). The mitochondrial pathway is also characterized by mitochondrial membrane permeabilization (MMP) and the release of proapoptotic proteins from the mitochondrial intermembrane space (IMS) into the cytosol. These proapoptotic factors include cyt c, apoptosis-inducing factor (AIF), Omi, endonuclease G, caspase-9, and Smac (Haeberlein et al., 2004; Kroemer et al., 2007; Henry-Mowatt et al., 2004; Parone et al., 2006).

The complete manifestation of apoptosis appears to be highly dependent on activation of a specific family of intracellular cysteine-aspartic proteases known as the caspases. Caspases generally act as signal transducing molecules grouped into “initiator” caspases (e.g. caspases-2, -8, -9 and -10) that function to cleave and thereby activate various protein and enzymatic substrates. These substrates include the second group of “effector” caspases (e.g. caspases-3, -6 and -7), which mediate the characteristic widespread protein degradation seen during apoptosis. Both extrinsic and intrinsic pathways converge at caspase-3, the activation of which promotes the final proteolytic phase of apoptosis (Haeberlein et al., 2004, Porter et al., 1999; Henry-Mowatt et al., 2004; Reed and Pellecchia, 2005).

The extrinsic and intrinsic pathways of apoptosis are not mutually exclusive, as products from the extrinsic pathway can promote activation of the intrinsic pathway. Caspase-8 cleavage of Bid to truncated Bid (tBid), for example, allows for the association of tBid
with the mitochondrial membrane, thereby stimulating, through Bax/Bak pore formation, the release of cyt c. Mitochondria are thus important regulators of apoptosis as they represent a convergence point for both extrinsic and intrinsic pathways (Basu et al., 2006; Kroemer et al., 2007; Reed and Pellecchia 2005).

Regardless of whether cell death is induced by intrinsic or extrinsic factors, the physical and biochemical hallmarks for apoptosis remain the same. These include cellular vacuolization, chromatin condensation and DNA fragmentation, plasma membrane blebbing, and cell shrinkage and disintegration into packaged apoptotic bodies that may later be engulfed by surrounding phagocytic cells, in order to circumvent inflammatory responses. (Kroemer et al 2007; Henry-Mowatt et al 2004; Ly et al., 2003).

Due to its involvement in tissue homeostasis, apoptosis is controlled at multiple steps throughout the process. Mitochondria are important at these apoptotic checkpoints as they are involved in integration of a number of signals from both endogenous and exogenous factors. Mitochondria undergo outer MMP when the level of pro-apoptotic signals predominate over anti-apoptotic signals, at the level of the mitochondrial membrane (Kroemer et al., 2007; Bossy-Wetzel & Green, 1999).

Apoptotic stimuli, including Bcl-2 family proteins (Bax, Bak, tBid), calcium and cytosolic metabolites, and activation of OXPHOS-targeting caspases promote changes in mitochondrial transmembrane potential. The resultant loss of mitochondrial transmembrane potential often leads to increased ROS production and concomitant saturation of antioxidant systems, thereby hindering mitochondrial function through impairment of oxidative phosphorylation and downstream effects on opening of the permeability transition pore complex (PTPC). Opening of the PTPC leads to equilibration of ions between the matrix and cytoplasm, resulting in transmembrane potential depolarization, ATP hydrolysis, and mitochondrial swelling. As a result of the influx of solutes into the matrix and unfolding of the MIM, the OMM ruptures and releases the proapoptotic proteins found within the IMS (Zoratti et al., 1994; Haeberlein et al., 2004). As the mitochondria are responsible for the release of several proapoptotic
proteins in response to stress signals, it is critical not only that they are able to perceive stress signals properly but also to have proper control over release of these proteins. These functions highlight the critical importance of mitochondrial integrity as well as the organelle’s critical role in the control of programmed cell death.

Neurons are cells with limited capacity to replace themselves, and as such, apoptosis in neurons subsequent to disease or injury is different from programmed cell death in that it ultimately results in a loss of function. Extensive apoptosis of neuronal cells is observed after stroke and trauma, and is the basis for a number of progressive neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), PD, and AD. (Nijhawan et al., 2000; Pettman and Henderson, 1998).

1.6 Objectives of this thesis

Mitochondrial dynamics play a central role in neuronal injury and several neurodegenerative diseases. The precise physiological role of mitochondrial fission and fusion in cell function and survival, however, is poorly understood. Furthermore, the exact mechanisms underlying the pathology of neurodegenerative diseases associated with mitochondrial defects are currently unknown. SLP-2 has been identified as a mitochondrial protein reported to interact with and stabilize a number of proteins involved in mitochondrial dynamics, and has been implicated in the regulation of mitochondrial functions (Da Cruz et al., 2008; Christie et al., 2011; Tondera et al., 2009; Artal-Sanz and Tavernarakis, 2009). In a microarray study examining transcriptional changes in neurons, we have found that SLP-2 expression is increased in response to a variety of stresses. Although SLP-2 may contribute to many cell signaling functions through its modulation of mitochondrial function and dynamics, the exact role of SLP-2 in mitochondrial stress responses remains to be discovered. Given the importance of proper mitochondrial function and dynamics to normal neuronal function and
neurodegenerative processes, we sought to investigate the role of SLP-2 in neuronal injury.

Through its involvement in mitochondrial dynamics, most notably the hyperfusion stress-response (SIMH), and interactions with integral mitochondrial proteins, SLP-2 may provide a putative link between mitochondrial stress responses, bioenergetics and apoptosis. Thus, we hypothesized that SLP-2 regulates neuron survival in response to stressful stimuli. In order to address this hypothesis, my objectives were to determine the effect of altered SLP-2 expression levels, through deletion and up-regulation, on mitochondrial biogenesis and function in neurons, and to examine the role of SLP-2 in the neuronal stress-response. The specific aims of this study are to: 1. Examine the effects of SLP-2 deletion on primary cortical neurons, and 2. Examine the role of SLP-2 in neuronal survival under various conditions of stress.
Chapter 2: Experimental Procedures

2.1 Mice

Mice heterozygous for the SLP-2 floxed allele (SLP-2\textsuperscript{lox/wt}), were generously provided by Dr. Joaquin Madrenas’ Lab at the University of Western Ontario (Montreal, QC). These mice were generated in the C57/BL6 background (Christie et al., 2012).

2.1.1 Generating forebrain-specific SLP-2 knockout mice

Germline deletion of SLP-2 results in embryonic lethality at the pre-implantation stage (Christie et al., 2012). Therefore, we employed a conditional Cre/\textit{lox}P recombination method (Kuhn & Torres, 2002; Le & Sauer, 2000) to generate a conditional SLP-2 knockout mouse (Appendix A).

SLP-2\textsuperscript{lox/wt} mice were bred to produce mice that were homozygous for the SLP-2 floxed allele (SLP-2\textsuperscript{lox/lox}). PCR analysis (Section 2.9) was employed to confirm the genotype of SLP-2\textsuperscript{lox/lox} mice, which were then crossed with C57BL/6 mice transgenic for Cre recombinase under the control of the Brain Factor-1 (Foxg1) promoter. Brain Factor-1 (BF-1) is specifically expressed in the telencephalon, beginning at around E9.5, and persisting in the adult brain in structures derived from the telencephalon including the cerebral cortex, hippocampus, and basal ganglia (Hebert and McConnell, 2000; Ferguson et al., 2002). Therefore, this model allows for forebrain-specific expression of the Cre gene, as it develops in the mouse embryo. The offspring of this breeding pair (SLP-2\textsuperscript{lox/lox} x SLP-2\textsuperscript{wt/wt/Cre}) were thus selected for both heterozygous expression of the floxed SLP-2 allele and expression of Cre recombinase (SLP-2\textsuperscript{lox/loc/Cre}). SLP-2\textsuperscript{lox/loc/Cre} mice were then crossed with SLP-2\textsuperscript{lox/loc} mice, by pairing males and females for a timed period of 3-days, in order to obtain forebrain-specific SLP-2 knockout embryos (SLP-2\textsuperscript{lox/loc/Cre}) at E14.5-15.5 (Appendix A).
2.2 Genotyping

Tail samples from each mouse or embryo were placed in a separate 1.5mL microcentrifuge tube and dissolved in a solution of 500µL DNA Lysis buffer [DNA LB; 100mM Tris buffer, 5mM EDTA, 0.2% SDS, 200mM NaCl] and 10µL of 0.2 mg/mL Proteinase K (Invitrogen), and placed over night on a 55°C heat block to ensure proper digestion.

To extract the DNA, 500µL phenol-chloroform [phenol:chloroform:iso-amyl alcohol (25:24:1)] was added to each tail sample. Samples were shaken thoroughly for 1 minute and centrifuged at room temperature at 12,000xg for 10 minutes. The upper aqueous phase was then transferred to a new set of tubes, while carefully avoiding contamination with the interphase. Following addition of 500µL of cold 95% isopropanol, each tube was shaken vigorously until a DNA precipitate was seen. Tubes were centrifuged at 12,000xg for 10 minutes at 4°C. Next, the supernatant was carefully aspirated and 1mL of 70% ethanol was added to each tube to rinse the DNA pellet. Samples were centrifuged 12,000xg for 5 minutes at 4°C. The excess supernatant was removed, leaving the DNA pellet damp, and tubes were left open for 10 minutes on a 55°C heat block to allow the remainder of the ethanol to evaporate. Upon drying, pellets were resuspended in 40µL 1x TE buffer (pH 8.0) and incubated for 20 minutes on a 55°C heat block to ensure the DNA completely dissolved. DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.2.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was employed to genotype each sample. The reagents, cycling conditions, and expected products for each PCR reaction are outlined in Appendix B. Once the PCR was complete, each sample was loaded, along with a 1kb+ ladder (Fermentas) onto a 1.5% agarose gel bathed in 1 x TAE buffer. The gel was run for 40 minutes at 130V, and PCR products were visualized using a Gel Doc imager (BioRad) under UV light.
2.3 Cell culture

2.3.1 Primary Cortical Neurons

Primary cortical neurons were dissected from E14.5-15.5 mouse embryos of timed pregnant SLP-2\textsuperscript{lox/lox} mice that had been crossed with an SLP-2\textsuperscript{lox/wt/Cre} male (or pregnant SLP-2\textsuperscript{lox/wt/Cre} mice that had been crossed with an SLP-2\textsuperscript{lox/lox} male) (Section 2.1, Appendix A). An intra-peritoneal injection of euthanyl was used to sacrifice the female. Embryos were dissected immediately after and placed in 1x Hanks’ Balanced Salt Solution (HBSS; Gibco\textsuperscript{®}, 14170-112). A dissecting microscope was used to visualize the embryos in order to dissect and remove the meninges from cerebral cortices. Cerebral cortices were kept separate for processing due to variances in genotype. A tail sample and the remainder of the brain of each embryo were also retained separately for genotyping purposes.

To dissociate the cortical neurons, individual cortices were trypsinized with 300µL of HBSS solution supplemented with 1.2 mM MgSO\textsubscript{4} and 75 mg/mL trypsin (Sigma), and placed in a 37\textdegree C incubator, on a rotor, for 25 minutes. To halt trypsinization, 600µL of a HBSS solution supplemented with 1.2mM MgSO\textsubscript{4}, 0.25 mg/mL DNase I, and 0.2 mg/mL trypsin inhibitor was added to each sample and mixed thoroughly by inversion for 2 minutes. Next, the cell solutions were centrifuged for 5 minutes at 400xg and the supernatant was carefully removed. The remaining cell pellets were resuspended in HBSS solution supplemented with 3mM MgSO\textsubscript{4}, 1mg/mL trypsin inhibitor and 0.65mg/mL DNase I.

To ensure dissociation, the cells were triturated 15-20 times through a flame polished glass pipette, and then centrifuged at 400xg for 5 minutes. The supernatant was carefully aspirated and the cell pellets were resuspended in 2mL complete neurobasal media (NBM; Gibco\textsuperscript{®}, 21103-049) [C-NBM; 1x B27 and N2 supplements, 1mM glutamax, and 50 U/mL penicillin:50µg/mL streptomycin].
Cell density was determined using Trypan Blue (Invitrogen) and a haemocytometer under a light microscope. Cell suspensions were diluted in C-NBM to a concentration of 1 million cells/mL and plated in either 60mm (5mL; 5x10^6 cells/dish), or 4-well (500µL; 3x10^6 cells/well) dishes. All dishes were previously coated with poly-L-ornithine (Sigma) (diluted 1:10 in ddH2O) and cultures were maintained in a humidified 37°C incubator with 5% CO₂.

2.3.2 SN56 Neuronal Cell Line

SN56 cells were generously provided by Dr. Jane Rylett (Hammond et al., 1986; Le et al., 1997; Blusztajn et al., 1992). The SN56 neuronal cell line is a hybrid cell line resulting from the fusion of dissociated embryonic mouse septal neurons with murine neurobastoma (N18TG2) cells. These cells were maintained in complete Dulbecco’s Modified Eagle Medium (DMEM: Gibco®, 11965-092) [C-DMEM; 10% FBS, Gentamicin, and in 50 U/mL penicillin:50µg/mL streptomycin] in T-75 flasks (Thermo Scientific) and passaged every 3-4 days at 1:10. For experiments, SN56 cells were plated at 1:15 on either 4-well or 60mm dishes. After a minimum of 24 hours, when SN56 cells had attached to the bottom of the dish, C-DMEM was aspirated and replaced with serum-free DMEM [SF-DMEM], in order to induce differentiation. SF-DMEM was replaced each day in order to induce differentiation-associated changes including decreased cell division and extensive neuritic outgrowth. These changes were consistent with those documented in previous studies of SN56 cells (Barbosa et al., 1999; Blusztajn et al., 1992). The cultures were maintained in a humidified 37°C incubator with 5% CO₂.

2.3.3 Plasmids

Green fluorescent protein (GFP) cDNA and fused SLP-2-GFP cDNA were cloned into CMV-promoter based vectors (pcDNA3, Invitrogen), to generate pEGFP and pSLP-2-GFP plasmids that would fluoresce green upon protein expression. Stable transfectants were generated by nucleofection.
2.3.4 Transfection

A Calcium Phosphate transfection method was employed to transfet differentiated SN56 cells, with either pEGFP or pSLP-2-GFP. Stock solutions of 1x HBS [1x HBS; 5g HEPES Acid, 8g NaCl, 1g Dextrose, 3.7g KCl, 10mL Na$_2$HPO$_4$(7H$_2$O), 890mL ddH$_2$O. pH 7.1] and 2.5M CaCl$_2$ [2.5M CaCl$_2$; 27.75g anhydrous CaCl$_2$, 100mL ddH$_2$O] were prepared prior to transfection and stored at -20°C, while a working tube was kept at 4°C.

The following table denotes the amount of DNA and each reagent used relative to the surface area of the dish being treated. DNA was diluted in ddH$_2$O to a final volume of 15mL for each transfection.

**Table 1. DNA and Reagent Amounts for Calcium Phosphate Transfection Method**

<table>
<thead>
<tr>
<th>Dish</th>
<th>Culture Media</th>
<th>1x HBS</th>
<th>2.5M CaCl$_2$</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-well</td>
<td>500µL</td>
<td>18µL</td>
<td>1.1µL</td>
<td>1.1µg</td>
</tr>
<tr>
<td>60 mm</td>
<td>5mL</td>
<td>186µL</td>
<td>11µL</td>
<td>11µg</td>
</tr>
</tbody>
</table>

For each transfection, DNA was added to 1x HBS in a 1.5mL microcentrifuge tube and mixed thoroughly. Next, 2.5M CaCl$_2$ was added to the tube in a dropwise fashion and mixed carefully by inversion. The transfection mix was then added to plates in a dropwise fashion. Dishes were put back in a humidified 37°C incubator at 5% CO$_2$. Serum-free DMEM was changed after 16 hours and cells were either treated or harvested 48-72 hours later, once 30-40% transfection efficiency was reached.
2.4 Protein Extraction and Quantification

Following respective treatments and at appropriate time points, culture media was carefully removed from adhered cells on 60mm dishes. Cells were then washed twice with cold PBS and 100µL complete RIPA (Sigma) Lysis Buffer [RIPA: 100µL protease inhibitor cocktail (Sigma), 900µL RIPA Lysis Buffer (Sigma)] was added directly onto each culture dish. A cell scraper was used to carefully remove cells from each dish, and the resultant lysates were each passed through a pipette 5 times. The lysates were then transferred to separate 1.5mL microcentrifuge tubes and placed on ice for at least 20 minutes. The resulting mixture was centrifuged at 14,000xg for 12 minutes at 4°C. The supernatant from each tube was transferred to a fresh 1.5mL microcentrifuge tube and stored at -80°C until further analysis.

The Pierce® BCA Protein Assay Kit (Thermo Scientific) was used, according to manufacturer’s protocol, to determine the protein concentration of each sample. The absorbance at 562 nm was measured using the SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices) and SoftMaxPro V5 (Molecular Devices) software. A standard curve was generated by plotting absorbance at 562 nm versus concentration of BCA. The concentrations of the protein samples were then calculated by inserting their absorbance values into the equation of the standard curve.

2.5 Western Blot Analysis

Thirty micrograms of each protein sample were mixed with different volumes of complete RIPA Lysis Buffer in order to obtain a constant sample volume. Next, 1x SDS was added to each sample and the mixtures were heated at 37-100°C (depending on the protein of interest) for five minutes to reduce disulfide bonds and denature proteins. The samples were then loaded onto a 1.5mm 12% polyacrylamide mini-gel (Bio-Rad) and run at 100-125V. The protein was then transferred to a nitrocellulose membrane via semi-dry
electroblotting. The nitrocellulose membrane was then stained with Ponceau S (Sigma) to visualize total protein present, and then cleaned thoroughly by rinsing three times for 5 minutes with a wash buffer solution [WB: 150mM NaCl, 10mM Tris, and 0.05% Tween-20].

Next, the nitrocellulose membrane was incubated in a 5% blocking solution [5% powdered milk (w/v) in WB] for 1 hour at room temperature, after which the blocking solution was replaced with primary antibody diluted in a 5% wash buffer solution [%5 (w/v) powdered milk in WB], and incubated on an orbital shaker overnight at 4°C. All primary antibodies were diluted in 5% wash buffer solution. Primary antibodies used were rabbit polyclonal Calnexin antibody [1:500; Santa Cruz (H-70)], rabbit polyclonal Actin antibody [1:10,00; Santa Cruz (I-19)], mouse Total Oxidative Phosphorylation monoclonal antibody cocktail [1:250; Mito Sciences #MS604], mouse monoclonal NDUFS3 antibody [1:500; Abcam #14711], mouse monoclonal TOMM20 antibody [1:500; Abcam #56783] and rabbit polyclonal SLP-2 antibody [1:500; ProteinTech (1-AP) #10348]. Next, membranes were incubated with a secondary antibody diluted in 3% wash buffer solution [3% (w/v) powdered milk in WB] for 1 hour at room temperature. All secondary antibodies were diluted in 3% wash buffer solution. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-rabbit (GAR) IgG (H+L) [1:10,000, Bio-Rad] and goat anti-mouse (GAM) IgG (H+L) [1:10,000, Bio-Rad]. Excess secondary antibody was washed away with three 5 minute washes with WB. The membrane was then coated with a 1:1 mixture of Stable Peroxidase Solution and Luminol/Enhancer Solution from a SuperSignal® West Pico Chemiluminescent Substrate Kit (Thermo Scientific) for 5 minutes and then dried and wrapped with Saran wrap. Kodak BioMax maximum sensitivity film was pressed against the membrane in a dark room for 10 seconds to 15 minutes, depending on the antibody. Films were developed with a Micro-Max X-Ray Automatic Film Processor (Hope).
2.6 RNA extraction and Quantification

Culture media was carefully removed from adhered cells on 4-well or 60mm dishes. RNA was extracted from both primary cortical neurons and SN56 cells using Trizol® (Invitrogen) reagent, according to the manufacturer’s protocol.

RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Each sample was measured in duplicate, and the average concentration was calculated.

2.7 Quantitative RT-PCR

RNA samples were diluted to a working concentration of 10ng/µL in RNAse/DNAse free H2O (Gibco). The reagents, cycling conditions, and expected products for each quantitative real-time polymerase chain reaction (qRT-PCR) reaction may be found in Appendix B. Primer probes were selected for the housekeeping gene, ribosomal S12, which served as a comparison for the other genes that were analyzed. Four microliters of each RNA sample was mixed with 21µL of Master Mix for each primer probe. Samples were run on a Peltier-Thermal Cycler 200 connected to a Chromo 4 Continuous Fluorescence Detector (MJ Research) and data acquisition software Opticon Monitor 3. PCR efficiency was >90% for all amplicons. Melting curve analysis revealed a single peak indicating a single RT-PCR product for each gene, and products were confirmed via sequencing to determine primer specificity. The difference in target gene expression was calculated using the Delta-Delta CT Method, as per the following equation:

\[
\text{ratio} = \frac{(E_{\text{target}}) \Delta CT_{\text{target (control-sample)}}}{(E_{\text{ref}}) \Delta CT_{\text{ref (control-sample)}}}
\]
2.8 Drug treatments

Drug treatments were administered on day 5 in culture for primary cortical neurons and 48-72 hours following transfection in SN56 cells. Each drug was diluted with media from a stock solution to a desired treatment concentration. A non-competitive NMDA receptor antagonist, MK-801, was added to all treatments, except Camptothecin (CPT), in order to prevent secondary excitotoxic cell death. Details regarding each drug used are summarized in the following table:

Table 2. Drugs Used for Stress Treatments Experiments

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Effects</th>
<th>Company</th>
<th>Cat #</th>
<th>Stock [µM]</th>
<th>Solvent</th>
<th>Final [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-methyl-4-phenylpyridinium (MPP+)</td>
<td>Complex I (NADH dehydrogenase) inhibitor</td>
<td>Sigma</td>
<td>D048</td>
<td>100mM</td>
<td>Water</td>
<td>5-200µM</td>
</tr>
<tr>
<td>3-Nitropropionic Acid (3-NP)</td>
<td>Complex II (succinate dehydrogenase) inhibitor</td>
<td>Sigma</td>
<td>N5636</td>
<td>1M</td>
<td>Ethanol</td>
<td>0.5-10µM</td>
</tr>
<tr>
<td>Camptothecin (CPT)</td>
<td>DNA damage - Topoisomerase I inhibitor</td>
<td>Sigma</td>
<td>C9911</td>
<td>10mM</td>
<td>DMSO</td>
<td>40µM</td>
</tr>
<tr>
<td>MK-801</td>
<td>NMDA antagonist</td>
<td>Sigma</td>
<td>M107</td>
<td>10mM</td>
<td>Water</td>
<td>10µM</td>
</tr>
</tbody>
</table>

2.9 Cell death assessment

2.9.1 Primary Cortical Neurons

Following 24 hours treatment of 4-well dishes with either MPP+, 3-NP, or CPT, a Live/Dead Assay (Invitrogen) for cell viability was employed to assess the percentage of dead cells in a given cell population. Live cells have intracellular esterases that convert non-fluorescent, cell permeable calcein acetoxymethyl to a fluorescent (green) calcein. Dead
cells, as a result of having damaged membranes, allow for the ethidium homodimer-1 to enter damaged cells and fluoresce (red) upon binding to nucleic acid.

An Olympus IX70 Fluorescence Microscope, and Northern Eclipse software (v. 7.0) were used to perform imaging. Images were captured on 5-6 random locations per well at 400X magnification. Using ImageJ software, at least 500 cells were counted per treatment in each experiment. The percentage of cell death was calculated by dividing the number of dead (red) cells by the total number of cells (red and green).

2.9.2 SN56 Cells
Following 24 hours treatment of 4-well dishes with either MPP+ or CPT, cells were stained with Hoechst 33342 (1µM) dye (Sigma), incubated at 37°C for an optimized time of 15 minutes to minimize signal bleeding, and imaged immediately using fluorescence microscopy and Northern Eclipse 7.0 software. Images were captured on 5-6 random locations per well at 400x or 600x magnification. Using ImageJ software, at least 500 cells were counted per treatment in each experiment. Cells were considered apoptotic if nuclei displayed highly condensed chromatin that was uniformly and brightly stained by Hoechst 33342 (See Appendix D). Only GFP-positive cells (plasmid-expressing) were counted when assessing the percentage of apoptosis, which was calculated by dividing the number of apoptotic nuclei by the total number of healthy and apoptotic nuclei counted for each treatment.

2.10 Immunofluorescence
Primary cortical neurons and SN56 cells were plated on to 4-well dishes containing cover slips (coated with poly-L-ornithine for primary cortical neurons) and cultured in a humidified 37°C incubator with 5% CO₂. Following treatment, culture media was carefully removed from adherent cells and replaced with 500µL Lana’s Fixative [LF; 4% paraformaldehyde, 0.2% picric acid] for 20 minutes at room temperature. After the cells
had been fixed, they were rinsed three times in cold PBS. Cells were permeabilized with a mixture of 0.1% Triton prepared in PBS (pH 7.4) for 10 minutes. After three 5 minute washes with PBS, cells were treated with Blocking Solution [0.2% Triton X, 1x PBS] for at least 1 hour. The cells were then incubated with 500µL of primary antibody, diluted 1:500 in Antibody Diluent [1x PBS containing 0.2% Triton-X and 2% BSA], and stored overnight at 4°C. The next day, this solution was carefully aspirated and after three 5 minutes washes with 1x PBS, cells were incubated with a secondary antibody against mouse IgG [AlexaFluor 568 rabbit anti-mouse IgG (H+L) HRP conjugate; Molecular Probes], diluted 1:1000 in Antibody Diluent, for 1 hour at room temperature. To stain nuclei in fixed cells, samples were incubated with Hoechst 33358 (1µM) for 15 minutes at room temperature. After washing with 1x PBS, coverslips were carefully removed from 4-well dishes and placed cell-side down on slides with Immumount (Thermo Scientific). Cells were imaged using an Olympus IX70 Fluorescence Microscope, and Northern Eclipse software (v. 7.0).

2.11 Flow Cytometry

Primary cortical neurons, on day 5 in culture, were labeled with 10nM MitoTracker Red® CMXRos (Invitrogen), and incubated at 37°C for 20min. MitoTracker Red® CMXRos (MtkrRed) is a red-fluorescent dye that stains mitochondria in live cells. Once the mitochondria were labeled, C-NBM (Section 2.2.1) was carefully aspirated off of the adherent cells and diluted Ethylenediaminetetraaceitc acid [0.2% EDTA in 1x PBS] was used to remove adherent cells from each plate.

EDTA is a calcium chelator that binds Ca^{2+} ions which are required for cells to maintain adhesion. After 5 minutes, C-NBM was added to each plate to neutralize the EDTA, and samples were placed in separate 15mL conical centrifuge tubes and centrifuged at 100xg for 3 minutes. The supernatant was aspirated, the cells were rinsed with PBS, and the solution was centrifuged again at 100xg for 3 minutes. The supernatant was aspirated again, each sample was resuspended in 1mL PBS, and tubes were immediately covered in
foil and placed on ice. Fluorescence was detected by flow cytometry using the BD Acuri™ C6 Flow Cytometer (Biosciences) and FACS analysis was conducted using the accompanying BD Acuri™ C6 Software (Biosciences).

2.12 Brain Slice Preparation

2.12.1 Mouse Brain Perfusion and Preparation
Adult brains of age-matched (P70) litter mate mice were dissected following a standard protocol for perfusion fixation with 4% paraformaldehyde (PFA) through the heart (Gage et al., 2012). The brains were stored at 4°C in 4% PFA. Twenty four hours prior to sectioning, 4% PFA was replaced with a 30% sucrose solution [30% sucrose (w/v) in 4% PFA]. Cryostat frozen sectioning was used to produce thin (40µm), uniform, brain slices. Brain slices were stored in PBS at 4°C, until they were mounted on slides and prepared for staining.

2.12.2 Cresyl Violet Staining
Once placed on slides, brain slices were left to air-dry at room temperature for 6-8 hours, in order to encourage adherence of the tissue to the slide. Next, the slides were rehydrated with ddH₂O, and then submersed in Cresyl Violet Stain [CVS; 0.1% Cresyl Violet Acetate (w/v), 0.25% Glacial Acetic Acid (pure), ddH₂O] for 3-4 minutes. Excess stain was rinsed off in ddH₂O. Slides were dipped in 70% Ethanol for 2 minutes, followed by 95% Ethanol for 2 minutes, 100% Ethanol for 2 minutes, and Xylene for 2 minutes. Tissues were covered with ImmuMount (Thermo Scientific) and a cover slip, and dried overnight at room temperature. Slides were imaged using a Zeiss LSM 510 Meta Confocal Microscope and Zeiss LSM digital imaging software.
2.13 Statistical Analysis

All results are reported as mean and standard error (± SE). Each value represented for ‘n’ indicates an individual cortical neuronal culture or SN56 passage of the indicated genotype. A one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used to determine whether differences between more than three sample means were significant (Figures 6, 9, 11, 16, and 18). An unpaired Student’s t test was used to determine whether differences in RNA levels, Mitotracker Red fluorescence, and expression of SLP-2 protein and levels of electron transport chain proteins were significant when comparing wild-type and SLP-2 knockout cortical neurons under basal conditions (Figures 8, 10, 11 and 18). A P value less than 0.05 was considered significant, and all statistical analyses were conducted using GraphPad Prism software version 6.0.
Chapter 3: Results

3.1 Experimental model for generating SLP-2-deficient primary cortical neurons

3.1.1 Successful knockout of SLP-2 gene

Germline SLP-2 deletion results in embryonic lethality in mice (Christie et al., 2012). We therefore employed a conditional knockout approach to study the effects of SLP-2 depletion in mouse primary cortical neurons (See section 2.1 and Appendix A). Under the control of the Brain Factor-1 (FoxG1) specific promoter, Cre recombinase coupled with a genome-wide loxP insertion flanking the SLP-2 gene (SLP-2\_\text{lox}) was expected to result in a forebrain-specific knockout of the SLP-2 gene as it develops in the mouse embryo. With our results, we were able to demonstrate successful knockout of SLP-2 in E14.5-15.5 forebrain cortical neurons of SLP-2\_\text{lox/lox/Cre} mice (Figure 4).

Although conditional gene targeting by the Cre/\text{lox}P system is considered an effective method of specific gene excision, cre transgene recombination efficiency has been shown to possess variability and unexpected expression patterns depending on the target gene. In both the male and female germlines, cre may mediate non-homogeneous mutations aside from the desired conditional mutation. In most cases, this results when both the \text{lox}P allele and the cre allele are inherited from the same parent (Schmidt-Supprian & Rajewski, 2007). Given that our breeding paradigm is at risk of this phenomenon, and that a germline deletion of SLP-2 on one allele would not be detected with either SLP-2\_\text{lox} or SLP-2\_\text{wt} primers, we generated a primer for an ‘SLP-2 deletion’ allele (SLP-2\_\text{del}; see Appendix B for sequence), to confirm specificity of the conditional knockout model herein employed, and ensure proper genotyping of each sample.

Additionally, in order to confirm that the ‘wild-type’ (SLP-2\_\text{lox/wt}) employed in our experiments was comparable to a wild-type mouse lacking the flanked sequences, an
SLP-2^{wt/wt/Cre} mouse was crossed with a mouse heterozygous for the SLP-2^{lox} allele (SLP-2^{lox/wt}). Tail samples obtained from E14.5-15.5 mice were used to extract DNA and determine genotypes by PCR and agarose gel electrophoresis analysis (Figure 4a). Whole cell protein lysates were prepared from primary cortical neurons on day 5 in culture and compared by Western Blot analysis (Figure 4b). As a result we were able to compare relative SLP-2 protein expression in ‘wild-type’ genotypes and confirm SLP-2 deletion in our conditional knockout mouse model (Figure 4b).
To study the effects of enforced expression of SLP-2, we adopted a SN56 neuronal cell line. Whole cell lysates were prepared from primary cortical neurons on day 5 in culture. SLP-2 expression served as a loading control.

Additionally, in order to confirm that the 'wild-type' (SLP-2 wildtype (control) mice; SLP-2 wt/wt Cre+ mouse expressed Cre recombinase under the control of a brain factor-1 specific promoter. Calnexin expression served as a loading control.

**Figure 1a.** Representative agarose gel (1.5%) electrophoresis illustrating PCR products for i) Cre recombinase (Cre) and ii) SLP-2 wild-type (SLP-2 wt), SLP-2 floxed (SLP-2 lox) and SLP-2 null (SLP-2 del) alleles. DNA extracts were prepared from tail samples obtained at E14.5-15.5. Genotype is provided by combining results from lanes corresponding to the same number: Lane 1) SLP-2 knockout (SLP-2 del), 2) SLP-2 knockout (SLP-2 del/lox/Cre), 3) homozygous floxed SLP-2 not expressing Cre (SLP-2 flox/lox), 4) heterozygous floxed SLP-2 not expressing Cre (wild-type, SLP-2 flox/wt) and 5) heterozygous floxed SLP-2 expressing Cre (SLP-2 flox/wt/Cre).

**Figure 1b.** Representative Western blot analysis of SLP-2 protein expression. Whole cell lysates were prepared from primary cortical neurons on day 5 in culture. SLP-2 flox/wt corresponds to the wild-type genotype employed in our experiments as control; SLP-2 wt/wt corresponds to a wild-type C57/BL6 mouse lacking the SLP-2 flox mutation; Cre+ mice express Cre recombinase under the control of a brain factor-1 specific promoter. Calnexin expression served as a loading control.

**Figure 4. Confirmation of SLP-2 deletion.** (a) Representative agarose gel (1.5%) electrophoresis illustrating PCR products for i) Cre recombinase (Cre) and ii) SLP-2 wild-type (SLP-2 wt), SLP-2 floxed (SLP-2 lox) and SLP-2 null (SLP-2 del) alleles. DNA extracts were prepared from tail samples obtained at E14.5-15.5. Genotype is provided by combining results from lanes corresponding to the same number: Lane 1) SLP-2 knockout (SLP-2 del), 2) SLP-2 knockout (SLP-2 del/lox/Cre), 3) homozygous floxed SLP-2 not expressing Cre (SLP-2 flox/lox), 4) heterozygous floxed SLP-2 not expressing Cre (wild-type, SLP-2 flox/wt) and 5) heterozygous floxed SLP-2 expressing Cre (SLP-2 flox/wt/Cre). (b) Representative Western blot analysis of SLP-2 protein expression. Whole cell lysates were prepared from primary cortical neurons on day 5 in culture. SLP-2 flox/wt corresponds to the wild-type genotype employed in our experiments as control; SLP-2 wt/wt corresponds to a wild-type C57/BL6 mouse lacking the SLP-2 flox mutation; Cre+ mice express Cre recombinase under the control of a brain factor-1 specific promoter. Calnexin expression served as a loading control.
3.2 Analysis of the effects of SLP-2 deletion on primary cortical neurons

3.2.1 Forebrain morphology in wild-type and SLP-2 knockout mice
Macroscopic examination of brains from age-matched litter-mate wild-type (SLP-2\textsuperscript{lox/wt}) and knockout (SLP-2\textsuperscript{lox/lox/Cre}) adult (P70) mice revealed no major abnormalities. Additionally, coronal cryostat sections (40\(\mu\)m) were prepared from the cerebral cortex, and labelled with Cresyl Violet Acetate (CVA). CVA is a basic aniline dye that binds readily to acidic components of neuronal nuclei and cytoplasm, thereby highlighting important neuronal features such as the rough endoplasmic reticulum (Nissl bodies) and nuclei. Representative confocal images (Figure 5) reveal similar cell layering and morphology in forebrains of SLP-2 knockout and wild-type mice.

3.2.2 Neuron viability under normal cell culture conditions
In order to assess and compare cell viability between SLP-2 knockout (SLP-2\textsuperscript{lox/lox/Cre}) and wild-type (SLP-2\textsuperscript{lox/wt}) primary cortical neurons, a Live/Dead Assay (Invitrogen) was employed to obtain data for the percentage of cell death in cell cultures at days 3, 5, and 7 under standard culture conditions [37\(^\circ\)C humidified incubator, 5\% CO\(_2\), C-NBM]. The analysis was repeated five times for each genotype and day in culture, and results were analyzed using a one-way ANOVA and Tukey’s multiple comparisons test. There was no significant difference (P> 0.05, n=5) in the percentage of cell death between SLP-2 knockout and wild-type (control) cultures at either time point (Figure 6).

3.2.3 Neuritic outgrowth
Dendrite development and synapse formation are important for and indicative of proper neuronal function as they allow neurons to form functional networks and communicate between each other. In order to visualize neuritic outgrowth in both SLP-2 knockout (SLP-2\textsuperscript{lox/lox/Cre}) and wild-type (SLP-2\textsuperscript{lox/wt}) primary cortical neurons, cells were labeled with anti-microtubule-associated protein 2 (MAP2). Proteins of this family are thought to be involved in microtubule stabilization and assembly, which are essential for the
development and maintenance of proper neuronal morphology (Matus et al., 1981; Caceres et al., 1984, 1986; Peng et al., 1986). Representative fluorescence images (Figure 7) demonstrate that both SLP-2 knockout and wild-type cortical neurons are MAP-2 positive, and display competent neuritic outgrowth and dendrite formation.
Figure 5. Histology of adult (P70) wild-type (SLP-2^{lox/wt}) and SLP-2 knockout (SLP-2^{lox/lox/Cre}) mouse forebrains. Representative confocal images of Cresyl-Violet-stained coronal hemi-sections (40 µm) through cerebral cortex at different rostral forebrain levels. Cell layering (1-6) appears similar in both wild-type (c) and SLP-2 knockout (f) cortices, and morphology appears unaltered in wild-type (a and b) and SLP-2 knockout (d and e) rostral forebrains. (c) and (f) are magnified images of sections outlined in (b) and (e) respectively. CP: caudate putamen; LV: left ventricle; RV: right ventricle; Sep: septum. Scale bars: 1mm.
Figure 6. SLP-2 deficient primary cortical neurons survive at a level comparable to wild-type primary cortical neurons under normal cell culture conditions. A Live/Dead Assay was employed to calculate the percentage of dead cells (% Cell Death) relative to the total number of cells in wild-type (SLP-2\textsuperscript{lox/wt}) and knockout (SLP-2\textsuperscript{lox/lox Cre+}) E14 primary cortical neuronal cultures on days 3, 5, and 7 in culture. Values expressed as mean ± SE for n=5 per group, P> 0.05.
Figure 7. SLP-2 deficient primary cortical neurons demonstrate neuritic outgrowth. Representative fluorescence images (600X) of (a) wild-type (SLP-2\textsuperscript{lox/wt}) and (b) SLP-2 knockout (SLP-2\textsuperscript{lox/lox/Cre}) primary cortical neurons fixed on day 5 in culture. Cells were labeled with an antibody against microtubule-associated protein 2 (anti-MAP2, green) to detect dendrites and Hoechst 33258 (blue) to detect nuclei. Both (a) wild-type (b) SLP-2 knockout primary cortical neurons are MAP2-positive and display dendritic development. Scale bars: 20\textmu M.
3.2.4  Analysis of mitochondrial mass - Mitotracker Red fluorescence

Mitochondrial dysfunction can have a number of severe cellular consequences that are closely linked with neurological disorders. Through mitochondrial biogenesis, however, cells may increase their mitochondrial mass in an effort to counteract a decline in mitochondrial function, or in response to various cellular stresses, as is seen in the case of SIMH. Given that SLP-2 has an implicated role in stabilizing mitochondrial fusion proteins, most notably during the mitochondrial stress-response (SIMH) (Szabadkai et al., 2004; Da Cruz et al., 2010; Tondera et al., 2009; Rambold et al., 2011), we sought to investigate the effect of SLP-2 deficiency on mitochondrial mass in primary cortical neurons.

Mitotracker (MtrkR) is a red fluorescent membrane-dependent probe for staining mitochondria in live cells, and a useful tool for studying the mitochondrial mass within a given cell or cell population. To determine whether SLP-2 deficiency has an effect on mitochondrial mass or membrane potential, the level of MtrkR fluorescence in both SLP-2 knockout (SLP-2\textsuperscript{lox/lox/Cre}) and wild-type (SLP-2\textsuperscript{lox/wt}) primary cortical cell cultures was analyzed. Cultures were labelled with 10nM MtrkR, harvested, washed with and resuspended in PBS, and subjected to fluorescence-activated cell sorting (FACS) using a flow cytometer (BD Accuri\textsuperscript{TM} C6, Biosciences). Mean MtrkR fluorescence (FL3-H) values were normalized to unstained controls within each genotype. The analysis was repeated four times for each genotype and results were analyzed using an unpaired t-test. FACS analysis revealed a significantly decreased MtrkR fluorescence in SLP-2 knockout primary cortical neuronal cultures when compared to wild-type (P< 0.05, n=4) (Figure 8).

3.2.5  Analysis of mitochondrial mass - TOM20 protein expression

Our results obtained by flow cytometry and FACS analysis (Figure 8) indicate that there could be a decline in the overall mitochondrial mass in SLP-2 knockout primary cortical cultures. However, given that the accumulation of MtrkR in mitochondria depends on mitochondrial membrane potential, a decline in the level of MtrkR fluorescence signal
could also be indicative of mitochondrial membrane potential depolarization. Translocase of outer mitochondrial membrane 20 (TOM20) is a subunit of the receptor complex responsible for recognition and translocation of cytosolically-produced mitochondrial precursor proteins. TOM20 serves as an established marker for mitochondrial mass and biogenesis (Whitaker-Menezes et al., 2011). The effect of SLP-2 deficiency on mitochondrial mass and biogenesis was thus further assessed by analyzing the level of TOM20 expression in both SLP-2 knockout (SLP-2<sup>lox/lox/Cre</sup>) and wild-type (SLP-2<sup>lox/wt</sup>) primary cortical neurons.

Whole cell protein lysates were prepared from SLP-2 knockout and wild-type cultures that had been under normal culture conditions for 5 days. Western Blot analysis was employed to assess the level of TOM20 protein expression in both genotypes. A single band representing TOM20 was present around 15kDa in all sample lanes. Relative expression of the protein was determined by densitometry analysis of Western Blots that were performed for three replicates for each genotype. Densitometry values were normalized to Calnexin (90kDa) control, an endoplasmic reticulum (ER) membrane protein, and results were analyzed with one way ANOVA and Tukey’s multiple comparisons test. There was no significant difference (P> 0.05, n=5) in TOM20 protein levels between SLP-2 knockout and wild-type cultures under normal culture conditions (Figure 9).
Figure 8. Flow Cytometry and FACS analysis reveal decreased mean Mitotracker Red fluorescence (FL3-H) in SLP-2 knockout primary cortical neurons. Wild-type (SLP-2<sup>lox/wt</sup>) and SLP-2 knockout (SLP-2<sup>lox/lox Cre+</sup>) primary cortical neurons were labeled with 10nM Mitotracker Red. Fluorescence Units are normalized to unstained controls within each genotype. Values expressed as mean ± SE for n = 4 per group, *p < 0.05.
Figure 9. SLP-2 depletion does not have an effect on mitochondrial mass in primary cortical neurons under normal culture conditions. Representative Western blot (a) and densitometry analysis (b) of TOM20 protein expression in wild-type (SLP-2<sup>lox/wt</sup>) and SLP-2 knockout (SLP-2<sup>lox/lox/Cre</sup>) primary cortical neurons. (a) Whole cell protein lysates were prepared on day 5 in culture. (b) Densitometry values for TOM20 protein expression were normalized to the loading control, Calnexin. Values are expressed as mean ± SE for n= 5, p > 0.05.
3.2.6 *Analysis of mitochondrial respiration - Proteins involved in Oxidative Phosphorylation*

Mitochondrial membrane potential provides a measure of the level of ion transport, and is therefore a prime indicator of mitochondrial health, metabolic activity and membrane integrity (Acton et al., 2003). Our analyses of MtrkR fluorescence and TOM20 protein expression in primary cortical neurons (Sections 3.2.4 and 3.2.5) indicate that SLP-2 depleted neuronal mitochondria likely possess depolarized membranes. Mitochondria establish and maintain membrane potential mainly through proper function of the RC and OXPHOS along the inner mitochondrial membrane. This is especially important for neurons, as they rely heavily on OXPHOS to produce the vast majority of their cellular ATP.

The effect of SLP-2 deficiency on the levels of different protein complexes of the mitochondrial respiratory chain was thus assessed in the neuronal cell type. Whole cell protein lysates were prepared from SLP-2 knockout (SLP-2\(^\text{lox/lox/Cre}\)) and wild-type primary cortical neuronal cell cultures. A total oxidative phosphorylation monoclonal antibody cocktail (MitoSciences) targeting Complex II subunit 30kDa (30kDa), Complex III subunit Core 2 (47kDa), Complex IV subunit I (39kDa) and ATP synthase subunit alpha (53kDa) as well as a separate monoclonal antibody (Abcam) for Complex I subunit NDUFS3 (30kDa) were used to assess, by Western Blot analysis, the protein levels of various subunits of RC complexes involved in oxidative phosphorylation (Figure 10a). Densitometry analyses of Western Blots were performed for 5-7 replicates of each respiratory complex for primary cortical neurons of both genotypes. Densitometry values were normalized to Calnexin (90kDa) control, and results were analyzed using unpaired t-tests. There was a significant decrease (Complex I, P< 0.05, n=5; Complex II, P < 0.05, n=7; Complex III, P< 0.05, n=7; Complex IV, P< 0.05, n=7; Complex V, P< 0.05, n=6) in protein levels of all five subunits of complexes of the respiratory chain examined in SLP-2 knockout samples when compared to wild-type (Figure 10b-f).
Figure 10. Depletion of SLP-2 is associated with decreased OXPHOS protein levels in primary cortical neurons. Representative Western blot (a) and densitometry (b-f) analysis for Complex I (subunit NDUFS3), Complex II (subunit 30kDa), Complex III (subunit Core 2), Complex IV (subunit I), and Complex V (subunit alpha) of the electron transport chain in wild-type (SLP-2<sup>lox/lox</sup>) and SLP-2 knockout (SLP-2<sup>lox/lox Cre<sup>+</sup></sup>) primary cortical neurons. Whole cell protein lysates were prepared on day 5 in culture. Densitometry values are normalized to the loading control, Calnexin. Values are expressed as mean ± SE for n= 5-7, *p< 0.05.
3.3 Examining the role of SLP-2 in neuronal mitochondria under various conditions of stress

3.3.1 SLP-2 up-regulation in response to oxidative stress and DNA damage

We have identified SLP-2 as a stress-inducible transcript in a previous microarray study employing a variety of stressors (unpublished data). We therefore sought to further characterize the stress-responsive expression pattern of SLP-2 in primary cortical neurons, by examining its expression specifically in response to drug-induced oxidative stress and DNA damage. The mitochondrial stressor MPP+ is a Complex I (NADH dehydrogenase) inhibitor that can mimic the symptoms of PD when administered to rodents or primates (Braungart et al., 2004; Sherer et al., 2003). MPP+ functions to interfere with oxidative phosphorylation in mitochondria, resulting in oxidative stress, depletion of ATP and cell death. Camptothecin (CPT), on the other hand, is a class I DNA topoisomerase inhibitor that promotes DNA breakage, and induces a host of cellular activities that ultimately lead to apoptosis (Sen et al., 2004).

Whole cell RNA and protein lysates were prepared from both SLP-2 knockout (SLP-2^lox/lox/Cre) and wild-type (SLP-2^lox/wt) primary cortical neurons following 24 hours treatment with either 100µM MPP+ or 40µM CPT. qRT-PCR and Western blot analysis were employed to analyze changes in SLP-2 mRNA and protein levels respectively.

Expression levels of SLP-2 mRNA were determined by qRT-PCR, and changes in mRNA expression levels were calculated as fold change expressed relative to an untreated control within each genotype, using the delta-delta CT method. The analysis was repeated four times for each genotype (SLP-2 knockout data not shown) following each treatment, and results were analyzed using unpaired t-tests. There was a ~3-fold increase in SLP-2 mRNA levels following both MPP+ and CPT treatments in wild-type cortical neurons (Figure 11a).

Western blot and subsequent densitometry analysis was used to compare the level of SLP-2 protein expression following MPP+ and CPT treatments. The analyses were
repeated three times for each genotype following each treatment and results were analyzed using a one-way ANOVA and Tukey’s multiple comparisons test. There was a significantly increased level of SLP-2 protein expression in wild-type cultures following both MPP+ and CPT treatments (P<0.05, n=3) and as expected, SLP-2 could not be detected in extracts from SLP-2 knockout cultures (Figure 11b).
Figure 11. SLP-2 expression is up-regulated in primary cortical neurons in response to oxidative stress and DNA damage. SLP-2 mRNA expression (a) and representative Western blot and densitometry analysis (b) for SLP-2 protein expression in wild-type (SLP-2<sup>lox/WT</sup>) and SLP-2 knockout (SLP-2<sup>lox/lox/Cre</sup>) primary cortical neurons. Densitometry analysis was only preformed for cells expressing SLP-2 (SLP-2<sup>lox/WT</sup>). Cells were treated on day 5 in culture, and whole cell RNA and protein lysates were prepared following 24 hours 100µM MPP+ or 40µM CPT treatments. (a) mRNA levels are expressed relative to untreated (control) cells within each genotype. (b) Control (Ctrl) cells were untreated and (c) densitometry values were normalized to the loading control, Calnexin. Values expressed as mean ± SE for (a) n = 4 per group, and (b) n=3 per group, *p< 0.05.
3.3.2 Analysis of mitochondrial biogenesis in response to oxidative stress and DNA damage

When cells are exposed to selective stresses, they may undergo SIMH. As mentioned (Section 1.2.1), this process involves increased mitochondrial biogenesis and ATP production, and confers on cells a resistance to stress (Tondera et al., 2009; Rambold et al., 2011). SLP-2 is required to stabilize the fusion mediator L-OPA1 during SIMH (Szabadkai et al., 2004; Da Cruz et al., 2010; Tondera et al., 2009; Rambold et al., 2011). Given that our results indicate that SLP-2 expression is induced in response to both MPP+ and CPT treatments (Section 3.3.1, Figure 11), we sought to gain further insight into the role of SLP-2 in mitochondrial biogenesis, by examining the effects of these same treatments on mitochondrial mass in the neuronal cell type, both in the presence and absence of SLP-2.

The level of TOM20 protein expression is a suitable measure of mitochondrial mass and biogenesis, as previously described (see Section 3.3.5) for experiments conducted in primary cortical neurons under normal culture conditions. Thus, the effect of SLP-2 deficiency on mitochondrial mass and biogenesis was further assessed by analyzing the level of TOM20 expression in both SLP-2 knockout (SLP-2lox/lox/Cre) and wild-type (SLP-2lox/wt) primary cortical neurons following 24 hours treatment with either a mitochondrial stressor (MPP+) or DNA-damaging agent (CPT).

Whole cell protein lysates were prepared from SLP-2 knockout and wild-type cultures under normal culture conditions and following 24 hours treatment with either 100µM MPP+ or 40µM CPT. Relative expression of TOM20 protein was determined by densitometry analysis of Western Blots that were performed for three replicates for each genotype and drug treatment. Densitometry values were normalized to Calnexin (90kDa) control, and results were analyzed with one way ANOVA and Tukey’s multiple comparisons test.

Similar to the data obtained from analysis of TOM20 expression under normal culture conditions (Figure 9), there was no significant difference (P> 0.05, n=5) in TOM20
protein levels between SLP-2 knockout and wild-type cultures following either MPP+ or CPT treatments (Figure 12). There was a significant increase in TOM20 expression following both MPP+ and CPT treatments within each genotype (P<0.05, n= 3 for MPP+, n= 4 for CPT).
Figure 12. SLP-2 depletion does not have an effect on mitochondrial mass in primary cortical neurons following stressful stimuli. Representative Western blot (a) and densitometry analysis (b) of TOM20 protein expression in wild-type (SLP-2lox/wt) and SLP-2 knockout (SLP-2lox/loxCre) primary cortical neurons. Cells were treated on day 5 in culture, and whole cell protein lysates were prepared following 24 hours 100µM MPP+ or 40µM CPT treatments. Control (Ctrl) cells were untreated and densitometry values were normalized to the loading control, Calnexin. Values are expressed as mean ± SE for n= 5 per control group, p > 0.05; n= 3-4 per treatment group, p > 0.05.
3.3.3 Analysis of PGC-1α mRNA expression following oxidative stress and DNA damage

PGC-1α is a master co-activator and regulator of mitochondrial biogenesis and energy expenditure (Ciron et al., 2012; Hempenstall et al., 2012; Puigserver et al., 2003), found up-regulated under conditions of increased cellular energy demand, such as in response to gluconeogenesis, thermogenesis, and exercise (Christie et al., 2011; Yoon et al., 2001). Additionally, PGC-1α regulates the activity of a number of transcription factors involved in a number of metabolic pathways. This includes downstream induction of replication of the mitochondrial genome, resulting in stimulation of mitochondrial biogenesis and respiration (Scarpulla et al., 2008; Hempenstall et al., 2012; Puigserver et al., 2003).

Our results indicate that both oxidative stress (MPP+) and DNA damage (CPT) result in increased mitochondrial biogenesis in neurons, as demonstrated by an increase in mitochondrial mass (Figure 12). Previous studies have implicated a role for SLP-2 in SIMH in a number of non-neuronal mammalian cell-lines and mouse cell types (Tondera et al., 2009; Christie et al., 2011). In this study, we have shown that there is an increase in SLP-2 expression in neurons, in response to stress induced by both oxidative stress and DNA damage (Figure 11). Interestingly, our data further illustrates that SLP-2 is not required for the induction of mitochondrial biogenesis in neurons, in response to stress induced by MPP+ or CPT (Figure 12). Previous studies examining the role of SLP-2 during T-Cell activation have shown that SLP-2 up-regulation precedes PGC-1α up-regulation and mitochondrial biogenesis (Christie et al., 2011). In an effort to further characterize the stress-induced up-regulation of SLP-2, and gain further insight into the mechanism by which a concomitant increase in mitochondrial mass occurs, we sought to determine whether SLP-2 may induce the transcription of mitochondrial target genes in neurons. We therefore examined the expression of PGC-1α in primary cortical neurons following treatment with MPP+ and CPT.

Total RNA was extracted from both SLP-2 knockout (SLP-2lox/lox/Cre) and wild-type (SLP-2lox/wt) primary cortical neurons treated with either 100µM MPP+ or 40µM CPT for
24 hours. Expression levels of PGC-1α mRNA were determined by qRT-PCR, and changes in mRNA expression levels were calculated as fold change expressed relative to an untreated control within each genotype, using the delta-delta CT method. The analysis was repeated five times for wild type cultures and four times for SLP-2 knockout cultures and results were analyzed using an unpaired t-test. Following CPT treatment, there was significantly greater fold increase in PGC-1α mRNA expression (P<0.05, n=4) in SLP-2 knockout cultures relative to wild-type cultures. There was no significant difference (P>0.05, n=4) in PGC-1α mRNA levels between SLP-2 knockout and control cultures following MPP+ treatment (Figure 13).
Figure 13. **PGC-1α is up-regulated in primary cortical neurons in response to DNA damage.** PGC-1α mRNA expression in wild-type (SLP-2^lox/wt) and SLP-2 knockout (SLP-2^lox/lox/Cre) primary cortical neurons was measured by qRT-PCR. Cells were treated on day 5 in culture, and whole cell RNA lysates were prepared following 24 hours 100μM MPP+ or 40μM CPT treatments. mRNA levels are expressed relative to untreated (control) cells within each genotype. Values expressed as mean ± SE for n = 4 per group, *p < 0.05.
3.3.4 Analysis of the effects of SLP-2-depletion on neuron survival following oxidative stress and DNA damage

Our results indicate that SLP-2 knockout primary cortical neurons survive under normal cell culture conditions at a level that is comparable to wild-type primary cortical neurons (Figure 6). Additionally, we have shown that SLP-2 is up-regulated in primary cortical neurons in response to both oxidative stress and DNA damage (Figure 11). In an effort to determine whether up-regulation of SLP-2 occurs as a pro-survival response in neurons, and further characterize the role of SLP-2 in the neuronal stress-response, we sought to examine the effects of SLP-2 depletion on neuronal cell survival in response to a number of cell stressors.

In addition to MPP+ and CPT, we employed 3-NP in our experiments, as a secondary model of mitochondrial inhibition and oxidative stress. 3-NP is a complex II (succinate dehydrogenase) inhibitor and has been previously used to produce animal models of Huntington’s Disease (Huang et al., 2006; Beal et al., 1993; Borlongan et al., 1997).

Following 24 hour treatments with 100µM MPP+, 10µM 3-NP, or 40µM CPT, the percentage of cell death in both SLP-2 knockout (SLP-2\textsuperscript{lox/lox/Cre}) and wild-type (SLP-2\textsuperscript{lox/wt}) primary cortical cultures was assessed using a Live/Dead Assay (Invitrogen). The analysis was repeated six times for each genotype following each drug treatment, and results were analyzed using a one-way ANOVA and Tukey’s multiple comparisons test. Following treatment with mitochondrial stressors, MPP+ and 3-NP, a significantly lower percentage of cell death (P< 0.05, n=6) was observed in SLP-2 knockout cultures relative to wild-type cultures (Figure 14a and 14b). Conversely, following treatment with a DNA-damaging agent, CPT, a significantly greater percentage of cell death (P< 0.05, n=6) was observed in SLP-2 knockout cultures relative to wild-type cultures (Figure 14c).
Figure 14. SLP-2 depletion has a differential effect on survival in primary cortical neurons in response to oxidative stress and DNA damage. A Live/Dead Assay was employed to calculate the percentage of dead cells (% Cell Death) relative to the total number of cells following 24 hours treatment with a) 100μM MPP+, b) 10μM 3-NP, and c) 40μM CPT. Both wild-type (SLP-2lox/wt) and knockout (SLP-2lox/lox/Cre) primary cortical cultures were treated on day 5 in culture. Control (Ctrl) cells were untreated. Values expressed as mean ± SED for n=6 per group, *p < 0.05.
3.3.5 Analysis of the effects of SLP-2 induction on neuron survival following oxidative stress and DNA damage

Our results indicate that SLP-2 is up-regulated in primary cortical neurons in response to both oxidative stress and DNA damage (Figure 11). SLP-2 depletion, however, appears to have a differential effect on cell survival in response to both of these stresses (Figure 14). In an effort to further characterize the potentially pro-survival induction of SLP-2 expression in neurons in response to stressful stimuli (MPP+, 3-NP, CPT), we sought to examine whether preemptive over-expression of SLP-2 would have a protective effect in neurons exposed to similar stresses.

We therefore adopted an SN56 neuronal cell line to study the effects of enforced expression of SLP-2 on cell survival. When differentiated, SN56 cells possess neuronal morphology and a cholinergic phenotype (Krieglstein et al., 2008). Serum withdrawal was employed to induce differentiation in SN56 cells. Subsequently, either GFP plasmid (pEGFP) or SLP-2-GFP plasmid (pSLP-2-GFP), was transfected into SN56 cell cultures, using a calcium phosphate transfection method as outlined in section 2.2.4. Whole cell protein lysates were extracted from SN56 cells following expression of SLP-2-GFP or EGFP in cultures demonstrating 30-40% transfection efficiency. The relative expression levels of SLP-2 protein in transfected SN56 cell cultures were examined by Western Blot analysis, which revealed increased SLP-2 protein levels in SN56 cultures transfected with pSLP-2-GFP when compared to those that were un-transfected or transfected with pEGFP (Figure 15a).

SN56 cells transfected with pSLP-2-GFP display a punctate and cytoplasmic GFP signal that appears to co-localize with the mitochondrial mass (Figure 15b, top panels). Conversely, SN56 cells transfected with pEGFP display a diffuse GFP signal throughout the cytoplasm and nucleus (Figure 15b, bottom panels).

Due to the highly proliferative and metabolically active nature of the SN56 cell line, a dose-responsive range for treatment with 3-NP was not attainable (data not shown). Following 24 hours treatment with 5µM and 50µM MPP+, and 40µM CPT, however, the
percentage of apoptotic cells in cultures of differentiated SN56 cells expressing either EGFP or SLP-2-GFP was assessed using Hoechst 33342 staining to visualize apoptotic nuclei. A representative fluorescence image of Hoechst 33342 staining used to distinguish characteristic apoptotic nuclei is included in Appendix D.

The analysis was repeated four times for each plasmid following each drug treatment, and results were analyzed using a one-way ANOVA and Tukey’s multiple comparisons test. Following treatment with mitochondrial stressor MPP+, a significantly greater percentage of apoptosis (P< 0.05, n=4) was observed in cells expressing SLP-2-GFP relative to those expressing EGFP (Figure 16a). Conversely, following treatment with the DNA-damaging agent, CPT, a significantly lower percentage of apoptosis (P< 0.05, n=4) was observed in cells over-expressing pSLP-2-GFP relative to cells over-expressing pEGFP (Figure 16b).

3.3.6 Analysis of mitochondrial respiration - Proteins involved in Oxidative Phosphorylation

Our results indicate that SLP-2 depletion has a significant effect on the protein levels of subunits from all five complexes of the mitochondrial respiratory chain (Figure 10). We therefore sought to further characterize the role of SLP-2 in stabilizing mitochondrial OXPHOS proteins by employing our model of enforced SLP-2 expression in SN56 cells, to examine whether SLP-2 up-regulation, in neurons, was associated with changes in protein levels of subunits of RC complexes involved in mitochondrial respiration.

Whole cell protein lysates were prepared from SN56 cells transfected with either pEGFP or pSLP-2-GFP. Similar to our previous experiments in primary cortical neurons (Section 3.2.6), a total oxidative phosphorylation monoclonal antibody cocktail (MitoSciences) and a separate monoclonal antibody for Complex I subunit NDUFS3 (Abcam) were used to detect, by Western Blot analysis, levels of specific proteins involved in oxidative phosphorylation (Figure 17a). Densitometry analysis of Western Blots was performed for 3 replicates for SN56 cells for both transfected plasmids.
Densitometry values were normalized to Calnexin (90kDa) control, and results were analyzed using unpaired t tests. There was a significant increase in Complex I NDUFS3 subunit protein levels (P<0.05, n=3) in SN56 cells over-expressing SLP-2GFP relative to those over expressing EGFP. There was no significant difference in protein levels for subunits of complexes II-V in SN56 cells expressing SLP-2-GFP relative to those expressing EGFP (Figure 17b-f).
Figure 15. Confirmation of SLP-2-GFP expression. (a) Representative Western blot analysis of pSLP-2-GFP plasmid expression in differentiated SN56 cells. Whole cell protein lysates were prepared following differentiation induced by serum-withdrawal, and transfection by a calcium phosphate method. Calnexin serves as loading control. (b) Representative fluorescence images showing co-localization (yellow) of SLP-2-GFP and mitochondria in SN56 cells transfected with SLP-2-GFP plasmid (pSLP-2GFP, top panels). SN56 cells transfected with GFP plasmid (pEGFP, bottom panels) display a diffuse GFP signal. Hoechst 33258 (blue), a nuclear marker, is shown in the first panel; TOM20 (red), a mitochondrial marker, is shown in the second panel; GFP (green), plasmid protein expression marker, is shown in the third panel; and the fourth panel shows the first three panels merged together. Scale bars: 10µm.
Figure 16. Induction of SLP-2 has a differential effect on SN56 cell survival in response to oxidative stress and DNA damage. A Live/Dead Assay was employed to calculate the percentage of apoptotic cells relative to the total number of cells following 24 hours treatment with a) 5μM and 50μM MPP+ and b) 40μM CPT in differentiated SN56 cells transfected with EGFP plasmid (pEGFP) and SLP-2-GFP plasmid (pSLP-2GFP). Control (Ctrl) cells were untreated. Values expressed as mean ± SE for n = 4 per group. Treatment effects *p < 0.05.
Figure 17. Induction of SLP-2 is associated with increased OXPHOS Complex I protein levels in SN56 cells. Representative Western blot (a) and densitometry (b-f) analysis of protein levels for Complex I (subunit NDUFS3), Complex II (subunit 30kDa), Complex III (subunit Core 2), Complex IV (subunit I), and Complex V (subunit alpha) of the electron transport chain in differentiated SN56 cells expressing either GFP plasmid (pEGFP) of SLP-3-GFP plasmid (pSLP-2-GFP) DNA. Whole cell protein lysates were prepared following successful transfection of differentiated SN56 cells. Densitometry values are normalized to the loading control, Calnexin. Values are expressed as mean ± SE for n= 3, *p< 0.05.
Mitochondria are central organelles for a number of critical cellular functions. Due to a number of high-energy demanding neuronal processes, neurons rely heavily on mitochondrial function and proper mitochondrial dynamics to maintain proper cellular function and homeostasis. Studies focused on isolated mitochondria and dissociated cell cultures have provided insight to the function of this organelle, and have highlighted a number of integral proteins involved in the stabilization and proper organization of mitochondrial components. SLP-2 has been shown to be closely linked with mitochondrial dynamics, most notably the stress-response, thereby implicating a role for this protein in mitochondrial biogenesis, energy production, and programmed cell death.

4.1 Establishment of a model to study the effects of SLP-2 deletion and SLP-2 up-regulation

A systemic deletion of SLP-2 has been shown to result in embryonic lethality (Mitsopoulos et al., 2012). In an effort to characterize SLP-2 deficiency in neurons, we employed a conditional knockout mouse expressing Cre recombinase under the control of the brain factor 1 promoter. We have shown that this model results in successful forebrain-specific deletion of the SLP-2 gene as it develops in the mouse embryo, from which we were able to obtain SLP-2 deficient E14.5-15.5 primary cortical neurons. Additionally, C57BL/6 mouse embryos carrying one wild-type SLP-2 allele (SLP-2$^{wt}$) and one flanked SLP-2 allele (SLP-2$^{lox}$), express a comparable amount of SLP-2 protein relative to wild-type C57BL/6 mouse embryos and therefore serve as a suitable model for wild-type controls in our experiments.

In an effort to characterize the effects of SLP-2 up-regulation in neurons, we enforced the expression of SLP-2-GFP plasmid DNA in the SN56 neuronal cell line. In agreement
with previous studies conducted in a number of non-neuronal cell lines (Wang et al., 2009; Hajek et al., 2007; Da Cruz et al., 2008, Christie et al., 2012) we have shown that SLP-2 co-localizes with the mitochondrial marker TOM20, in the neuronal cell type.

4.2 Conditional SLP-2 knockout mice are similar to wild-type mice: Brain morphology, neuron viability and neuritic outgrowth

Forebrain-specific SLP-2 knockout mice survive to adulthood (P70+), and display no significant gross morphological alterations to the brain, when compared to wild-type (SLP-2^{lox/wt}) mouse brains. At 10 weeks of age, forebrains of SLP-2 knockout mice appear to have a normal gross neuroanatomy that is devoid of neurodegeneration, and Nissl staining of coronal brain sections through the cortex reveal cell density and layering that is similar to wild-type forebrain sections. Although our studies on forebrain-specific SLP-2 knockout mouse brains indicate no significant morphological or neuroanatomical abnormalities, further microscopic analyses, including immunohistochemical characterization of neuronal morphology, perhaps additionally at various stages of development, would be useful to rule out potential alterations to other areas or cellular components of the brain. Furthermore, behavioral experiments with adult mice would be useful for assessing overall neurotransmission and brain functionality in an effort to determine, for example, whether learning, locomotion, sensory capabilities, or drug responses are altered as a result of a forebrain-specific SLP-2 deletion. SLP-2 knockout and wild-type adult brains could also be used for further study of anatomical, biochemical, and degenerative changes related to lesions associated with stroke, epilepsy, or major progressive neurodegenerative diseases such as PD, HD or AD.

In addition, we have shown that in vitro, SLP-2 knockout neurons survive under normal culture conditions at levels comparable to wild-type primary cortical neurons. Moreover, we have shown that SLP-2 knockout neurons are MAP2 positive, thereby demonstrating dendritic development. Additional studies on cytoskeletal organization, neuritic
branching, branch length, and complexity of neuronal dendritic arbors would be necessary to further characterize the functionality of SLP-2 knockout neurons.

4.3 SLP-2 contributes to stabilization of mitochondrial respiratory chain proteins

In this study we have shown that SLP-2 is required to stabilize normal levels of a number of protein subunits of mitochondrial respiratory complexes. This is consistent with previous studies in HeLa cells, employing short hairpin RNA (shRNA) (Da Cruz et al., 2008), and KYSE 150 cells (an ESCC cell line), employing small interfering RNA (siRNA) (Wang et al., 2009), that have demonstrated a decrease in protein levels of subunits from Complexes I and IV of the respiratory chain as a result of SLP-2 depletion. Similarly, studies in SLP-2 deficient T lymphocytes (T-cells) (Christie et al., 2012) have demonstrated decreased protein levels of multiple subunits of Complex I, as well as decreased activity at complexes I, II and III of the mitochondrial respiratory chain (Christie et al., 2012).

Alterations to the ETC in T-cells have been associated with impaired compartmentalization of cardiolipin, an important IMM protein involved in promoting optimal mitochondrial function through the maintenance of membrane potential, fluidity, and osmotic stability (Christie et al., 2012). Cardiolipin is essential for the structural and functional stabilization of several mitochondrial respiratory chain complexes, and contributes to the regulation of cyt c (Ott et al., 2002; Tuominen et al., 2002; Schlame et al., 2005). Cardiolipin has also been shown to play a critical role in the assembly of ETC supercomplexes, as well as mitochondrial biogenesis (Robinson et al., 1993; Schlame et al., 2000; Chico and Sparagna, 2006).

Here we show a significant decrease in protein levels of subunits from all five complexes of the respiratory chain [Complex I (subunit NDUFS3), Complex II (subunit 30kDa),
Complex III (subunit Core 2), Complex IV (subunit I), and Complex V (subunit alpha)] in SLP-2 deficient primary cortical neurons. This suggests that SLP-2 also contributes to the maintenance of mitochondrial membrane potential in neurons, is involved in mitochondrial biogenesis, and could perhaps serve a larger role in OXPHOS maintenance in neurons than in other cell types, given that we are the first to report a link between SLP-2 deficiency and changes in protein levels of subunits from complexes II, III, and V.

In support of this, we have found that in SN56 cells over-expressing SLP-2 there is also a significant increase in the level of Complex I (NDUFS3) protein levels. Additionally, although there was no significant difference seen in the protein levels of subunits from Complexes II-V, the levels of these proteins do appear to be elevated in SN56 cells over-expressing SLP-2, relative to control.

Recent structural and functional evidence has suggested that mitochondrial complexes associate with one another to form supramolecular structures, known as supercomplexes, in order to carry out their roles as “respirasomes” (Acin-Perez 2008). These supercomplexes seem to offer structural and functional benefits to the OXPHOS system by stabilizing and helping to prevent degradation of RC complexes. This appears to improve substrate channeling and electron transport efficiency, and contributes to reduced levels of mitochondrial electron and proton leakage (Moran et al., 2012; Koopman et al., 2010; Lenaz et al., 2010; Wittig et al., 2009).

Due to the structural interdependence among RC complexes however, structural changes or defects affecting one particular complex may have a significant effect on the stability of others. As a result, genetic deficiency of a single complex can result in multiple RC enzyme deficiencies (Acin-Perez et al., 2004; Li et al., 2007; Schagger et al., 2001)

Complexes I/III have been shown to form the most abundant and stable supercomplexes, particularly in mouse liver mitochondria were the majority of Complexes I and III are found in supercomplex form. Additionally, associations of Complexes III/IV, I/III/IV, II/III/IV and I/II/III/IV have also been observed, however Complexes II and IV display a
larger unassociated fraction than the other respiratory complexes. Some supercomplexes additionally include CoQ and cyt c, and retain the ability to transfer electrons from NADH or Complex II to oxygen, thereby indicating a functional role for these respirasomes (Schagger and Pfeiffer, 2001; Schagger, 2001; Cruciat et al., 2000; Acin-Perez et al., 2008).

Our results indicate that SLP-2 depletion in neurons is closely linked with decreased respiratory complex protein levels, which thereby indicates a decline in supercomplex formation and density. It is well documented that decreased expression of mitochondrial complexes is associated with a decline in mitochondrial respiration, ATP synthesis, and mitochondrial membrane potential as well as increased free-radical production most likely due to electron leakage. Given that neurons necessitate such a high amount of energy, and are so heavily dependent on functional mitochondrial dynamics and energy metabolism, such perturbations to mitochondrial function can have extremely detrimental effects. This is further supported by the prevalence of mitochondrial dysfunction as the basis for a number of neurodegenerative disorders (Section 1.1). Taken together, this evidence suggests that through its role in stabilizing mitochondrial respiratory complexes, SLP-2 may thereby contribute to maintenance of proper mitochondrial functions and bioenergetics, and potentially serve an overall neuroprotective role.

4.4 SLP-2 contributes to maintenance of mitochondrial membrane potential

Our results demonstrate that SLP-2 knockout neuronal cultures display a significantly reduced MtrkR fluorescence signal relative to wild-type neuronal cultures. Given that the uptake and accumulation of MtrkR is dependent on mitochondrial membrane potential, a decline in the level of MtrkR fluorescence could indicate not only a decrease in mitochondrial mass and density, but also signal to a decline in mitochondrial membrane potential. The level of expression of outer mitochondrial membrane protein TOM20, however, is considered a reliable indicator of mitochondrial mass. When we examined the level of membrane potential-independent TOM20 expression in these cells, we were
able to conclude that the mitochondrial mass in SLP-2 knockout neuronal cultures is not significantly different from that observed in wild-type cultures. This suggests that the decrease in MtkR fluorescence signal observed in SLP-2 knockout cultures is likely due to a decline in mitochondrial membrane potential, and correlates with our data regarding SLP-2 depletion-associated decreased ETC Complex protein levels.

Maintenance of the mitochondrial membrane potential is central to the control of calcium accumulation within the mitochondrial matrix, mitochondrial respiration and ATP synthesis (Ward et al., 2007; Nichols and Budd 2000). Although establishing the mitochondrial membrane potential involves ion transport and therefore requires energy expenditure, once this potential is established it is further used to transport additional ions and metabolites and drive ATP synthesis. Neurons are highly metabolic cells that rely critically on the energy produced by OXPHOS, as they require large amounts of ATP for maintenance of membrane excitability in order to carry out neurotransmission and plasticity (Kann and Kovacs, 2007; Billups and Forsythe, 2002). Taken together, this suggests that SLP-2 also contributes to neuronal signaling through stabilizing mitochondrial respiratory proteins and maintaining the mitochondrial membrane potential.

Additionally, studies in MEFs (Tondera et al., 2009) have shown that active oxidative phosphorylation is required for SIMH to occur, thereby revealing that disrupting the mitochondrial membrane potential prevents SIMH. A decline in mitochondrial membrane potential in SLP-2 knockout neurons, and a resultant inability to undergo protective SIMH, may therefore contribute to increased sensitivity to a number of cell stressors in this genotype.

Mitotracker Green FM (MtkG) is a green-fluorescent dye that localizes to mitochondria, in live cells, in a membrane potential-independent manner. Although our experiments with MtkR and TOM20 indicate that a decline in mitochondrial membrane potential in SLP-2 knockout neurons is likely, repeating Flow Cytometry and FACS analysis experiments with a probe such as MtkG would be a useful method to confirm our results.
4.5 SLP-2 is up-regulated and associated with a number of sub-cellular changes in neurons following stressful stimuli

In this study, we have shown that SLP-2 is up-regulated in primary cortical neurons under conditions of direct mitochondrial inhibition and stress, as it is induced by treatment with MPP+. Although this is consistent with previous studies in HeLa cells (Da Cruz et al., 2009), we have also found that contrary to HeLa cells, in primary cortical neurons, this increase in SLP-2 expression was also seen following treatments with the DNA damaging agent, CPT. This could indicate that in neurons, SLP-2 may play a more broad-spectrum role in cellular stress-responses.

4.5.1 Increased mitochondrial mass

When primary cortical neurons are subjected to stressful stimuli that result in increased SLP-2 expression, we also see a concomitant increase in mitochondrial mass as indicated by increased expression of mitochondrial protein marker TOM20. This is consistent with studies conducted in T-cells which demonstrated an increase in the mass of metabolically active mitochondria following up-regulation of SLP-2 (Christie et al., 2011). Studies in MEFs (Tondera et al., 2009) have indicated that SLP-2 is required for SIMH to occur in response to a number of selective stresses. The apparent increase in mitochondrial mass following stress treatments in our experiments, however, is also observed in SLP-2 knockout neuronal cell cultures, and not significantly different from what is observed in wild-type neuronal cell cultures. This suggests that SLP-2 is therefore, not absolutely required for the induction of mitochondrial biogenesis that results in response to stress, in neurons.

4.5.2 Differential PGC-1α expression

The expression of a key transcriptional co-activator and regulator of mitochondrial biogenesis, PGC-1α, is also up-regulated following CPT treatment, but not following MPP+ treatment in neuronal cultures. This up-regulation is also significantly greater in SLP-2 knockout neurons, which are also more sensitive, relative to wild-type neurons, to
the effects of CPT. Interestingly, these findings are not consistent with experiments conducted in T lymphocytes (Christie et al., 2011) that show that SLP-2 over-expressing cells display increased PGC-1α expression levels, thereby suggesting that there may be a differential link, between SLP-2 expression levels and other mitochondrially targeted proteins such as PGC-1α, in neurons.

PGC-1α is known to play a prominent role in controlling the expression of genes involved in the mitochondrial respiratory chain (Schreiber et al., 2004; Wu et al., 1999), as well as in promoting the expression of critical ROS-detoxifying enzymes (St-Pierre et al., 2006). Studies conducted in a colorectal tumor cell line (Srivastana et al., 2007) have indicated that PGC-1α up-regulation is associated with OXPHOS rescue, through stimulation of mitochondrial respiration and expression of several OXPHOS genes, in OXPHOS deficient cells. The increase in PGC-1α expression in response to CPT treatment in SLP-2 knockout neurons may therefore represent a cellular effort to improve mitochondrial function and protect against the effects of CPT. This could explain the increase in PGC-1α expression in response to stress treatments is similarly associated with increased levels of OXPHOS proteins we have reported as part of the SLP-2 knockout neuronal phenotype.

Based on these findings, it would be interesting to look at the relative protein levels of OXPHOS proteins following MPP+ and CPT treatments, to determine whether the induction of PGC-1α in response to stress treatments is similarly associated with increased levels of OXPHOS proteins in neurons.

Despite an observed resultant increase in mitochondrial mass, PGC-1α up-regulation in neurons, as well as in cardiac and muscle tissue, has also been shown to result in a significant decline in both respiratory reserve capacity and mitochondrial membrane potential (Russel et al., 2004; Ciron et al., 2011). Additionally, it has been linked with alterations in axonal trafficking and mitochondrial movement in dopaminergic neurons, which can ultimately result in axonal degeneration and cell death (Ciron et al., 2011). Taken together, these findings may explain the increased sensitivity of SLP-2 knockout
neurons to the effects of CPT as perhaps a result of, or contributing effect of, PGC-1α up-regulation. Additionally, the lack of PGC-1α up-regulation in response to MPP+ treatment may indicate a form or mechanism of cellular defense in SLP-2 knockout neurons in an effort to prohibit or circumvent the potential negative effects of PGC-1α up-regulation.

Furthermore, PGC-1α is considered one of the major controllers of mitochondrial biogenesis and mtDNA transcription. The action of PGC-1α includes downstream activation of other regulators of mitochondrial biogenesis such as Nuclear respiratory factors 1 (NRF1) and 2 (NRF2), and mitochondrial transcription factor A (Tfam). Recent studies in mice have shown that observed increases in both NRF1 and Tfam, as a result of exercise, can occur independently of PGC-1α activation or ROS, thereby indicating that these regulatory proteins may be up-regulated by other mechanisms (Koltai et al., 2012). Conversely, upstream regulators of PGC-1α such as the calcineurin-A- and CaMK-mediated, or protein kinase B/Akt-mediated pathways can, respectively, activate or suppress PGC-1α transcription (Czubryt et al., 2003; Handschin et al., 2003; Scheffer et al., 2003; Herzig et al., 2001).

Our results indicate that in both wild-type and SLP-2 knockout neurons, PGC-1α does not contribute to the increase in mitochondrial mass observed following treatment with MPP+. Additional studies aimed at examining the expression levels of other co-activators or inducers of mitochondrial biogenesis (such as NRF1/2 and Tfam) or upstream regulators of PGC-1α expression (such as transcription factors involved in the calcineurin A-, CaMK-, or protein kinase B/Akt-mediated pathways), under conditions or following treatments similar to those employed in our experiments, could provide further insight to the mechanisms of mitochondrial biogenesis in neurons with different expression levels of SLP-2 and in response to drugs with different targets.

4.5.3 Differential survival response to stress induced by drug treatments

In this study we have shown that SLP-2 knockout neurons are significantly more prone to
cell death resulting from the detrimental effects of DNA damage (CPT). These results are consistent with previous studies, in SLP-2 depleted MEFs (by shRNA), that have indicated that mitochondria may undergo protective SIMH in response to a number of apoptosis-inducing cell stressors, such as UV irradiation or Actinomycin D (Tondera et al., 2009). This suggests that a lack of SIMH, as a result of SLP-2 depletion, could contribute to the increased sensitivity of SLP-2 knockout neurons to the effects of CPT.

The treatments used in the experiments examining SIMH (Tondera et al., 2009) however, examined the effects of a number of DNA damaging agents and inhibitors of protein and RNA synthesis, like CPT, but did not examine the effects of direct mitochondrial stressors, such as MPP+ or 3-NP, which were employed in our experiments. Interestingly, we show that SLP-2 knockout neurons are more resistant to the effects of MPP+ and 3-NP. This may suggest, that under these conditions, depletion of SLP-2 confers on cells a specific resistance to the stress resulting from direct mitochondrial inhibition. Furthermore, our results have shown that SLP-2 knockout neurons display significantly decreased protein levels of subunits of all five complexes involved in OXPHOS. A decline in OXPHOS results in diminished ATP production, reduction in the activity of ATP synthase in maintaining the mitochondrial membrane potential, and thereby results in decreased ROS production and decreased apoptotic sensitivity (Wu et al., 2006). Up-regulation of SLP-2 has been closely linked with a number of metastatic cancers, and associated with increased tumor cell motility, proliferation, and chemotherapeutic resistance (Wang et al., 2009).

In line with our experiments conducted in primary cortical neurons, we found that relative to control SN56 cells, SN56 cells that over-express SLP-2 are more sensitive to the effects of MPP+ and more resistant to the effects of CPT. This suggests that up-regulating SLP-2 in the neuronal cell type results in capacity to withstand cellular stress that results from DNA damage, which could be explained due to perhaps a resultant increase in mitochondrial biogenesis and function, as is seen in the SIMH response that is shown to require SLP-2 (Tondera et al., 2009). The increased sensitivity of SN56 cells
over-expressing SLP-2, however, could be explained by an increase in OXPHOS protein levels, with a potential increase in ROS production, thereby rendering cells more apoptosis-prone. Figures 18 and 19 provide a schematic overview summarizing our main findings from survival response experiments employing MPP+, 3-NP and CPT.
Figure 18. The level of SLP-2 expression alters the level of mitochondrial ROS production induced by mitochondrial stressors MPP+ and 3-NP in neurons. Relative to wild-type neurons (panel II), SLP-2-depleted neurons (panel I, SLP-2 knockout neurons) display decreased levels of mitochondrial RC complex subunits, while SLP-2 over-expressing SN56 cells (panel III) display increased levels of mitochondrial RC complex subunits. As a result, SLP-2 knockout neurons (panel I) treated with inhibitors of mitochondrial respiration (either MPP+ targeting complex I, or 3-NP targeting complex II) display decreased production of ROS (HO\textsuperscript{.}, O\textsubscript{2}\textsuperscript{−}, and H\textsubscript{2}O\textsubscript{2}) by OXPHOS, relative to wild-type (bottom panels I and II). Conversely, SLP-2 over-expressing neurons (panel III) treated with MPP+ (3-NP data not available) may lead to enhanced ROS production (bottom panel III) and oxidative stress due to the increased levels of OXPHOS proteins. As a result of altered ROS production, SLP-2 knockout neurons (panel I) are more resistant to the effects of mitochondrial stress (induced by MPP+ and 3-NP) while SLP-2 over-expressing neurons (panel III) are more sensitive to the same effects (induced by MPP+), relative to wild-type (panel II).
Figure 19. The level of SLP-2 expression alters mitochondrial dynamics in response to DNA damage induced by CPT in neurons. In our study we have shown that the level of SLP-2 expression does not have an effect on mitochondrial mass under normal cell culture conditions (top panels I-III). When DNA damage is induced by treatment with CPT, mitochondria of SLP-2 knockout neurons (bottom panel I) are unable to undergo SIMH (due to the absence of SLP-2), which may result in increased mitochondrial fragmentation, rendering these neurons more susceptible to cell death, relative to wild-type (panel II). Conversely, mitochondria of SLP-2 over-expressing neurons (panel III), may undergo a hyperfusion (SIMH) response that is even greater than that which is observed in wild-type neurons (panel II) as a result of greater stabilization of critical SIMH proteins (such as L-OPA1) due to increased availability of SLP-2. As a result, due to perhaps the protective effects of SIMH, SLP-2 over-expressing neurons are more resistant to the effects of CPT, relative to wild-type.
Additional experiments examining the relative levels of ATP production and ROS production in both SLP-2 depleted and SLP-2 over-expressing cells, not only under normal culture conditions, but in response to the stress treatments employed in our experiments, would help determine whether the differential susceptibility of neurons to the induction of apoptosis in our experiments is dependent on the level of mitochondrial stability and OXPHOS function. Additionally, examining mitochondrial network organization by immunohistochemistry or Mitotracker fluorescence, both at baseline and under stress conditions, may provide further insight to whether or not neurons undergo mitochondrial network reorganization or SIMH as a result of differential expression of SLP-2, or in response to stressful stimuli.

Further investigation of our findings, by exposing both SLP-2 knockout and wild-type primary cortical neurons to alternative mitochondria- and DNA-damaging agents, are necessary to determine whether the effects observed in our experiments are consistent depending on drug targets or unique to the treatments that were herein employed.

4.6 Conclusion

In this study, we have for the first time shed light on the role of SLP-2 in mitochondrial stress responses, bioenergetics, and apoptosis in the neuronal cell type. In support of our hypothesis, we have shown that SLP-2 plays a role in altering cellular responses and the ability of neurons to either withstand or succumb to the effects of various cell stressors, depending on their targets. We have been able to conclude that in neurons, SLP-2 is involved in stabilizing a number of mitochondrial respiratory chain proteins, thereby contributing to maintenance of mitochondrial membrane potential, and differentially altering cellular responses to stress. Furthermore, although SLP-2 is not required for neuronal development, differential expression of this protein in neurons appears to be
closely linked with both apoptosis induction and suppression, depending on the insult to the cell.

This knowledge of the SLP-2 knockout neuronal phenotype will improve our understanding of neuronal stress responses and proteins necessary for development. Studies aimed at characterizing cellular responses to conditions of stress often look at changes that influence, or occur as a result of, differential levels of protein expression. Identifying and investigating proteins such as SLP-2, that are expressed differentially under various stress conditions, may be useful for development of therapeutic strategies to target invasive cells in tumors, or to evade the onset or progression of neurodegeneration.
References


Kukidome, D., et al., *Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes...*


Rambold, A.S., et al., Together we are stronger: fusion protects mitochondria from autophagosomal degradation. Autophagy, 2011. 7(12): 1568-1569.


Appendix A  Conditional SLP-2 Knockout Mouse Breeding Paradigm

Note: SLP-2<sup>lox/wt</sup> denotes loxP sites flanking SLP-2 on one allele (heterozygous); SLP-2<sup>lox/lox</sup> denotes loxP sites flanking SLP-2 on both alleles (homozygous); SLP-2<sup>wt/wt/Cre+</sup> denotes a transgenic mouse expressing cre recombinase (Cre+) under the control of the BF-1 promoter

E14.5-15.5: embryonic day 14.5-15.5 (calculated based on when male and female were paired)

♂/♀ indicates that genotype not sex-specific - either male or female mouse in the breeding-pair could present this genotype, so long as both genotypes indicated are present in the pair

PCN: Primary cortical neuron
### Appendix B  Qiagen PCR reagents, cycling conditions, and primers for mouse genotyping

Table 3. Qiagen PCR reagents and volumes for SLP-2 primers

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
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<tr>
<td>QIAGEN 10x Buffer</td>
<td>2.5µL</td>
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<tr>
<td>25mM MgCl₂</td>
<td>1.75µL</td>
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<tr>
<td>2.5mM dNTPs</td>
<td>2.0µL</td>
</tr>
<tr>
<td>5µM SLP-2 1a</td>
<td>2.0µL</td>
</tr>
<tr>
<td>5µM SLP-2 1b</td>
<td>2.0µL</td>
</tr>
<tr>
<td>5µM SLP-2 1c</td>
<td>2.0µL</td>
</tr>
<tr>
<td>Q-Solution</td>
<td>5.0µL</td>
</tr>
<tr>
<td>H₂O (RNAse/DNAse free)</td>
<td>5.5µL</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.25µL</td>
</tr>
<tr>
<td>Total Master Mix</td>
<td>23µL</td>
</tr>
<tr>
<td>Sample DNA (~0.5µg/µL)</td>
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</tr>
<tr>
<td>Total</td>
<td>25µL</td>
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</table>

Table 4. PCR Cycling Conditions for SLP-2 Primers

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<tr>
<th>Temperature (℃)</th>
<th>Time (minutes)</th>
<th>Duration (cycles)</th>
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</thead>
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<td>4:00 x 32</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>∞ x ∞</td>
</tr>
</tbody>
</table>

**SLP-2 primers**

SLP-2 forward: 5’-ACT TCC ACC CTT CAG TCC AGG TCG-3’
SLP-2 reverse: 5’-ACT TGG ATT CTG TGA AAG CAG ACA C-3’
SLP-2 deletion: 5’-GGA GTG GAC CAG CTG AAG GAG-3’
Table 5. Qiagen PCR reagents and volumes for Cre primers

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAGEN 10x Buffer</td>
<td>2.5µL</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>2.0µL</td>
</tr>
<tr>
<td>2.5mM dNTPs</td>
<td>2.0µL</td>
</tr>
<tr>
<td>5µM Cre 2a</td>
<td>2.0µL</td>
</tr>
<tr>
<td>5µM Cre 2b</td>
<td>2.0µL</td>
</tr>
<tr>
<td>Q-Solution</td>
<td>5.0µL</td>
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<tr>
<td>H₂O (RNAse/DNase free)</td>
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<td>Taq DNA Polymerase</td>
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<tr>
<td>Total Master Mix</td>
<td>23µL</td>
</tr>
<tr>
<td>Sample DNA (~0.5µg/µL)</td>
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</tr>
<tr>
<td>Total</td>
<td>25µL</td>
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Table 6. PCR Cycling conditions for Cre primers

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<th>Time (minutes)</th>
<th>Duration (cycles)</th>
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<td>5:00</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>∞</td>
</tr>
</tbody>
</table>

*Cre primers*

Cre forward: 5’-GGA CAT GTT CAG GGA TCG CCA GGC G-3’

Cre reverse: 5’-GCA TAA CCA GTG AAA CAG CAT TGC TG-3’
### Appendix C  Qiagen RT-PCR reagents and primers for SLP-2, PGC-1α, and S12 genes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
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<tbody>
<tr>
<td>2x QuantiTect SYBR Green RT-PCR Master Mix</td>
<td>12.5μL</td>
</tr>
<tr>
<td>5μM Forward primer</td>
<td>3μL</td>
</tr>
<tr>
<td>5μM Reverse primer</td>
<td>3μL</td>
</tr>
<tr>
<td>H₂O (RNAse/DNAse free)</td>
<td>2.25μL</td>
</tr>
<tr>
<td>QuantiTect Fast RT mix</td>
<td>0.25μL</td>
</tr>
<tr>
<td><strong>Total Master Mix</strong></td>
<td>21μL</td>
</tr>
</tbody>
</table>

**SLP-2 Primers**
SLP-2 forward: 5’-GTG AC TCT CGA CAA TGT AAC-3’
SLP-2 reverse: 5’-TGA TCT CAT AAC GGA GGC AG-3’

**PGC-1α Primers**
PGC-1α forward: 5’-TCA GTC CTC ACT GGT GGA CA-3’
PGC-1α reverse: 5’-TGC TTC GTC GTC AAA AAC AG-3’

**S12 Primers**
S12 forward: 5’-GGA AGG CAT AGC TGC TGG-3’
S12 reverse: 5’-CCT CGA TGA CAT CCT TGG-3’
Appendix D  Representative fluorescence images demonstrating apoptotic nuclei as determined by Hoechst 33342 staining

Differentiated SN56 cells were stained with 1µM Hoechst 33342 fluorescent dye. Apoptotic nuclei are distinguished by characteristic condensed, pyknotic nuclei (arrows) and a brighter fluorescence emission. Arrow heads provide examples of normal nuclear morphology. Scale bar: 10µm.
Curriculum Vitae

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