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Regulation of Mitochondrial Fission and Fusion in Neuronal Injury

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Regulation of Mitochondrial Fission and Fusion in Neuronal injury

Arezu Jahani-Asl

This thesis is submitted as a partial fulfillment of the requirements for the degree of
Doctorate of Philosophy in Neurosciences

Department of Neurosciences

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ABSTRACT

Mitochondria are dynamic organelles meaning that they constantly fuse with each other, divide and move. The creation of such dynamic nature of mitochondria seems to be very purposeful. For example mitochondrial fusion is identified as a repair mechanism to dilate damaged mitochondria while mitochondrial fission results in the mitochondrial biogenesis and generates smaller mass mitochondria that can move faster to strategic locations within cellular compartments. In mammals, Mitofusin 2 (Mfn2) an outer mitochondrial membranes (OMM) protein and Optic atrophy 1 (Opa1), found in the inter mitochondrial space in association with the inner mitochondrial membrane (IMM), regulate OMM and IMM fusion, respectively. In non neuronal cells, the key components of mitochondrial dynamics have been linked to the regulation of cell death induced by apoptotic signaling. Neurons possess unique morphological complexities and undergo cell death by distinct mechanisms which are far more complicated than just apoptosis. A fundamental question that has arisen from the previous studies is whether mitochondrial fission and fusion machineries impact neuronal survival and function. The first goal of my PhD thesis has been to tackle the important questions of whether mitochondrial morphology defects are associated with neuronal demise and whether components of mitochondrial fusion can rescue neuronal loss in physiologically relevant models. The results of my studies have culminated in a number of key findings. First, mitochondrial morphological defects have been identified as early events following different modes of neuronal injury such as DNA damage (induced by camptothecin), oxidative stress (induced by H₂O₂) and calcium toxicity (induced by overactivation of glutamate receptors). While mitochondrial fission contributes to the dramatic mitochondrial

fragmentation following neuronal death, a defective mitochondrial fusion and loss of IMM integrity are identified as two of the major mechanisms contributing to the mitochondrial dysfunction and neuronal demise. Second, the fusion proteins Mfn2 and Opa1 are shown to confer neuroprotection in response to multiple cell death stimuli. Mfn2 was identified as an antiapoptotic protein that functions upstream of cytochrome c release to attenuate neuronal loss, whereas Opa1 functions at the IMM level to maintain mitochondrial cristae morphology. Third, mitochondrial remodeling as a result of loss of Opa1 oligomers is identified as hallmarks of excitotoxic neuronal injury. Opa1 is essential for neuroprotection by inhibition of calpain (a calcium activated protease) as calpastatin, an endogenous inhibitor of calpains, fails to protect against excitotoxicity following Opa1 knockdown. Our findings are the first to identify Opa1 as a key regulator of neuronal fate following calcium deregulation associated with excitotoxic neuronal injury. The second goal of my PhD thesis has been to study the regulation of mitochondrial fission in post mitotic neurons. In mammals, Dynamin related protein 1 (Drp1), a cytosolic protein, is recruited to the mitochondrial OMM to induce mitochondrial fission. Here, a novel mechanism has been identified for the regulation of Drp1 recruitment and mitochondrial fission. A physical and functional interaction has been documented between Drp1 and cyclin dependent kinase 5 (Cdk5), an important regulator of neuronal plasticity and neuronal loss. Cdk5 phosphorylates Drp1 at a conserved serine residue (Ser585) and results in Drp1 recruitment from the cytoplasm to the mitochondria to induce its fission. These findings suggest a regulatory mechanism through which Cdk5 performs its multiple functions during neuronal development and disease through modulation of mitochondrial shape. In conclusion, this research identifies

a missing link between mitochondrial fission and neuronal development and disease through a Drp1-Cdk5 cross talk. These findings have broad implications for reassessment of fundamental concepts in neurobiology such as synaptic plasticity and strength, axonal growth, neuronal demise and injury associated mitochondrial dysfunction from a different angle. At the same time this research offers the components of the *mitochondrial fusion* machinery as promising targets in rescuing neuronal loss associated with a wide range of neurological disorder.

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DEDICATION

I dedicate this thesis to the memories of two people I've never stopped loving:

My late brother, Manuchehr Jahani-Asl, who left us in 1991 at the age of 21. In one of his letters to me when I was just a little girl, he wrote “I am a strong believer that you will get that PhD degree”.

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LIST OF MANUSCRIPTS

- I. **Jahani-Asl A**, Cheung EC, Neuspiel M, MacLaurin JG, Fortin A, Park DS, McBride HM, Slack RS (2007) Mitofusin 2 protects cerebellar granule neurons against injury-induced cell death. *J Biol Chem.* 282(33):23788-98.
- II. **Jahani-Asl A**, Xu William, Pilon Larose K, Park DS, McBride HM, Slack RS (2009). Regulation of mitochondrial dynamics during excitotoxic cell death (Submitted, *J. Cell Biol. Submission number: JCB_2052491*)
- III. **Jahani-Asl A**, Rashidian J, Irrcher I, Ishihara N, Park DS, Slack RS. **Cdk5 Regulates Dynamin related protein 1 (Drp1) and Mitochondrial Fission in Postmitotic Neurons.** (In Preparation)

APPENDED ARTICLES

- IV. Arbour N, Vanderluit JL, Le Grand JN, **Jahani-Asl A**, Ruzhynsky VA, Cheung EC, Kelly MA, MacKenzie AE, Park DS, Opferman JT, Slack R.S. (2008) Mcl-1 is a key regulator of apoptosis during CNS development and after DNA damage. *J Neurosci.* 28(24):6068-78.
- V. **Jahani-Asl A.** and Slack RS (2007) The phosphorylation state of Drp1 determines cell fate. *EMBO Rep*, 8(10):912-3.

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LIST OF ABBREVIATIONS

A- β	amyloid β
AD	Alzheimer's disease
Ad	Adenovirus
ADOA	Autosomal Dominant Optic Atrophy
Ala	Alanine
AIF	Apoptosis Inducing Factor
AMPA	α -amino-3-hydroxy-5methyl-4-isoxazolepropionic acid
ANOVA	Analysis of Variance
ANT	Adenine Nucleotide Translocase
Apaf1	Apoptotic peptidase activating factor 1
APP	Amyloid precursor protein
BSA	Bovine Serum Albumin
Caf4	Caffeine Resistant 4
CAG	Codon that encodes for the amino acid glutamine
CamKII α	Calcium/Calmodulin dependent protein Kinase I α
CBUL	Cullin-F-Box Ubiquitin Ligase
Ca ⁺⁺	Calcium ion
Calp	Calpastatin
CGN	Cerebellar Granule Neurons
CJ	Cristae Junctions
CM	Cristae Membrane
CMT II	Charcot Marie Tooth neuropathy type II

Cdk1	Cyclin dependent kinase 1
Cdk5	Cyclin dependent kinase 5
CycD	Cyclophilin D
Cyt C	Cytochrome C
DAP3	Death Associated Protein 3
DIV	Days In Vitro
DN	Dominant negative
Drp1	Dynamin related protein 1
EDTA	Ethylenediamine tetra-acetic acid
ER	Endoplasmic Reticulum
Fis1	Fission 1
FZO1	Fuzzy Onion 1
GDAP1	Ganglioside-induced Differentiation Factor 1
GED	GTPase Effector Domain
FADD	Fas-Associated protein with Death Domain
HD	Huntington's disease
h-Fis1	Human Fis1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H ₂ O ₂	Hydrogen peroxide
htt	Huntington
IBM	Inner Boundary Membrane
IMS	Intermembrane Space
IMM	Inner Mitochondrial Membrane

IS	Intracristal Space
K ⁺	Potassium ion
kDa	Kilo Dalton
LacZ	β-galactosidase
LETM1	Leucine zipper EF hand containing transmembrane protein 1
L-Opa1	Long (mature) Opa1
Mdv1p	Mitochondrial Division 1
MEF	Mouse Embryonic Fibroblasts
Mfb1	Mitochondrial F box protein 1
Mfns	Mitofusins
Mfn1	Mitofusin1
Mfn2	Mitofusin2
Mfn2 _{RasG12V}	Constitutively activated mutant of Mfn2
Mfn2 ^{-/-}	Mfn2 knock out
MgCl ₂	Magnesium Chloride
Mgm1	Mitochondrial genome maintenance 1
MIB	Mitofusin Binding Protein
MitoPLD	Mitochondrial Phospholipase D
MOMP	Mitochondrial Outer Membrane Permeability
MPP	Mitochondrial protein Peptidase
MTS	Mitochondrial Targeting Sequence
mPT	Mitochondrial Permeability Transition
MTP18	Mitochondrial protein, 18 kDa

MW	Molecular Weight
n	Number of independent experiment
NES	Nuclear Export Signal
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
n-NOS	neuronal-Nitric Oxide Synthase
N	Asparagine residue
NR1	NMDA receptor subunit 1
NR2	NMDA receptor subunit 2
OMM	Outer Mitochondrial Membrane
O ₂ ⁻	Superoxide anion
ONOO ⁻	Peroxy nitrite
Opa1	Optic Atrophy 1
P	Probability
PBS	Phosphate Buffered Saline
PFA	paraformaldehyde
PARL	Presenilin-associated rhomboid-like
PCP1	Protease of Cytochrome Peroxidase 1
PD	Parkinson's Disease
P-Drp1	Phosphorylated Drp1
PGT	Polyglutamine tract
PHD	Plckstrin Homology Domain
PKA	Protein Kinase A

PRD	Proline Rich Domain
PSD95	Post Synaptic Density protein 95
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
RT	Room Temperature
S	Cleavage sites within Opa1
S/Ser	Serine residue
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the mean
SER	Serine residue
S-Opa1	Soluble (short) Opa1
Sh-Opa1	Short hairpin Opa1
Slp2	Somatin like protein 2
SNEP5	Sentrin/SUMO-specific Protease 5
SUMO	Small Ubiquitin like Modifier
T	Threonine residue
TD	Transmembrane Domain
TOM20	Translocase of outer mitochondrial membrane 20
TPR	Tetratricopeptide Repeat
VDAC	Voltage-Dependent Anion Channel
WH	Wolf-Hirschhorn
XO	Xanthine Oxidase

THESIS FORMAT

As per guidelines from the Faculty of Graduate and Postdoctorate studies, this thesis is presented as a collection of manuscripts. Each manuscript has its own introduction and discussion sections. A general introduction section precedes the manuscripts and provides a review of current literature as relevant to the field of study. The thesis ends with an overall discussion that discusses the findings of the presented manuscripts and how these findings fit into recent advances in the field.

Chapter 1 is a literature update on three main areas: a) the key molecules identified to regulate mitochondrial fusion and fission, b) overview of the literature that suggests a link between mitochondrial dynamics and apoptotic signaling and c) a review on the mechanisms that govern neuronal death. The questions that have arisen from the previous studies are integrated throughout this introduction. This chapter ends with the hypothesis and objectives of this thesis.

Chapter 2 presents a paper entitled “Mitofusin 2 protects cerebellar granule neurons against injury-induced cell death”. This article was published in 2007 in the *J Biol Chem.*; 282(33):23788-98. The importance of this study was to identify Mfn2 as a novel antiapoptotic protein and a signaling GTPase which confers neuroprotection beyond its role in mitochondrial fusion.

Chapter 3 present a manuscript entitled “Regulation of mitochondrial dynamics during excitotoxic cell death”. This paper is submitted to *J cell Biol.* This work

investigated mitochondrial morphology defects and the role of key fusion proteins in response to calcium deregulation as it relates to a physiologically relevant model: Excitotoxicity. This work has established a very exciting link between Opa1, a mitochondrial fusion GTPase and calcium signaling in post mitotic neurons.

Chapter 4 is a manuscript entitled “Cdk5 Regulates Dynamin related protein 1 (Drp1) and Mitochondrial Fission in Postmitotic Neurons” This work which is in preparation, identifies an important mechanism through which Cdk5 may confer its multiple function by regulating Drp1 phosphorylation state and inducing mitochondrial fission.

Chapter 5 summarizes the findings of Chapter 2, 3, and 4 and discusses the overall significance of these results. It also puts these results into perspective and compares it with findings from other laboratories. Not only this chapter highlights the significance of this thesis and its contribution to the advancement of the field, it also puts forward the questions and concerns that are to be addressed in the future.

APPENDED ARTICLES

In an attempt to gain a broader understanding of mitochondrial dynamics and neuronal survival, I have had the pleasure of performing collaborative research with my colleagues at Drs. Slack and Park’s laboratories. Most of these articles are not yet published (Cheung et al, submitted to Mol. Cell; Irrcher et al, Submitted to PNAS).

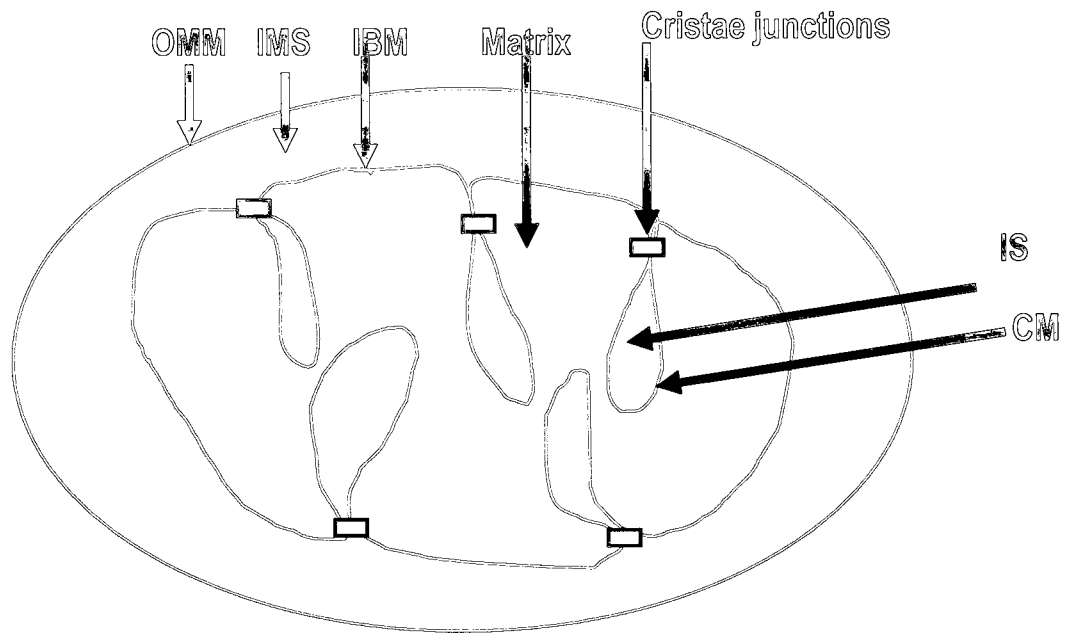
However, I have included two published articles (Arbour et al 2008, and Jahani-Asl et al 2007) which I have coauthored in appendix D.

CHAPTER 1
GENERAL INTRODUCTION

1. INTRODUCTION

Mitochondria are double membrane organelles that perform a wide array of important functions in the cells ranging from ATP production to the regulation of cell death and calcium homeostasis. The outer mitochondrial membrane (OMM) surrounds the inner mitochondrial membrane (IMM). The IMM has a large surface area and is highly folded into tubular shaped extrusions called cristae [reviewed in (Zick et al., 2009)]. The formation of cristae defines two compartments for the IMM: the inner boundary membrane (IBM) which is immediately adjacent to the OMM and the cristae membrane (CM) which represents invaginations of the IBM protruding into the matrix space (**Figure 1.1**). The IMM plays a critical role in the regulation of mitochondrial functions. First, the components of the respiratory chain complex are inserted in the IMM and as such the folding of the IMM into cristae may be essential in providing an efficient system for ATP production. Second, each crista houses important solutes that are critical to the regulation of the respiratory chain functioning and cell death depending on whether they are confined to or released from the cristae. It is therefore important that mechanisms have evolved in order to regulate cristae morphology and content. Each crista is connected to the IBM through structures called crista junctions (CJ). Maintaining a tight CJ promotes survival while their widening leads to loss of cristae content and cell death. The electron microscopy studies have revealed that the IMM can take on a variety of shapes to generate a mosaic ultrastructure and to better accommodate the organelle's metabolic needs at different respiratory states [reviewed in (Mannella, 2006)].

A.



B.

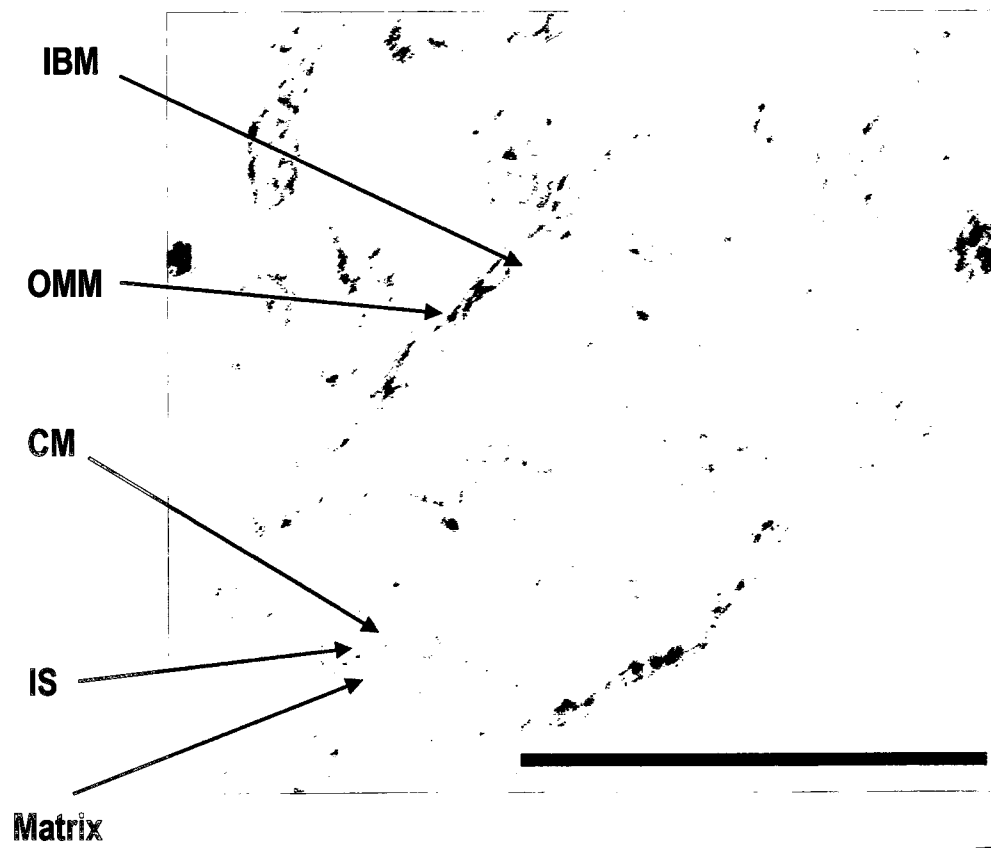


Figure 1.1

Figure 1.1. Morphological features of mitochondria

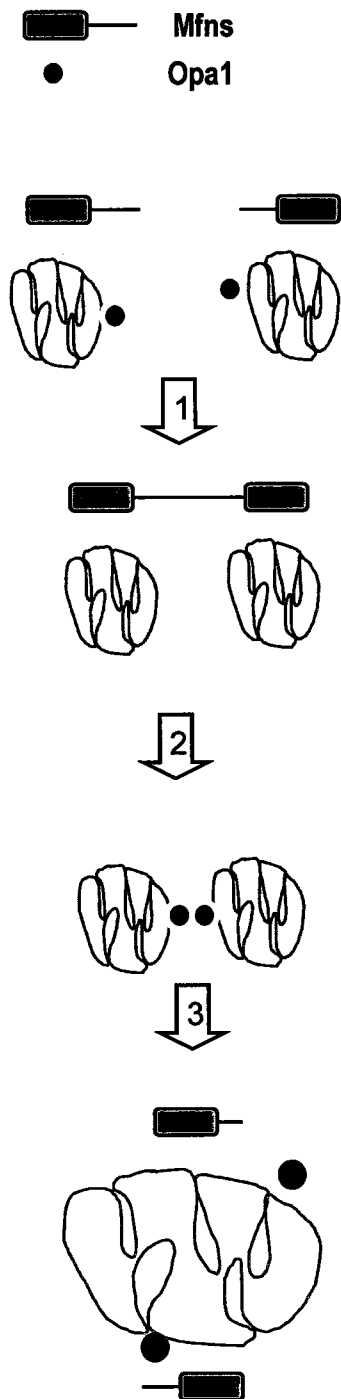
Mitochondria are double membrane organelles. (A) The outer mitochondrial membrane (**OMM**) surrounds the inner mitochondrial membrane (**IMM**). The IMM is highly folded into tubular shaped structures called cristae. The folding of IMM generates two membrane compartments: The inner boundary membrane (**IBM**) and the cristae membrane (**CM**). Each crista is connected to IBM through cristae junctions. Other abbreviations: **IS**, Intracristal Space; **IMS**, Intermembrane Space. (B). Electron microscopy image of mitochondria depicting IBM, OMM, CM, IS and matrix of mitochondria in 7 days in vitro (DIV) cerebellar granule neurons (CGN).

Mag Bar, 500 nm

A novel field in the study of mitochondrial shape has emerged, that of mitochondria dynamics. With the help of advanced imaging techniques and genetic screens we now know that mitochondria are not bean-shaped static entities within the cell, instead they are very motile organelles that continuously divide and fuse [reviewed in (Soubannier and McBride, 2009)]. Mitochondrial fusion is the process of combining two or more mitochondria into a single elongated mitochondrion. Mitochondrial fission refers to scission of a mitochondrion into two or more pieces (**Figure 1.2**). These two events generate many shapes of mitochondria and seem to play an important role in the regulation of vital cellular functions. For example, mitochondrial fusion allows rapid mixing of mitochondrial membranes and contents in order to buffer localized substrate deficits or damaged mitochondrial DNA and ensure the homogeneity of the population (Chen and Chan, 2005; Chen et al., 2005). Mitochondria undergo fission during cell division to reproduce themselves for transfer into daughter cells (Yaffe, 1999; Merz et al., 2007).

Mitochondrial fusion and fission are essential for transportation of the mitochondria to the areas of cell with high metabolic demands (Bereiter-Hahn and Voth, 1994). This is particularly important in specialized cells such as neurons. First, neurons have multiple neurites (axon and dendrites) processes that extend away from the cell body (soma). These processes have distinct energy requiring functions. Second, the firing of a neuron is critically regulated by ionic flow. Maintaining ionic homeostasis is essential in areas enriched with active channels such as the nodes of Ranvier and synapses. Given the multitasking property of the mitochondria, its positioning

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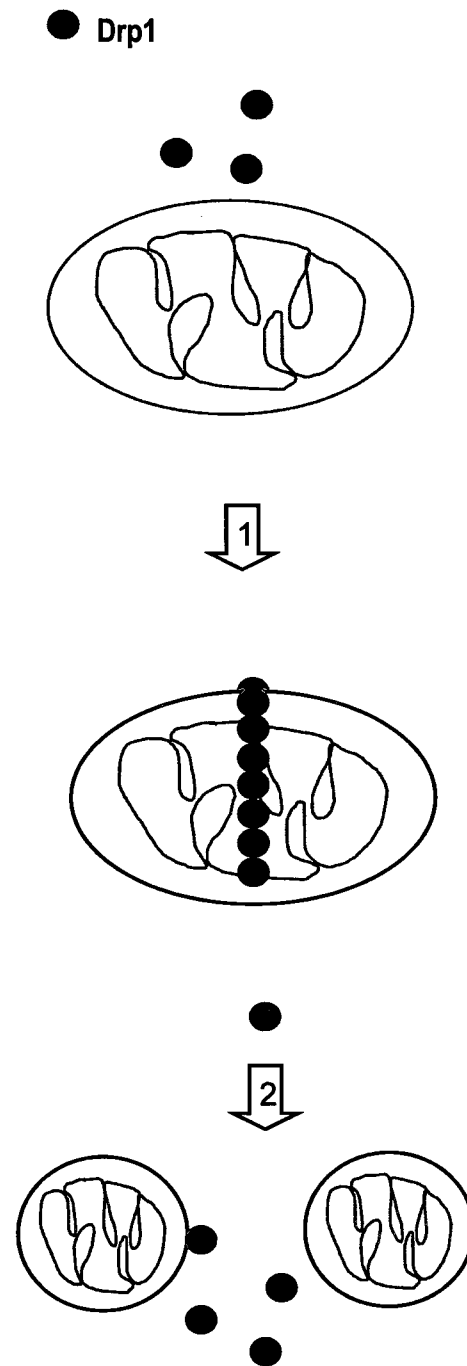


Figure 1.2

Figure 1.2. Mitochondria are dynamic organelles that continuously divide and fuse

(A) Mitochondrial fusion refers to adjoining of two or more mitochondria into a single elongated mitochondrion. Mitofusins (Mfn: Mfn1 and Mfn2) are located on the outer mitochondrial membranes (OMM) and regulate fusion of the OMM. Mfns interact on apposing mitochondria to mediate their tethering (Arrow 1). This results in OMM fusion (Arrow 2). Opa1, an intermembrane space GTPase which is found in association with the inner mitochondrial membrane (IMM), mediates fusion of IMM (Arrow 3). Opa1 is (B). Mitochondrial fission refers to scission of a single mitochondrion into two or more pieces. The key regulator of mitochondrial fission is dynamin related protein 1 (Drp1). Drp1 is a cytosolic GTPase which is recruited to the mitochondria at the site of scission (Arrow 1). Drp1 forms a ring like structure on the mitochondria surface. The energy from the GTP hydrolysis drives constriction of the membranes (Arrow 2).

within different compartments of neurons may play a crucial role in providing local ATP and buffering calcium transients (Hollenbeck and Saxton, 2005; Verstreken et al., 2005). This may explain why mitochondrion is one of the major organelles found in axons, dendrites, dendritic spines, and soma.

In addition to their role in ATP synthesis and ionic regulation, mitochondria are the key conductors of cell death signaling. Multiple cell death pathways converge on the mitochondria and induce the release of pro-death factors that is maintained in the mitochondrial cristae and IMS [e.g. Apoptosis inducing factor (AIF) and cytochrome c (cyt c)]. Following their release, AIF and cytochrome c activate different mechanisms to succumb neurons to death. For example cytochrome c forms a complex with Apaf1 and procaspase 9 at the cytoplasm to initiate activation of caspases, while AIF is translocated to the nucleus to induce large scale DNA damage.

In response to the environmental and molecular cues that trigger cell death, a switch in the mitochondrial morphology is well documented (Bossy-Wetzel et al., 2003; Youle and Karbowski, 2005; Cheng et al., 2006; Suen et al., 2008). These morphology defects are linked to an imbalance in the mitochondrial fusion and fission machineries. Identification of the molecular interfaces between mitochondrial shape and function are just beginning to emerge. Although mitochondrial fragmentation is shown as an upstream early event in the progression of apoptosis, future study is required to substantiate the temporal and spatial relationship of the key regulators of mitochondrial fusion and fission in these signaling pathways.

The aim of this chapter is to present a literature update on:

- Regulation of mitochondrial fusion with special focus on key proteins that have been conserved throughout evolution: **Mitofusin2 (Mfn2, Fzo1 in yeast), and Optical atrophy 1 (Opa1, Mgm1 in yeast)**. A list of proteins that directly or indirectly regulate mitochondrial fusion is included in Table 1.
- Regulation of mitochondrial fission with special focus on the key fission protein **Dynamin related protein 1 (Drp1, Dnm1 in yeast)**. Other regulators of mitochondrial fission in mammals and yeast are summarized in Table 2.
- The evidence suggesting an important role for the mitochondrial fusion and fission in the regulation of cell death. This includes a summary of the data obtained from the study of the fission and fusion proteins in cell lines and lower eukaryotes.
- The underlying molecular mechanism of cell death following acute neuronal injury. This section covers two major pathways of **intrinsic (mitochondrial) apoptotic program, and excitotoxic signaling**.

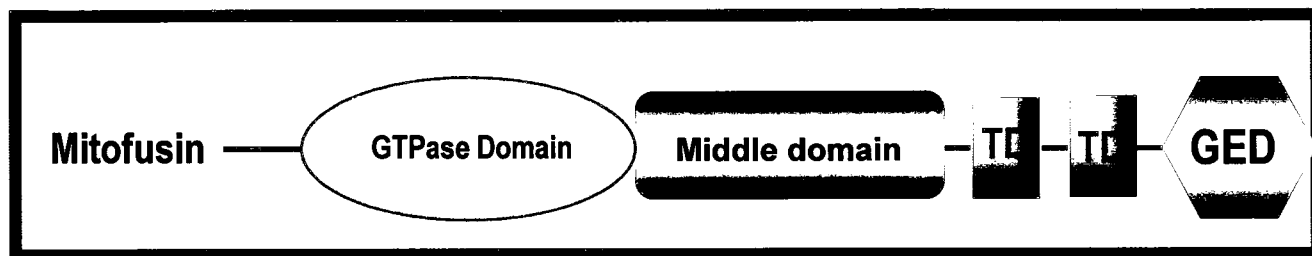
This literature review leads to the objectives of this PhD thesis with its main theme being how the key fusion and fission proteins impact neuronal survival and function.

1.1 Molecular Machineries that Regulate Mitochondrial Fusion

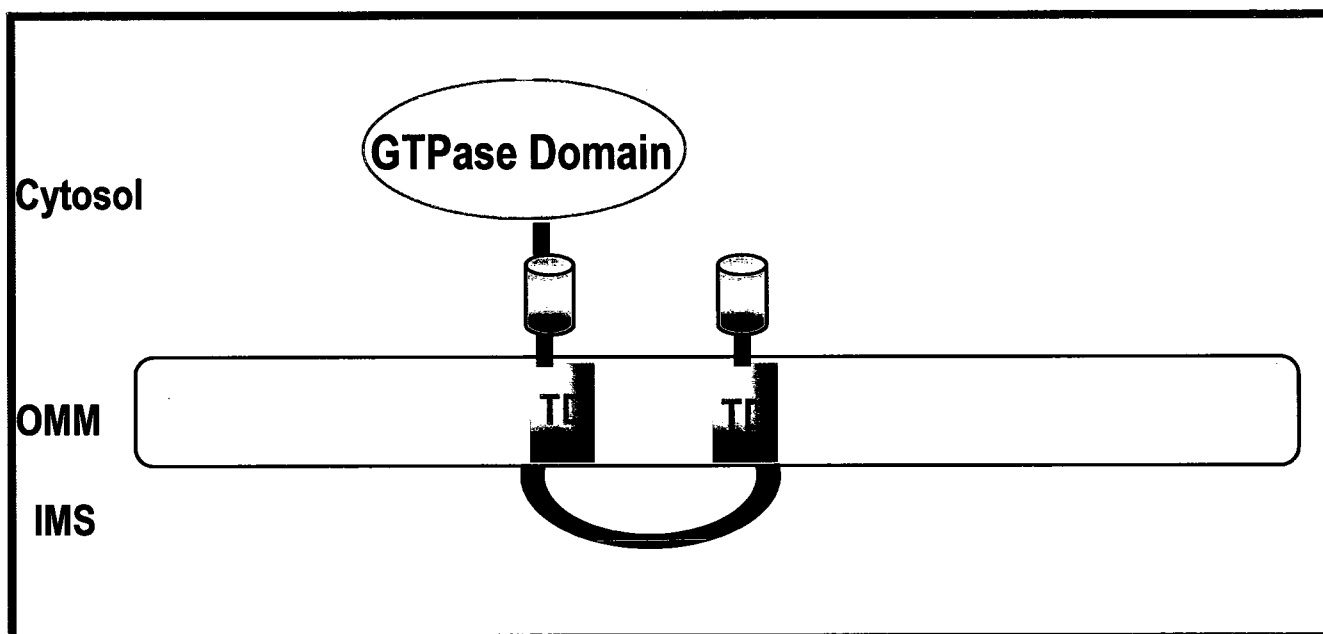
The machineries that regulate OMM and IMM fusion are distinct (Meeusen et al., 2004; Meeusen et al., 2006). In mammals, **mitofusins (Mfns)** are the key components of OMM fusion and **Optic atrophy 1 (Opa1)** is the main regulator of IMM fusion.

Mfns belong to the family of large GTPases and exist as two homologues: **Mitofusin 1(Mfn1)** and **Mitofusin 2 (Mfn2)**. Mfn1 and Mfn2 have structural similarities. The amino (N) terminal of both molecules contains a conserved GTPase domain and their carboxy (C) terminal region consists of a coiled coil structure. The N terminal is separated from the C terminal by a U- shaped transmembrane region that is inserted in the OMM (**Figure 1.3**) (Rojo et al., 2002). Mfn1 and Mfn2 have been found to form homo-oligomeric and hetero-oligomeric complexes in trans (Chen et al., 2003; Ishihara et al., 2004; Koshiba et al., 2004). These interactions are proposed to initiate the tethering between the two apposing mitochondria (**Figure 1.2**) and initiate their fusion (Koshiba et al., 2004). Although Mfn1 and Mfn2 have overlapping functions (Chen et al., 2003), they have distinct GTPase activities. Mfn1 has higher affinity for nucleotide binding and a faster rate of GTP hydrolysis relative to Mfn2 (Ishihara et al., 2004; Neuspiel et al., 2005). In addition, it is mainly involved in the docking of two juxtaposed mitochondria (Koshiba et al., 2004) and has more efficacy towards mitochondrial tethering. Recent studies have shown that a hydrolysis deficient mutant of Mfn2 resulted in enhanced mitochondrial fusion, indicating that the GTP hydrolysis by Mfn2 is not

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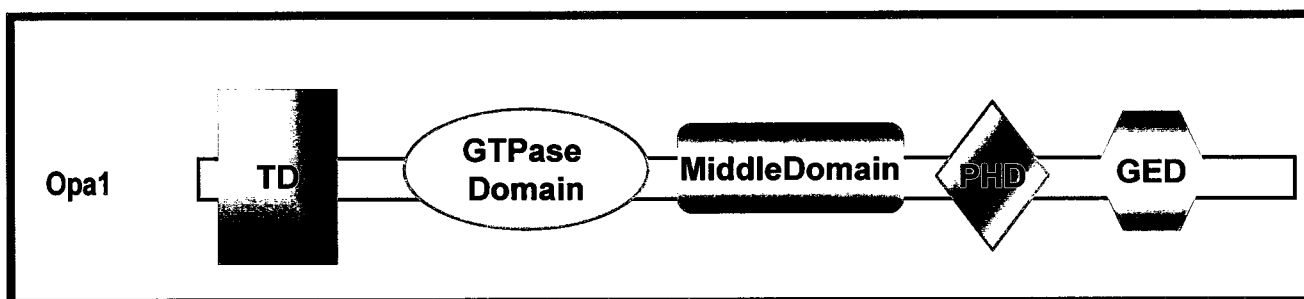


Figure 1.3

Figure 1.3. Structure of mitochondrial fusion proteins: Mfns and Opa1

(A) Schematic structure of Mfns is illustrated. Similar to dynamin family of GTPases, Mfns possess a GTPase domain, a middle domain, a transmembrane domain (**TD**), and a GTPase effector domain (**GED**). (B) Mitofusins are inserted in the OMM. The C terminal of Mitofusins contains a coiled coil region while its N terminal has a conserved GTPase domain. Both the C and N terminus are facing the cytoplasm to regulate mitochondrial fusion. These two domains are separated by a U- shaped transmembrane region inserted in the OMM. The TD spans the outer mitochondrial membranes. (C). Schematic structure of Opa1. Opa1 shares structural similarity to mitofusins in that it possesses a GTPase domain, a middle domain, GED and TD. In addition Opa1 contains a pleckstrin homology domain (PHD).

required for fusion (Neuspiel et al., 2005). These studies suggest that while Mfn1 may possess a dynamin like function, Mfn2 acts as a regulatory GTPase.

Our knowledge of the molecular events that regulate the activity of Mfns is limited. Studies performed on **Fzo1 (Fuzzy Onion 1)**, the only yeast homologue of Mitofusins, suggests a significant role for post translational modification. Fzo1 steady state concentration within a set point is critical for the fusion reaction to proceed. **Mdm30**, a member of Cullin-F-Box ubiquitin ligase family (CBUL) has been identified to play a role in the regulation of mitochondrial fusion (Fritz et al., 2003). The members of CBUL family are generally known for their function in turnover and degradation. By binding to Fzo1, Mdm30 targets Fzo1 to the 26S proteasome and controls its turnover (Fritz et al., 2003; Escobar-Henriques et al., 2006; Cohen et al., 2008). The absence of the F box protein Mdm30p leads to a fragmented mitochondria morphology. **Mfb1**, another mitochondrial associated F Box protein was identified to regulate mitochondrial morphology in yeast (Durr et al., 2006; Kondo-Okamoto et al., 2006). Mfb1 deficient cells exhibit a fragmented mitochondrial phenotype compared to their wild type counterparts. The presence of Mdm30 and Mfb1 in the mitochondria indicates an important role for **ubiquitination** in the regulation of Fzo1 and mitochondrial fusion. Whether mitofusins are also regulated by ubiquitination in mammals requires further study.

The fusion of the IMM requires **Optic atrophy 1 (Opa1)**, a GTPase found in the IMS in association with the IMM. Similar to Mfns, Opa1 protein contains a conserved

GTPase domain, a middle domain, and a GED (GTPase effector domain) or assembly domain (**Figure 1.3**) (Delettre et al., 2003). In addition Opa1 has a pleckstrin homology (PH) domain. This suggests that Opa1 may possibly play a role in the recruitment of proteins to the mitochondrial membranes or targeting some components of the mitochondrial signaling pathways to the appropriate sub-mitochondrial compartments. Upon import in the mitochondrial membrane through its N terminal, Opa1 is processed in the matrix by a mitochondrial processing peptidase (MPP)[reviewed in (Escobar-Henriques and Langer, 2006)]. MPP cleaves the mitochondrial targeting sequence (MTS) of Opa1 to generate mature Opa1 isoform L-Opa1 (**Figure 1.4**). Both mature Opa1 and its yeast homologue **Mgm1 (mitochondrial genome maintenance)**, undergo further proteolytic cleavage to form short/soluble isoforms (S-Opa1/S-Mgm1). Proteolysis of Opa1/Mgm1 is accomplished by a number of proteases including AAA and Rhomboid family. The inner membrane rhomboid protease **Pcp1 (protease of cytochrome c peroxidase 1)** cleaves L-Mgm1 to generate s-Mgm1 (McQuibban et al., 2003). **Presenilin-associated rhomboid-like (PARL)**, a human pcp1 homologue is also able to process yeast Mgm1 and human Opa1 (Cipolat et al., 2006; Gottlieb, 2006; Jeyaraju et al., 2006). Our knowledge of proteolysis of Opa1 is fast expanding. In addition to PARL, the inner membrane AAA proteases **Yme1** and the matrix AAA proteases such as **Afg3l2** are important in Opa1 processing (Duvezin-Caubet et al., 2007; Griparic et al., 2007; Song et al., 2007).

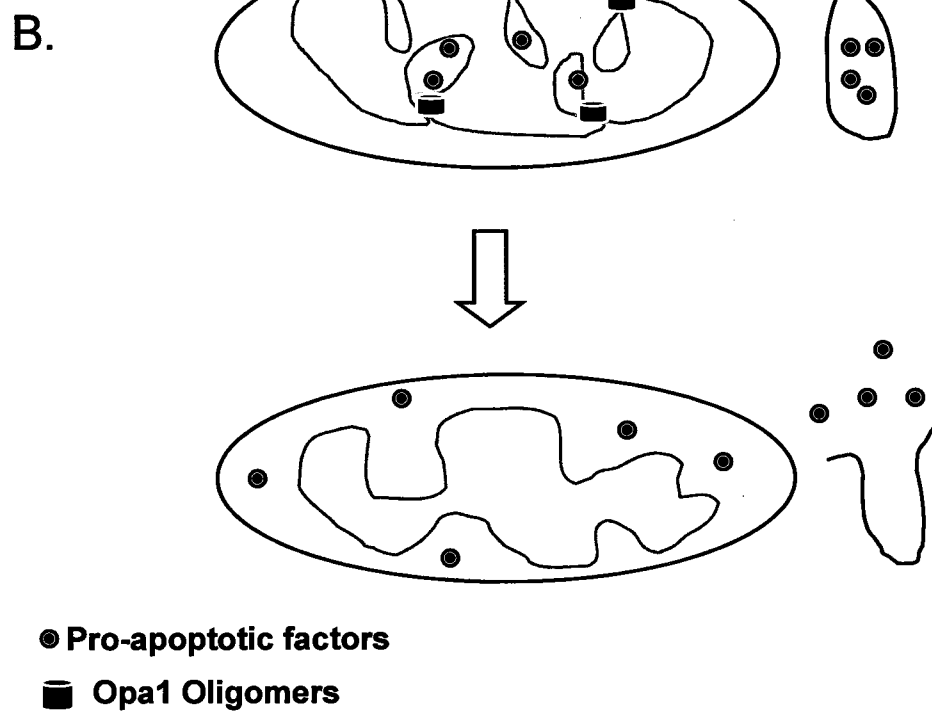
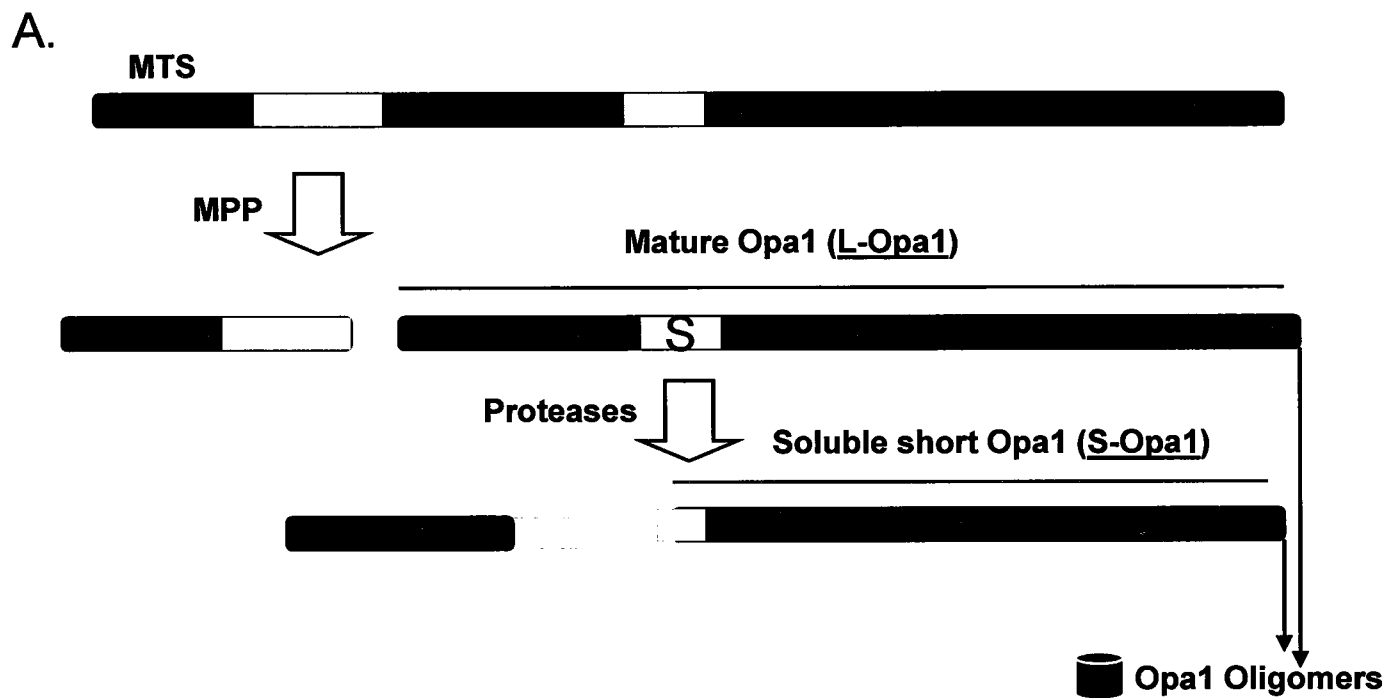


Figure 1.4

Figure 1.4. Opa1 processing generates long and short forms of Opa1

(A) Opa1 has a mitochondrial targeting sequence (**MTS**) that targets it to the mitochondria upon its synthesis. Once in the mitochondria, the MTS is cleaved by mitochondrial processing peptidase (**MPP**) in the matrix to generate the long or mature form of Opa1 (**L-Opa1**). In addition, Opa1 contains cleavage site/s (**S**) that is/are targeted by multiple proteases (e.g. Parl, Yme1) to generate a soluble or short form of Opa1 (**S-Opa1**). Short and long of Opa1 come together and form Opa1 oligomers. (B) Cristae junctions are regulated by Opa1 oligomers. The oligomers regulate the content of cristae such that their loss leads to the widening of cristae junctions, remodeling of cristae and release of the cristae solutes.

Studies from independent laboratories have demonstrated that both L-Opa1 and S-Opa1 are required for IMM fusion (Griparic et al., 2007; Song et al., 2007). In support of this, mitochondrial morphology defects are observed by the absence of the mitochondrial protease Yme1 or by defects in PARL mediated processing (Campbell et al., 1994; Jeyaraju et al., 2006) indicating that the tight regulation of Opa1 proteolysis is critical in maintaining mitochondrial integrity. An interesting question that remains to be addressed is whether proteolysis of Opa1 regulates the rate of its turnover. The distinction between proteolysis-mediated activation of Opa1 (generation of S-Opa1) and proteolysis-mediated degradation (into shorter dysfunctional fragments) remains to be established.

In addition to its role in IMM fusion, Opa1 is shown to be an essential factor in the regulation of cristae remodeling (Herlan et al., 2003; Frezza et al., 2006). In Opa1 knock down cells, mitochondria exhibit disorganized cristae (Olichon et al., 2003; Frezza et al., 2006). Opa1 seems to control cristae junctions and cristae morphology independent of its effect on IMM fusion [Reviewed in (Pellegrini and Scorrano, 2007)]. Importantly, similar to IMM fusion, maintenance of cristae junctions requires both of L- and S- Opa1 isoforms (Cipolat et al., 2006; Frezza et al., 2006). L- and S-Opa1 come together to form oligomers that are shown to be required for keeping the cristae junctions in check (**Figure 1.4**). The loosening of cristae junctions in the absence of the Opa1 oligomers results in the release of pro-death factors required to initiate cell death.

1.1.1. Coordination of IMM and OMM Fusion

Given the double membrane structure of the mitochondrion, fusion of two mitochondria into one is a complex process involving four adjacent membranes. It is not known how the orderly fusion of the OMM followed by IMM is coordinated in mammals. Although Opa1 has been shown to directly interact with Mfn1 to induce mitochondrial fusion, our knowledge of the mechanisms that coordinate a fusion event comes mostly from the studies performed in the yeast model. Fzo1 (the yeast homologue of Mfns) interacts with Mgm1 (the yeast homologue of Opa1) through a mitochondrial outer membrane protein termed **Ugo1**. The N terminal of Ugo1 faces the cytosol and its C terminal is in the intermembrane space (Sesaki et al., 2003; Wong et al., 2003). The cytoplasmic domain of Ugo1 directly interacts with Fzo1, whereas its intermembrane space domain binds to Mgm1 (Wong et al., 2003). A homologue of **Ugo1** in higher eukaryotes has not been identified and the interaction between Mfn2 and Opa1 remains elusive, however, **Slp2 (somatin like protein 2)**, an inner mitochondrial membrane protein facing the inner membrane space, interacts with Mfn2 (Hajek et al., 2007). It is tempting to speculate that Slp2 may potentially direct the signaling of the OMM fusion to that of IMM.

Two more interesting candidates that may be involved in mitochondrial fusion in mammals are **LETM1 (Leucine zipper-EF hand containing transmembrane protein 1)** and **MIB (Mfn-binding protein)**. LETM1 was identified to be required for maintenance of mitochondrial morphology (Dimmer et al., 2008). LETM1 is an IMM protein with two calcium binding EF hand motif (Endele et al., 1999) and its mutations

Fusion Factor	Species	Location	Potential function
Mitofusins (Fzo1)	Mammals (Yeast)	OMM	OMM fusion
Opa1 (Mgm1)	Mammals (Yeast)	IMM	IMM fusion, cristae remodeling
PARL (Pcp1)	Mammals (Yeast)	IMM	Opa1 cleavage
Afg3l2	Yeast	Matrix	Mgm1 cleavage
Ugo1	Yeast	OMM	OMM-IMM Fusion coordination
Mdm30	Yeast	Cytoplasm and OMM	Fzo1 turnover
Yme1	Mammals	IMS	Opa1 cleavage
Slp2	Mammals	IMM	Fusion coordination
LETM1	Mammals	IMM	IMM fusion
MIB	Mammals	IMM	Mfn2 interacting factor
MitoPLD	Mammals	OMM	Fusion

Table 1.1

Table 1.1. Molecules that regulate mitochondrial fusion

Mitochondrial fusion is mainly regulated by two key GTPases: Mitofusins (Mfn1 and Mfn2) and Optic Atrophy 1 (Opa1) in mammals. These proteins are evolutionary conserved from yeast to mammals. Fzo1 is the only yeast homologue of Mitofusins and Mgm1 is the yeast homologue of Opa1. Mitofusins/Fzo1 regulates OMM fusion. Opa1/Mgm1 regulates IMM fusion. Mitochondrial fusion also entails involvement of other proteins. This table includes the list of proteins that play a key role in these events either by mediating direct mitochondrial fusion or through regulating the components of the fusion machinery. Abbreviations: OMM, Outer mitochondrial membrane; IMM, Inner mitochondrial membrane; IMS, Intermembrane space.

leads to Wolf-Hirschhorn (WH) disease. WH patients exhibit epileptic seizures and growth retardation (Endele et al., 1999; Bergemann et al., 2005). Loss of LETM1 leads to a fragmented mitochondrial phenotype suggesting a role for LETM1 in mitochondrial fusion (Dimmer et al., 2008). **MIB** has been identified to interact with both Mfn1 and Mfn2 in HeLa cells (Eura et al., 2006). MIB is a cytosolic protein belonging to the family of dehydrogenase/reductase superfamily. Overexpression of MIB inhibits Mfn1 activity and induces mitochondrial fragmentation (Eura et al., 2006). In contrast, MIB induced mitochondrial fragmentation is prevented by coexpression of Mfn1, suggesting a functional interaction between MIB and Mfn1/2 proteins.

Finally, the lipid composition of the membranes and their contribution to the overall membrane architecture has been suggested to play a role in the regulation of mitochondrial fusion. **MitoPLD (mitochondrial phospholipase D)**, an enzyme inserted in the outer mitochondrial membrane facing cytoplasm, was shown to be required for mitochondrial fusion (Choi et al., 2006). This enzyme assists in hydrolysis of cardiolipin and formation of phosphatidic acid (PA). Mitochondrial fusion was blocked in the PA knocked down cells (Choi et al., 2006). This indicates that the membrane fluidity may play a significant role in mitochondrial fusion perhaps by modifying the membrane curvature and bringing the adjacent mitochondria in close apposition to each other.

Although the list of proteins involved in the regulation of mitochondrial fusion is rapidly increasing, *the focus of this thesis is on the key fusion proteins Mfn2 and Opa1. These proteins that are functionally conserved throughout the evolution play significant*

roles in the nervous system and mutations in these genes are proven to lead to severe neuropathies.

1.1.2 Physiological Importance of Mfn2 and Opa1 in the Nervous System

Point mutations in Mfn2 are associated with Charcot Marie Tooth Neuropathy type II (CMT II), a peripheral nerve neuropathy which represents primary disorders in motor neurons (Zuchner et al., 2004b). CMT patients exhibit progressive muscle weakness resulting in muscular atrophy. 15 different mutations in Mfn2 has been identified in 21 families, most of which are located on the GTPase domain (Zuchner et al., 2004a; Kijima et al., 2005). The mechanism by which these mutations lead to CMT IIA remains unclear, however, expression of neuropathy-associated forms of Mfn2 in cultured dorsal root ganglion neurons has been shown to induce mitochondrial fragmentation, abnormal mitochondrial clustering and distorted mitochondrial trafficking (Baloh et al., 2007). In addition, mitofusins are shown to be required during development (Chen et al., 2003). Conditional knock out studies has allowed an in depth study of mitofusins in the adult tissues and data from these studies indicate that the nervous system is severely affected by loss of Mfn2. Mfn2 is required for spine formation, dendrite growth and purkinje cell survival (Chen et al., 2007).

Like Mfn2, Opa1 plays a profound role in the nervous system. Northern Blot analysis of human RNA showed that the brain had the highest Opa1 transcript level after retina (Bette et al., 2005). In human, mutations in the Opa1 gene leads to autosomal

dominant optic atrophy (ADOA)(Kjer et al., 1996). ADOA is the most common form of inherited optic neuropathy (Kjer et al., 1996) and is characterized by a progressive degeneration of optic nerve, moderate to severe loss of visual acuity, and abnormalities of color vision. (Alexander et al., 2000; Delettre et al., 2000; Delettre et al., 2001; Delettre et al., 2002; Votruba, 2004). Many mutations have been reported in Opa1- coding sequence within the GTPase or GED domains of the protein. Mutations within GTPase domain may lead to mitochondrial fusion defects due to the impairment of the GTPase activity. Mutations identified within the GED domain of Opa1 are suggested to result in Opa1 inability to oligomerize which eventually disturbs the dynamin function. Finally, overexpression of the pathogenic alleles of Opa1 in Hela cells resulted in mitochondrial fragmentation and increased sensitivity to apoptotic stimuli (Olichon et al., 2007), consistent with the results obtained on the mitochondrial morphology defects in skin fibroblasts of ADOA patients (Olichon et al., 2007).

Together these studies suggest that defects in Mfn2 and Opa1 result in severe neuropathies in part through deregulation of the mitochondrial shape and function and that mitochondrial defects lead to increased sensitivity to cell death and loss of spines and dendrites in the expanse of the nervous system.

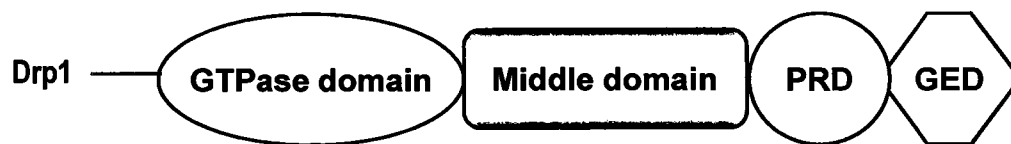
1.2 Molecular Machineries that Regulate Mitochondrial Fission

Dynamin related protein 1 (Drp1, Dnm1 in yeast) is the key regulator of mitochondrial fission. Drp1 is a cytosolic GTPase with an N terminal GTPase domain

and a C-terminal GTPase effector domain (GED) (**Figure 1.5**). Both, the GED and GTPase domains of Drp1 are reported to be required for mitochondrial fission (Zhu et al., 2004). In order for the fission event to proceed, Drp1 needs to be recruited from the cytosol to the mitochondria at the site of scission (**Figure 1.2**) (Yoon et al., 2001; Zhu et al., 2004). Although oligomerization of Drp1 on the mitochondrial surface is thought to provide a mechanical force to constrict the membranes (Ingelman et al., 2005), it remains unclear as to whether Drp1 functions as a mechanoenzyme or a regulatory GTPase.

In the yeast model, the recruitment of Dnm1 requires a second protein **Fis1** (**Fission 1**) (Mozdy et al., 2000). Fis1 is a transmembrane protein anchored to the outer mitochondrial membrane. Fis1 contains a tetratricopeptide repeat (TPR) domain which are packed spirals of anti-parallel α helices (**Figure 1.5**) (Suzuki et al., 2003; Dohm et al., 2004). TPR domains are involved in protein-protein interactions (D'Andrea and Regan, 2003) and as such Fis1 may play a significant role in the assembly of the mitochondrial fission complex. Two additional proteins, **Mdv1p** (**Mitochondrial division 1**) and **Caf4** (**Caffeine-resistant 4**) are shown to be required for mitochondrial fission by forming a complex with Dnm1 and Fis1 (Tieu and Nunnari, 2000; Griffin et al., 2005; Schauss et al., 2006; Motley et al., 2008). The human homologue of Fis1 is identified and named hFis1 (James et al., 2003). Although loss and gain of function studies indicate that hFis1 is an important fission factor (Stojanovski et al., 2004; Koch et al., 2005), it is not essential in Drp1 recruitment (Lee et al., 2004). It is therefore possible that hFis1 may serve as a scaffold for the formation of the mitochondrial fission complex through its TPR domains. To date, no homologues of Mdv1 and Caf4 are found in higher eukaryotes.

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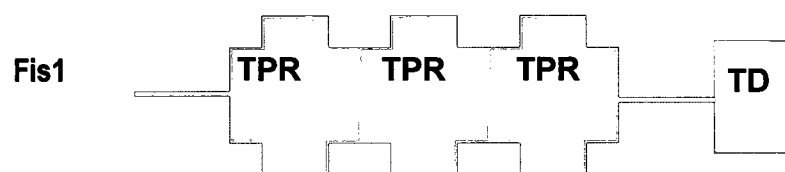


Figure 1.5

Figure 1.5. Schematic structure of the fission proteins Drp1 and Fis1

(A) Schematic structure of Drp1. Drp1 is a cytosolic protein that is recruited to the mitochondria to induce its fission. Similar to GTPases involved in the mitochondrial fusion, Drp1 contains a GTPase domain, a middle domain, and a GTPase effector domain (GED). The energy derived from the GTP hydrolysis is thought to drive mitochondrial constriction. In addition, Drp1 possesses a Proline Rich Domain (PRD). The PRD may play a role in Drp1 subcellular localization and enzymatic function. (B) Schematic structure of Fis1. Fis1 is a mitochondrial transmembrane protein that is inserted in the outer mitochondrial membrane (OMM). Fis1 is mainly composed of Tetratricopeptide Repeat (TPR) domains and Transmembrane Domain (TD).

It is thought that the fission of OMM is followed by the fission of IMM. **Mdm33**, a mitochondrial inner membrane protein regulates IMM fission in *Saccharomyces cerevisiae* (Messerschmitt et al., 2003). Similar to Dnm1, Mdm33 forms high order structures on the IMM and its loss leads to highly fused mitochondria (Messerschmitt et al., 2003). Overexpression of Mdm33 leads to aggregation of mitochondria, generation of aberrant inner membrane structures, loss of inner membrane cristae and inner membrane fragmentation. This indicates that Mdm33 plays a distinct role in the mitochondrial inner membrane to control mitochondrial morphology.

A number of novel proteins are identified to regulate mitochondrial fission in mammals. These include **MTP18 (mitochondrial Protein, 18 kDa)**, **Endophilin B1**, **GDAP1 (Ganglioside-induced differentiation protein 1)**, and **DAP3 (Death associated protein 3)** (Karbowski et al., 2004a; Mukamel and Kimchi, 2004; Tondera et al., 2004; Niemann et al., 2005). MTP18 is an inner membrane space protein anchored to IMM (Tondera et al., 2004; Tondera et al., 2005). Overexpression of MTP18 induces mitochondrial fission, while its downregulation results in fused mitochondria (Tondera 2005). In addition, Fis1 is not able to induce mitochondrial fragmentation in the absence of MTP18 indicating that MTP18 is required as part of the fission complex machinery. DAP3 and Endophilin B1 play a significant role in *cell death-induced- mitochondrial fission* and will be discussed in 1.3.

Fission regulator	Species	Location	Function
Drp1 (Dnm1)	Mammals (Yeast)	Cytoplasm	OMM fission
hFis1	Mammals	OMM	Fission complex assembly
Fis1	Yeast	OMM	Drp1 recruitment
Mdv1 and Caf4	Yeast	Cytoplasm	Fission complex assembly
Mdm33	Yeast	IMM	IMM fission
Bax	Mammals	Cytoplasm	Fission complex
MTP18	Mammals	IMS	Fission complex assembly
Endophilin B1	Mammals	Cytoplasm	Death induced fission
GDAP1	Mammals	OMM	Fission
DAP3	Mammals	Matrix	Death induced fission
SUMO	Mammals	Cytoplasm	Drp1 Sumoylation & recruitment
March5/Mitol	Mammals	Mitochondria	Fission
Cdk1, CamK1α	Mammals	Cytoplasm	Drp1 phosphorylation and Fission
PKA	Mammals	Cytoplasm	Drp1 phosphorylation and blocked fission

Table 1.2

Table 1.2. Molecules that regulate mitochondrial fission

Dynamin related protein 1 (Drp1, Dnm1 in yeast) is the key GTPase that regulates mitochondrial fission. Fis1 (yeast) mediates Drp1 recruitment while hFis1 (human) may be involved in the formation of the fission complex. Other proteins that are recently identified to regulate mitochondrial fission are summarized in this table. Of these, cyclin dependent kinase 1 (Cdk1), protein kinase K (PKA), Calcium/Calmodulin kinase I alpha (CamKI α) as well as SUMO are involved in Drp1's post translational modifications. Abbreviations: OMM, Outer mitochondrial membrane; IMS, Intermembrane space; IMM, Inner mitochondrial membrane.

1.2.1 Physiological Significance of Drp1 Activity in the Nervous System

Since the local need for ATP and calcium is especially great at the synapses, mitochondria are frequently found in the axonal terminals (Shepherd and Harris, 1998). In contrast to the abundance of mitochondria in the presynaptic terminals, mitochondria are rarely found in the post synaptic compartment (i.e. dendritic spines) of most excitatory synapses (Chicurel and Harris, 1992). An elegant study by Morgan Sheng's group has shed light on the significance of dendritic mitochondria for synapse development and activity (Li et al., 2004). Drp1-mediated distribution of mitochondria into the dendrites was shown to be essential and limiting for the support of synapses. While expression of a dominant negative Drp1 (Drp1-K38A) reduced the content of dendritic mitochondria and resulted in loss of synapses and dendritic spines, overexpression of Drp1 in hippocampal neurons resulted in the redistribution of the mitochondria into the dendritic protrusions in response to synaptic excitation, and increased synaptogenesis and spine formation (Li et al., 2004). Drp1 also affected synapse number. Primary neuronal cultures transfected with Drp1 or DnDrp1 resulted in an increase or decrease in the density of PSD-95 (a marker for excitatory synapses), respectively. These studies suggest that Drp1 is critical for the distribution of dendritic mitochondria and the regulation of excitatory synapses perhaps through facilitating mitochondrial fission.

In a follow up study, *Drosophila* Drp1 mutants were analyzed for the mitochondrial density at the synapses. These mutants exhibited synapses devoid of

mitochondria and had increased resting calcium levels (Verstreken et al., 2005). While basal synaptic transmission was normal in Drp1 mutant flies, these mutants failed to maintain neurotransmission during intense stimulation. In addition, in the *Drosophila* mutant, Milton, defective synaptic transmission was associated with the loss of mitochondria from axon terminals (Stowers et al., 2002), supporting the functional importance of presynaptically targeted mitochondria. In conclusion, Drp1-mediated regulation of mitochondrial density at pre and post synaptic compartment is essential for synapse strength and development. Identification of neuronal intrinsic mechanisms that regulates Drp1 activity and mitochondrial distribution will assist us in the better understanding of the mechanisms that control synaptic plasticity.

Despite the significance of Drp1 in the regulation of the steady state mitochondrial fission in the nervous system, downregulation of Drp1 has been shown to confer neuroprotection in response to NO-mediated neurotoxicity (discussed in detail in section 1.3). Identification of the mechanisms that regulate Drp1 activity under different physiological conditions will therefore shed light over the mechanism of action of this protein in the nervous system.

1.2.2. Regulation of Drp1 by post translational modification

Drp1 cycles between cytoplasm and mitochondria to regulate steady state mitochondrial fission. While our knowledge of the mechanisms that regulate the GTPase activity of Drp1 is limited, a number of recent studies have broadened our perspectives

on the regulation of Drp1 cycling between the cytoplasm and the mitochondria. These studies which together underscore the importance of post translational modifications of Drp1, define the underlying mechanisms for Drp1 recruitment and assembly at the mitochondrial scission site. First, Drp1 is shown to be sumoylated by **small ubiquitin like modifier (SUMO)** protein (Harder et al., 2004). Sumoylation is a form of protein modification that either affects the subcellular localization of the substrates or protects them from ubiquitin mediated degradation. Upon its sumoylation, Drp1 becomes stably associated with the mitochondrial membranes (Wasiak et al., 2007). SUMO-1 and its conjugating enzyme **Ubc9** stabilize Drp1 and drive mitochondrial fission (Harder et al., 2004). Conversely, **SNEP5 (the sentrin/SUMO-specific protease)** affects mitochondrial morphology through desumoylation (Zunino et al., 2007). SNEP5 overexpression rescues the SUMO1-induced mitochondrial fragmentation in cultured cells (Zunino et al., 2007). In addition to SUMO1, the mitochondrial **E3 ubiquitin ligase MARCH5/MITOL** was identified as a critical regulator of Drp1 recruitment. MARCH5 associates with Drp1 to promote mitochondrial fission (Karbowski et al., 2007) indicating that ubiquitination is involved in this process.

Importantly, multiple kinases have been recently identified to phosphorylate Drp1 at multiple sites and impact its recruitment (Chang and Blackstone, 2007b; Cribbs and Strack, 2007; Taguchi et al., 2007; Cereghetti et al., 2008; Han et al., 2008). The physiological outcome of the Drp1-mediated phosphorylation, however, seems to be more complex than that of Drp1 sumoylation/ubiquitination. First, there are multiple phosphorylation sites identified within Drp1 molecule. Second, these sites are

differentially targeted by multiple phosphatases/kinases. Third, depending on the physiological state of the cell (e.g. differentiating versus non- differentiating; steady state versus injury state), the impact of Drp1 phosphorylation can have opposing physiological consequences. For example, a conserved serine residue, Ser 585 (in rat, corresponding to Ser 616 in human) is shown to be phosphorylated by **cyclin dependent kinase 1 (Cdk1)** during mitosis (Taguchi et al., 2007). This phosphorylation results in enhanced fission which is perhaps required for mitochondrial biogenesis and transfer into daughter cells. Located in the proximity of Ser616 within the C terminal domain of Drp1, a second conserved residue Ser 637 is targeted by **Ca²⁺/calmodulin-dependent protein kinase Ia (CamK1 α)**, **protein kinase A (PKA)** and **calcineurin**. Similar to the effect of Cdk1-mediated Drp1 phosphorylation during mitosis, Drp1 phosphorylation at Ser637 by **CamK1 α** results in Drp1 translocation to the mitochondria and enhanced fission in post mitotic neurons. Surprising, phosphorylation of Ser 637 by PKA was reported to have an opposite physiological consequences and resulted in the elongation of mitochondrial length. The Ser637 is also targeted by the phosphatase **calcineurin**. Calcineurin mediated dephosphorylation of Drp1 was shown to induce its translocation from the cytoplasm to the mitochondria resulting in subsequent fission (Cribbs and Strack, 2007; Cereghetti et al., 2008). The underlying causes of these controversies remain to be studied, although the extent of phosphorylation under different physiological conditions and in different study models can provide a possible explanation. Finally, the mechanism that links Drp1 phosphorylation to mitochondrial fission at Ser 616 remains to be studied, however the PKA-mediated phosphorylation of Drp1 at Ser 637 is thought to inhibit the GTPase activity by decreasing the intermolecular interactions that direct GTP hydrolysis (Chang

and Blackstone, 2007a). Despite the controversies in the physiological outcome of Drp1 phosphorylation, these studies together support an important link between Drp1 phosphorylation status, mitochondrial fission and cell fate.

1.3 Mitochondrial Fission and Fusion in the Regulation of Cell Death

New evidence has suggested a novel requirement for the machinery that regulates mitochondrial fission in the progression of apoptosis in cell culture models and during development in *C. elegans* (Jagasia et al., 2005). The loss of DRP1 or expression of a dominant interfering mutant was shown to impede the apoptotic program by blocking mitochondrial fission. Furthermore, Drp1 and Bax, a proapoptotic member of Bcl2 family, has been found together on mitochondrial membrane at the site of scission (Karbowski et al., 2002). The proapoptotic Bax has been shown to regulate apoptosis-induced fission. Bax increases the rate of mitochondrial fission following apoptotic stimuli either directly (Frank et al 2001) or by inhibiting mitochondrial fusion (Karbowski et al 2004). However, the absolute requirement of Bax in this process is still unknown as mitochondrial fission occurred prior to Bax activation and translocation following Ca^{2+} efflux from the endoplasmic reticulum (Breckenridge et al 2003).

In addition, fission proteins Endophilin B1, DAP3, and Fis1 are shown to be involved in the regulation of mitochondrial morphology during cell death (Mukamel and Kimchi, 2004; Takahashi et al., 2007; Takahashi et al., 2009). For example Endophilin B1 is shown to be recruited to the mitochondria during autophagy. Endophilin B1

interacts with Bax and is associated with autophagy proteins such as Atg5, Atg9 and Beclin1. Loss of endophilin B1 has been shown to delay apoptosis (Takahashi et al., 2007; Takahashi et al., 2009). These findings suggest that Endophilin B1 mediated mitochondrial fission maybe involved in the regulation of autophagic cell death.

Dap3 which is a mitochondrial ribosome associated matrix protein is identified in the regulation of mitochondrial fragmentation under cellular stress (Mukamel and Kimchi, 2004). DAP3 leads to mitochondrial fission and loss of mitochondrial membrane potential. While in the absence of apoptotic stimuli mitochondria exhibited normal morphology in DAP3 Knock out cells, attenuated mitochondrial fission and decreased sensitivity to cell death was shown in response to staurosporine (Mukamel and Kimchi, 2004). This suggests that DAP3 is specifically involved in contributing to mitochondrial fragmented phenotype in apoptosis. Finally, depletion of Fis1 by RNAi resulted in fused and interconnected mitochondria and protection from various apoptotic stimuli in non-neuronal cells (Lee et al 2004), while overexpression of Fis1 induced mitochondrial fragmentation, release of cytochrome c and apoptosis (James et al 2003). Together, these findings represent another link between mitochondrial fission and apoptosis.

Consistent with the requirement for mitochondrial fission in the progression of apoptosis, it has been demonstrated that the rate of mitochondrial fusion is reduced as the apoptotic program progresses (Karbowski et al., 2004b). Loss of mitochondrial fusion proteins, Mfn1, Mfn2 and Opa1, has been linked to cellular death (Sugioka et al., 2004; Arnoult et al., 2005). Loss of mitofusins leads to mitochondrial fragmentation and

increased sensitivity to cell death stimuli (Sugioka et al., 2004). In contrast, overexpression of Mfn2 inhibits Bax activation and protects against permeability transition in cultured cell lines (Neuspiel et al., 2005). Similarly, loss of Opa1 induces mitochondrial fragmentation and cell death (Olichon et al., 2003; Lee et al., 2004). Expression of Opa1 in the mouse embryonic fibroblasts has been shown to protect against different modes of cell death including those induced by H₂O₂, etoposide, and staurosporine (Frezza et al., 2006). It was also demonstrated that Opa1 protects from apoptosis by attenuating cytochrome c release (Olichon et al., 2003; Frezza et al., 2006).

In conclusion, studies of the key fission and fusion proteins in cell lines exposed to multiple apoptotic stimuli have established an important role for the key fission and fusion proteins in the regulation of cell death. Whether components of mitochondrial fission and fusion machinery play a role following injury models relevant to stroke and trauma requires further investigation. Unlike cell lines, neurons have very high morphological complexities in which it is difficult to assess the consequences of improper mitochondrial targeting. Second, the underlying mechanisms of neuronal death are more complex and extend far beyond the classical apoptotic program. Therefore assessment of the mitochondrial fission and fusion following multiple mechanisms of neuronal injury such as excitotoxicity and oxidative stress requires special attention.

1.4 Molecular Mechanisms that Regulate Neuronal Death Following Acute Injury

One of the leading causes of neuronal death following acute neuronal injury is **excitotoxicity**. Excitotoxic cell death occurs as a result of overactivation of glutamate receptors accompanied by excessive influx of sodium and calcium ions. The disturbance in the ionic homeostasis is followed by an influx of water by osmosis that can lead to rupture and death of the cells by necrosis. At the same time, the increased calcium levels leads to the activation of two major events: 1) *activation of calcium dependent signalling pathways* and 2) *generation of reactive oxygen species (ROS)* (Zemke et al 2004). Generation of ROS can lead to an increase in the mitochondrial outer membrane permeability (MOMP) and activate the **classical apoptotic program** depending on the availability of ATP. In addition free radicals can cause further *damage to DNA* and other macromolecules. As a result, neurons undergoing excitotoxic injury exhibit a partial apoptotic and necrotic phenotype.

Apoptosis plays a major role in the disintegration of neurons following acute neuronal injury. Morphologically, cells undergoing apoptosis demonstrate nuclear/cytoplasmic condensation and membrane protrusions. These initial changes are followed by fragmentation of the nuclear contents and subsequent encapsulation of these fragments into apoptotic bodies (Kerr et al., 1972). Biochemically, apoptotic cells are characterized by: 1) Reduction in the mitochondrial transmembrane potential; 2) Intracellular acidification; 3) Production of reactive oxygen species; 4) Externalization of phosphatidylserine residues in membrane bilayers; 5) Degradation of DNA into high-molecular weight and oligonucleosomal fragments and 6) Selective degradation of structural components of the cytoskeleton and nucleus, as well as numerous proteins

involved in survival signaling pathways (Kayalar et al., 1996; Ku et al., 1997; Mashima et al., 1997). Loss of these essential components involves a family of serine-threonine proteases called caspases (Wyllie et al., 1980; Lazebnik et al., 1994; Alnemri et al., 1996; Datta et al., 1997).

Two major pathways of classical apoptosis are death receptor (extrinsic) and **mitochondrial (intrinsic) programs** (Salvesen and Dixit, 1997; Green, 1998; Song and Steller, 1999). Both of these pathways ultimately result in the activation of caspases (**Figure 1.6**). In addition, a third apoptotic pathway involving the Apoptosis-Inducing Factor (AIF) exists and appears to occur *independently of caspases*. This pathway of neuronal death is triggered by loss of AIF from the mitochondria and its translocation to the nucleus to execute large scale DNA damage. *The intrinsic apoptotic pathway and excitotoxicity are tightly regulated at the mitochondria to execute disintegration of the neurons. These two pathways will be therefore discussed in detail in the following sections.*

1.4.1 Intrinsic (Mitochondrial) Apoptotic Program

The proapoptotic members of Bcl2 family proteins, Bax and Bak, play pivotal role in the mitochondrial apoptotic program (Danial and Korsmeyer, 2004). Following neuronal injury such as stroke and trauma, one of the mechanisms that results in mitochondrial Bax/Bak activation is DNA damage (Cregan et al., 2004). For example, DNA damage is induced by the generation of reactive oxygen species (ROS) under hypoxic conditions. DNA damage in turn activates transcription factors such as p53 that

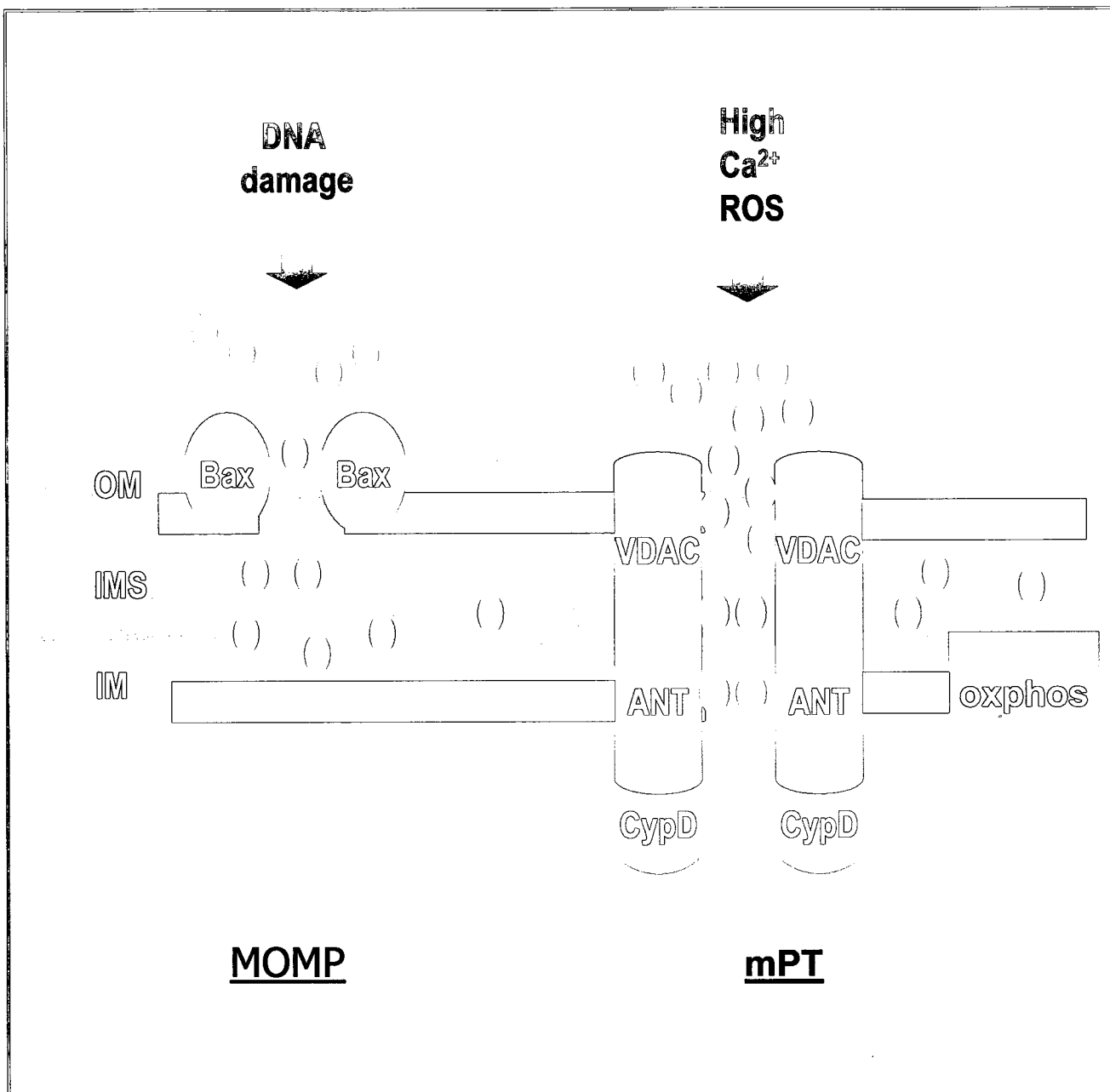


Figure 1.7

Figure 1.6. Two Major Apoptotic Pathways

Death receptor (extrinsic) and mitochondrial (intrinsic) pathways are two major apoptotic pathways, both of which result in a proteolytic cascade involving caspases. In both pathways the ultimate goal is cell death via activation of caspase-3 and caspase-3-mediated cleavage and activation of a number of pro-apoptotic proteins. Although the extrinsic pathway is beyond the scope of this thesis, this diagram is included to provide an overview when considering major apoptotic signaling. Briefly, intrinsic pathway signals by direct recruitment and activation of caspase-8. Once activated, caspase-8 activates caspase-3, which in turns leads to full implementation of the apoptotic program and the degradation phase of apoptosis. In the mitochondrial (drug-induced/intrinsic) apoptotic pathway which is one of the major pathways studied in this thesis, signals transmitted to the mitochondria trigger the release of cytochrome c which associates with the scaffolding protein, Apaf-1 to activate procaspase 9. Activated caspase-9 results in the proteolytic cleavage and activation of downstream caspases including caspase-3.

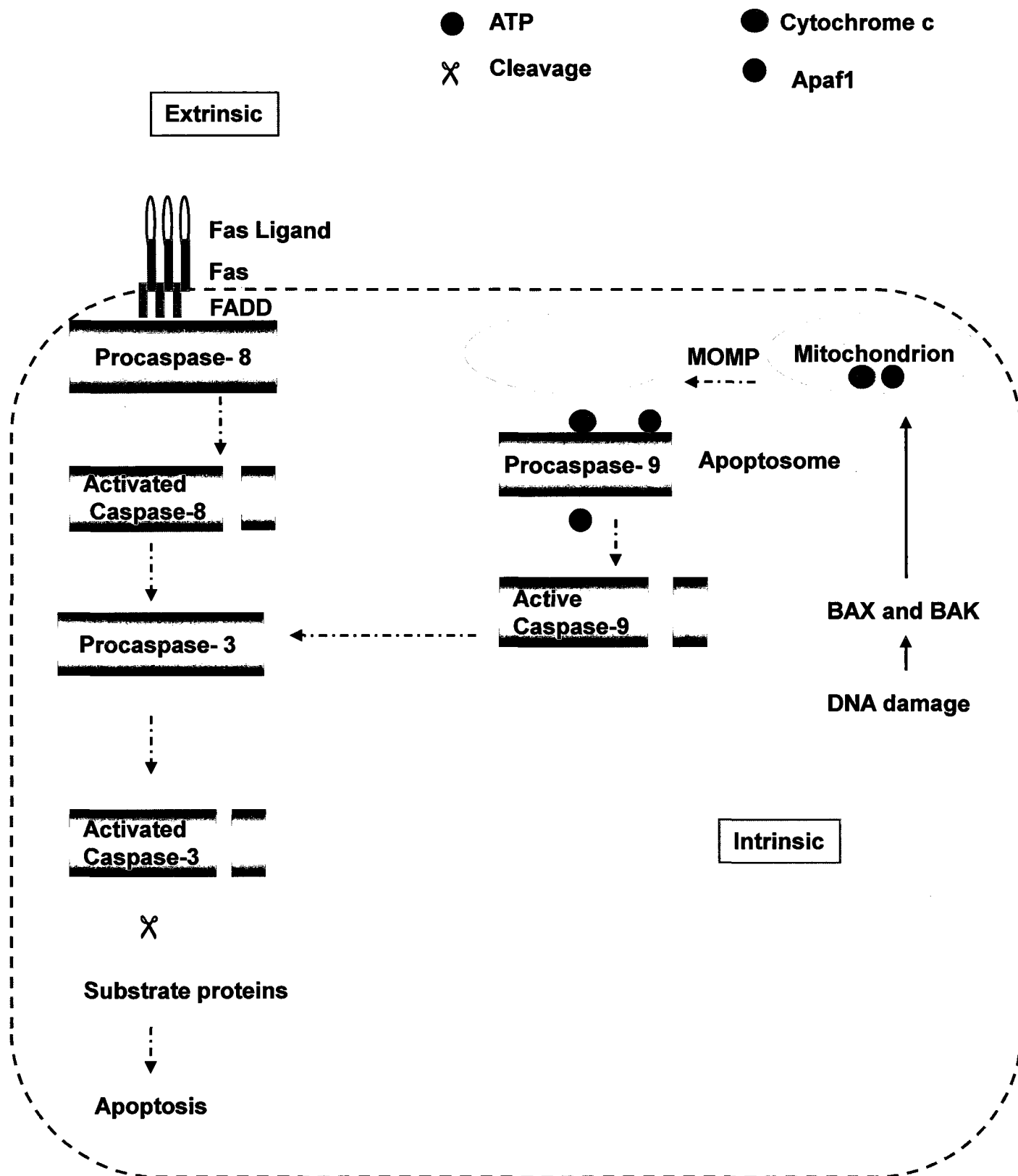


Figure 1.6

Figure 1.7. Mitochondrial outer membrane permeability (MOMP) and mitochondrial permeability transition result in the release of pro-death factors.

Activation of the multi domain Bcl2 proapoptotic proteins Bax and Bak contributes to the formation of distinct channels on the OMM. These channels facilitates the release of proapoptotic factors such as cytochrome c and AIF that is captured in the inter membrane space (IMS). This is referred to as mitochondrial outer membrane permeability (MOMP). Following acute injury associated with high calcium or generation of reactive oxygen species (ROS), channels are formed throughout the mitochondrial membranes. These channels require the assembly of a complex that includes the voltage-dependent anion channel (VDAC), mitochondrial Adenine nucleotide translocase (ANT) and mitochondrial matrix protein cyclophilin D (CycD). This event which is defined by the loss of mitochondrial membrane potential is referred to as mitochondrial permeability transition and profoundly facilitates the release of the pro-death factors that are captured in the cristae in addition to those confined to the IMS.

signals through Bcl2 family proteins [reviewed in (Cheung et al., 2007)]. Activation of the multi domain Bcl2 proapoptotic proteins Bax and Bak contributes to formation of channels on the OMM (MOMP) that facilitates the release of proapoptotic factors (cytochrome c, AIF, etc) captured in the inner membrane space (**Figure 1.7**). Upon its release, AIF translocates to the nucleus while cytochrome c associates with the scaffolding protein, Apaf-1 in the cytoplasm. Apaf-1 is a large, multi-domain protein which binds cytochrome c (Zou et al., 1997) and in the presence of dATP, forms a large oligomeric complex of about 700 kDa termed the apoptosome. Apoptosome recruits and activates procaspase-9 (Li et al., 1997; Zou et al., 1997). Upon activation, caspase-9 results in the proteolytic cleavage and activation of caspase-3 which in turns activates the caspase dependent degradation phase of apoptosis.

In summary, the intrinsic apoptotic pathway is a major mechanism by which neurons die following acute brain injury as blocking these pathways by expression of antiapoptotic Bcl2 family proteins Bcl-2 and Bcl-x_L have been shown to protect neurons against cerebral ischemia (Ouyang and Giffard, 2004).

1.4.2. Excitotoxic Pathway

Glutamate is the key excitatory neurotransmitter in the central nervous system of mammals and is required for a wide range of brain activities including regulation of synaptic strength and trophic developmental processes. This excitatory amino acid induces excitatory responses by binding to two classes of receptors: Ionotropic and

Metabotropic receptors (Hollmann and Heinemann, 1994). Three classes of ionotropic receptors include N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA), and Kainate. Binding of glutamate to ionotropic receptors results in enhanced permeability to cations including calcium, sodium and potassium (Arundine and Tymianski, 2004). Binding of glutamate to metabotropic receptors results in mobilization of calcium ions from the internal stores. In response to acute neuronal injury such as stroke and trauma, excessive amount of glutamate is released into the synapse. The excess of this excitatory amino acid leads to overactivation of its corresponding receptors and results in neuronal death termed excitotoxicity (**Figure 1.8**).

Extensive investigations have identified ionotropic receptors as major players in mediating excitotoxicity (reviewed in (Arundine and Tymianski, 2003). NMDA receptors (NMDAR) are the most extensively studied subtype of glutamate receptors and are most intimately linked to the excitotoxic response associated with calcium influx. NMDAR is either a hetero-tetramer or pentamer composed of multiple subunits (NR1, NR2A-D, and/or NR3) (reviewed in (Forder and Tymianski, 2009). On the cytoplasmic face, these receptors form large multiprotein complexes through interaction with numerous intracellular synaptic and cytoskeletal proteins (Sheng and Pak, 2000). One major family of protein interacting with NMDAR protein complex is postsynaptic density protein PSD95. By binding to the NR2 subunit of the NMDAR on one hand and through interaction with the neuronal nitric oxide synthase (nNOS) on the other, PSD95 mediates

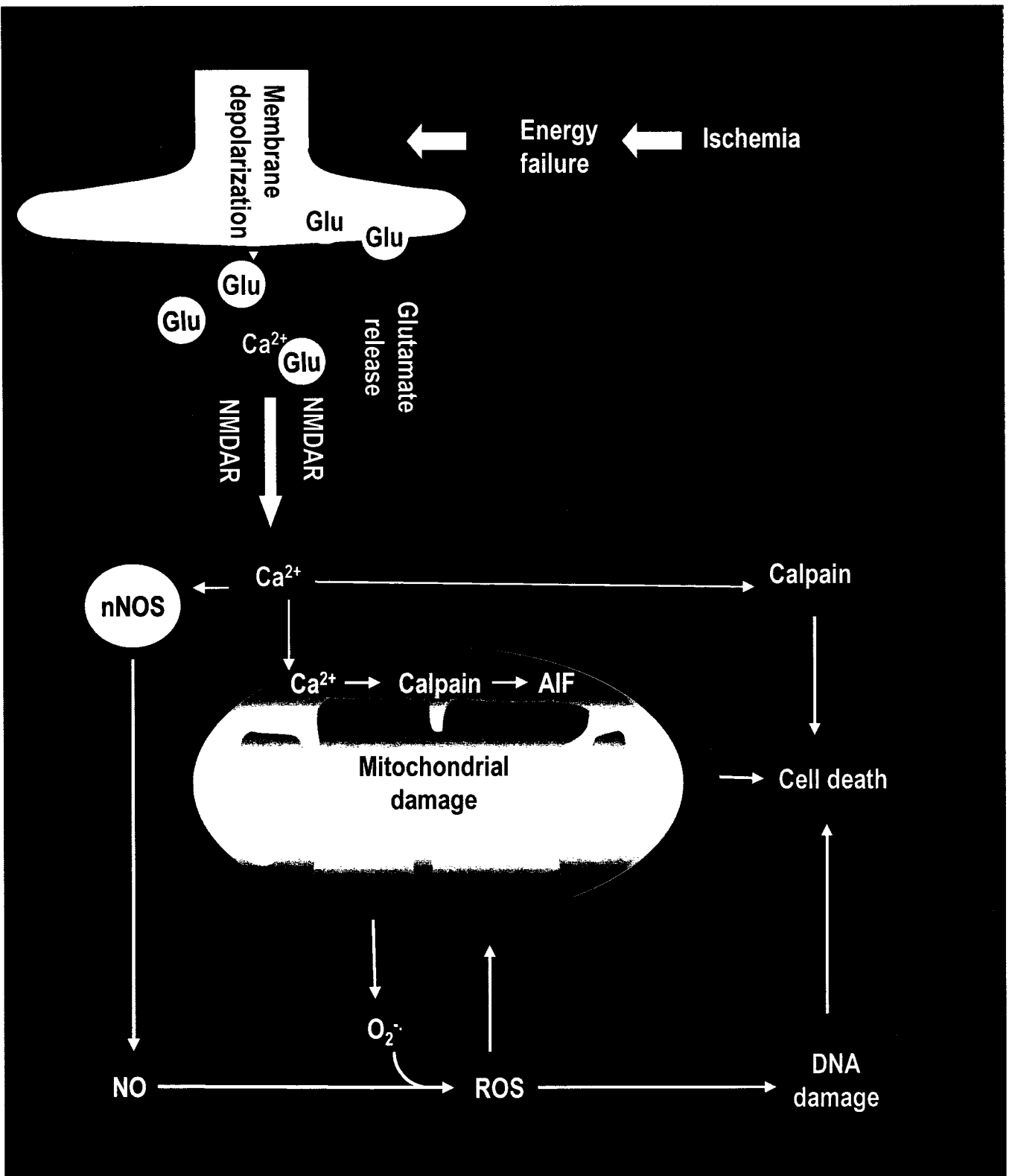


Figure 1.8

Figure 1.8. The mechanisms that converge on the mitochondria to induce neuronal death following excitotoxic injury

Glutamate release and overactivation of its corresponding receptors (e.g. NMDAR) leads to enhanced calcium influx and generation of nitric oxide. NO mediated neuronal toxicity ultimately converges on the mitochondria. This results in further generation of reactive oxygen species (ROS) leading to ATP depletion. While calcium activates calcium dependent kinases and proteases, a big portion of calcium will be taken up by the mitochondria. Once the buffering capacity of the mitochondria is saturated, calcium surplus results in the mitochondrial membrane depolarization, generation of ROS and energy failure. Calcium also activates important proteases such as calpain which induces neuronal loss at both cytoplasmic and mitochondrial levels. At the mitochondria, calpain assists in the truncation and release of AIF which eventually leads to large scale DNA fragmentation and neuronal demise.

generation of nitric oxide (NO) (Brenman et al., 1996; Aarts et al., 2002; Nelson et al., 2003). NO mediated toxicity is one of the mechanism contributing to neuronal loss following excitotoxicity (Sattler and Tymianski, 2001). A mechanism through which NO results in neurotoxicity is through a reaction with superoxide anion ($O_2^{\cdot -}$) to generate peroxy nitrite ($ONOO^{\cdot -}$). $ONOO^{\cdot -}$ destroys lipids, DNA and proteins and leads to ATP depletion [reviewed in (Arundine and Tymianski, 2004)]. Interestingly generation of $O_2^{\cdot -}$ which contributes to the formation of $ONOO^{\cdot -}$ is in part regulated by intracellular calcium. For example activation of calcium-mediated proteases results in production of xanthine oxidase (XO) within the cytoplasm which can act as an electron donor to generate more $O_2^{\cdot -}$ (Sussman and Bulkley, 1990). In addition to production of ROS at the cytoplasmic level, mitochondria are a major site of ROS production following excitotoxicity. The OMM is permeable to calcium ions and the IMM contains calcium uniporters that transfer calcium into the mitochondrial matrix. The IMM also contains calcium antiporters (sodium/calcium and hydrogen/calcium pumps) that require ATP and membrane potential gradients to move the calcium out of the mitochondria (Mattson et al 2008). Calcium is sequestered into the mitochondria matrix via a proton electrochemical gradient generated by the electron transport chain. This leads to mitochondrial depolarization, defects in the mitochondrial electron transport functioning and ROS accumulation. Mitochondrial ROS production is furthermore enhanced by $ONOO^{\cdot -}$. $ONOO^{\cdot -}$ is a potent free radical that is associated with mitochondrial depolarization and superoxide production. In addition, $ONOO^{\cdot -}$ induced mitochondrial depolarization results in the release of Apoptosis Inducing Factor (AIF) (among other proapoptotic factors) from the mitochondria. AIF translocation to the nucleus results in large scale DNA

fragmentation that is distinct from DNA damage induced by classical apoptotic cell death.

While the NR2 subunit of NMDAR regulate nNOS pathway and leads to NO production through interaction with PSD95, the NR1 subunit of the NMDAR governs the calcium permeability of these channels (Burnashev et al., 1992). Calcium permeability is controlled by an asparagine residue (N598) of the NR1 subunit of the channel pore. Importantly, NR1 subunit is required to form a functional NMDA receptor (Forrest et al., 1994), indicating the specificity of these channels for calcium flow. Among all other cations that NMDAR become permeable to, calcium has been recognized as a predominant mediator of excitotoxicity. For example, ion substitution experiments indicated that although removal of sodium ions attenuates the acute neuronal swelling following excitotoxicity, it failed to prevent long term neuronal degeneration, while removal of extracellular calcium ions could significantly attenuate this process (Choi, 1987; Choi et al., 1987). NR1 also interacts with an actin binding cytosolic protein called α -actinin. Disrupting this interaction or inducing actin depolymerization uncouples the NMDA receptors from the actin cytoskeleton and may results in redistribution of NMDA receptors from synaptic to extrasynaptic sites. Although both the synaptic and extrasynaptic NMDA receptors are involved in mediating neurotoxicity following glutamate release, the extrasynaptic NMDAR are proposed to have more deleterious effects. One hypothesis that could explain this event could be linked to decreased number of mitochondria at the non synaptic sites and lack of immediate calcium buffering.

Another issue to take into consideration is the density of mitochondrial pool close to calcium microdomains. For example the presence of the mitochondria at the route of calcium entry (ex: extrasynaptic versus synaptic; ER-mediated versus plasma membrane-mediated entry), impact activation of alternative calcium regulated pathways at the cytoplasmic or nuclear level. It is therefore a very challenging task to anticipate the spatial and temporal occurrence of the events that lead to excitotoxicity without taking a system biology approach. Of the calcium- dependent kinases and proteases [(reviewed in (Won et al., 2002))] that are deregulated following excitotoxicity, calpains have been found to play an important role in the death of neurons. Recent research suggests that calpains may be tightly linked to the regulation of mitochondrial morphology and ultrastructure.

1.4.2.1 Calpains

Calpains are a family of calcium-activated cystein proteases (Suzuki et al., 2004) and play a significant role in neuronal death through cleavage of key structural proteins and release of pro-death factors from the mitochondria (Bever and Neumar, 2008). The predominant calpains in the central nervous systems are the classical m-calpain and μ -calpain. These are composed of a large 80 kDa catalytic subunit and a small 28 kDa regulatory subunit. Although the m- and μ - calpains have been thought to be localized to the cytoplasm, an association of these calpains with subcellular organelles such as ER and Golgi apparatus has been observed (Hood et al., 2003). Interestingly, calpain like activity has been shown in both mitochondrial matrix and intermembrane space of rat

liver mitochondria (Tavares and Duque-Magalhaes, 1991; Garcia et al., 2005). In addition, an atypical calpain, calpain 10, was localized to the mitochondrial matrix (Arrington et al., 2006). Overexpression of calpain 10 resulted in mitochondrial swelling and dysfunction through the cleavage of Complex I subunits and activation of MOMP. Most recently, both the large and small subunits of μ -calpain were found in the mitochondrial IMS (Badugu et al., 2008). It was also shown that the N-terminus of Calpain 1 large subunit contain a mitochondrial targeting sequence. These findings together indicate an important role for calpains at the mitochondrial level suggesting a potential role in the regulation of mitochondrial morphology and function.

Importantly, in isolated liver or brain mitochondria calpain I induced cleavage of AIF and its release from the mitochondria (Polster et al., 2005). In addition, an increase in intracellular calcium levels was reported to be essential for both activation of mitochondrial calpains and AIF release following Bax dependent mechanisms of cell death (Norberg et al 2008). A link between calpain activation and AIF release was furthermore documented in primary neurons. In this study, activation of calpain was shown to be required for the truncation of AIF and its release from the mitochondria to execute neuronal death (Cao et al., 2007). Together these studies suggest an important role for calpain in the regulation of mitochondrial dysfunction following excitotoxicity.

In further support of an important role for calpains in neuronal injury following NMDAR overactivation, calpains have been identified to regulate the activity of **cyclin dependent kinase 5 (Cdk5)** (Hung et al., 2005; Wang et al., 2007b; Maestre et al.,

2008), an important player in neuronal injury [reviewed in (Zhang and Herrup, 2008)]. Increased calpain-dependent cleavage of the Cdk5 regulatory subunit, p35, to the constitutively active isoform, p25 has been documented following overactivation of NMDAR (Hung et al., 2005; Wang et al., 2007b). Phosphorylation of Cdh1 by the Cdk5-p25 complex was also shown to be necessary and sufficient for neuronal death after NMDAR stimulation (Maestre et al., 2008). Cdk5 is therefore a key molecule in the regulation of excitotoxic injury following enhanced calcium influx. Importantly, Cdk5 deregulation upon neurotoxic insults results in reactive oxygen species (ROS) accumulation and mitochondrial dysfunction (Sun et al., 2008). Whether Cdk5 is linked to the regulation of mitochondrial dynamics remain to be investigated.

1.4.2.2 Cyclin Dependent Kinase 5 (Cdk5)

Cdk5 is a proline-directed kinase that phosphorylates serine and threonines on the consensus sites (S/T)PX(K/H/R). A proline residue at position +1 is required and a basic amino acid at position 3 is preferred (Beaudette et al., 1993). Numerous substrates have been identified to be targeted by Cdk5. Similar to other cyclin dependent kinases (CDKs), Cdk5 alone shows no kinase activity. Association of Cdk5 with a regulatory substrate is required for its activation. Two non cyclin proteins p35 and p39 are identified to target and activate Cdk5 [reviewed in (Dhavan and Tsai, 2001)]. P35 and P39 have highest expression pattern in the post mitotic neurons (Zheng et al., 1998) and their functions seem to be somehow complementary (Dhavan and Tsai, 2001). While P39 can

compensate for some functions of P35 (Ko et al., 2001), it is not clear whether p35 and p39 can confer distinct substrate specificity for Cdk5.

Cdk5 plays an essential role in the development of the nervous system. The cdk5 knock out mice die embryonically or shortly after birth and have defects in different regions of the brain including cerebral cortex, hippocampus, cerebellum and olfactory bulb (Ohshima et al., 1996; Gilmore et al., 1998; Ohshima et al., 1999). Although Cdk5 was first identified to be a key regulator of synaptic plasticity (Dhavan and Tsai, 2001; Angelo et al., 2006; Cheung et al., 2006; Hawasli and Bibb, 2007), it has been clearly documented that Cdk5 plays an important role in the regulation of neuronal death (Green et al., 1997; Hung et al., 2005; Mitsios et al., 2007; Wang et al., 2007b; Maestre et al., 2008; Sun et al., 2008).

It was recently shown that mitochondrial fission contributes to the Cdk5-mediated neurotoxicity (Meuer et al., 2007). In RNAi knock down experiments, loss of cdk5 inhibited apoptosis-associated mitochondrial fission (Meuer et al., 2007). Interestingly, Cdk5 has identical substrate specificity to Cdk1 [reviewed in (Dhavan and Tsai, 2001)]. In cycling cells, Cdk1 was identified to regulate Drp1 and mitochondrial fission through its phosphorylation (Taguchi et al., 2007). Whether Cdk5 regulate the function of key fission proteins in post mitotic neurons remain to be studied.

In conclusion, mitochondria are dynamic organelles that continuously divide and fuse. Mitochondrial fission and fusion are identified to regulate classical apoptotic cell

death in non neuronal cell. Whether mitochondrial fission and fusion or their key components regulate multiple mechanisms that lead to neuronal loss in acute injury (e.g. calcium mediated excitotoxicity, oxidative stress, and DNA damage) is an interesting field of research that needs intensive investigation.

1.5 Hypothesis

The key players of mitochondrial dynamics, Mfn2, Opa1, and Drp1 are directly involved in the regulation of neuronal survival and function.

1.6. Thesis Objectives

The overall objective of this PhD thesis has been to investigate the role of mitochondrial dynamics in the regulation of neuronal survival and function.

The three main objectives of this PhD thesis have been:

- To investigate whether changes in the mitochondrial morphology impact neuronal death following injury models such as DNA damage, oxidative stress and excitotoxicity.
- To investigate whether the key molecules regulating **mitochondrial fusion** can confer neuroprotection following acute neuronal injury.
- To identify neuronal intrinsic mechanisms for the regulation of mitochondrial **fission protein** Drp1.

CHAPTER 2: Mitofusin 2 protects cerebellar granule
neurons against injury induced cell death
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Fortin, David S. Park, Heidi McBride, Ruth S. Slack

2.1. STATEMENT OF AUTHOR CONTRIBUTION

I performed 90 % of the experimental work and all statistical analyses as well as 100% of the revisions following submission. Experimental design, interpretation of the results was a joint effort between me, Dr. Slack and Dr. McBride. I wrote the manuscript with input for revisions from Drs. Slack and McBride. Dr. Eric C.C. Cheung and Jason G. MacLaurin provided assistance with the neuronal survival and mitochondria counts following oxidative stress model. Dr. Margaret Neuspiel assisted me with the use of confocal microscopy (Olympus model) in the laboratory of Dr. McBride. Dr. Andre Fortin trained me on how to perform primary neuronal culture and provided intellectual input for this study. Dr. Park provided intellectual input and advice in these experiments.

Mitofusin 2 Protects Cerebellar Granule Neurons Against Injury Induced Cell Death

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Running Title: Role of Mitofusin 2 in Neuronal Injury

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Key Words: Mitochondrial fusion, Mitofusin 2, Oxidative stress, DNA damage, Neuroprotection

2.2. ABSTRACT

Of the GTPases involved in the regulation of the fusion machinery, mitofusin 2 (Mfn2) plays an important role in the nervous system as point mutations of this isoform are associated with Charcot Marie Tooth neuropathy. Here, we investigate whether Mfn2 plays a role in the regulation of neuronal injury. We first examine mitochondrial dynamics following different modes of injury in cerebellar granule neurons. We demonstrate that neurons exposed to DNA damage or oxidative stress exhibit extensive mitochondrial fission, an early event preceding neuronal loss. The extent of mitochondrial fragmentation and remodeling is variable and depends on the mode and the severity of the death stimuli. Interestingly, whereas mitofusin 2 loss of function significantly induces cell death in the absence of any cell death stimuli, expression of mitofusin 2 prevents cell death following DNA damage, oxidative stress, and K^+ deprivation induced apoptosis. More importantly, whereas wild-type Mfn2 and the hydrolysis-deficient mutant of Mfn2 (Mfn2_{RasG12V}) function equally to promote fusion and lengthening of mitochondria, the activated Mfn2_{RasG12V} mutant shows a significant increase in the protection of neurons against cell death and release of proapoptotic factor cytochrome *c*. These findings highlight a signaling role for Mfn2 in the regulation of apoptosis that extends beyond its role in mitochondrial fusion.

2.3. INTRODUCTION

It has been recently demonstrated that the apoptotic program includes the regulated induction of mitochondrial fragmentation (1, 2). In addition, it has been shown that the rate of mitochondrial fusion is reduced early in the apoptotic program (3, 4), which together with the activation of the fission machinery leads to a morphological shift of the mitochondria to the fragmented state. The regulatory mechanisms and functional importance of these events during death remain unclear. For example, it is not clear whether the inhibition of mitochondrial fusion is an essential step in apoptosis or if fragmentation is promoted mainly because of the increase in fission. In addition, it has been shown that the loss of either Drp1 or hFis1 delayed, but did not block cell death, questioning the importance of the mitochondria morphological shift in the apoptotic cascade (5, 6). In this context, there is an emerging emphasis on the examination of mitochondrial fusion in the context of cell death.

Mitochondrial fusion is regulated by at least three essential GTPases, the outer membrane-anchored proteins mitofusin 1 (Mfn1),³ and mitofusin 2 (Mfn2) along with the intermembrane space GTPase, Opa1 (7, 8). Although the functions of Mfn2 overlap with Mfn1 in the process of mitochondrial fusion (9), there are clear distinctions between these two GTPases. Perhaps most informative of their distinct function is their biochemical difference in nucleotide binding and hydrolysis properties, where Mfn1 has a faster GTPase hydrolysis rate and higher affinity for nucleotide relative to Mfn2 (10, 11). In addition, Mfn1, but not Mfn2, has been shown to genetically interact with Opa1 (12), a

member of the dynamin family of mechanoenzymes. This relationship with Opa1 would suggest that Mfn1 plays a central role with Opa1 in the fusion process. In an *in vitro* mitochondrial docking assay, expression of Mfn1 significantly enhanced the tethering reaction, whereas Mfn2 resulted in tethering with low efficiency, suggesting a secondary role for Mfn2 in docking (10).

Recently, compelling evidence has emerged supporting additional roles for Mfn2 that goes beyond the regulation of the mitochondrial fusion. First, Mfn2 is colocalized in punctate with Bax and Drp1 at sites of future fission (13). This suggests that Mfn2 activity may affect mitochondrial recruitment of Bax or Drp1 during cell death. Second, Mfn2, but not Mfn1, can interact directly with Ced9 or Bclxl in HEK293 cells, suggesting a mechanism for cross-talk with antiapoptotic Bcl family proteins (3). Third, Mfn2, not Mfn1, has been shown to modulate metabolism through function of complex 1, IV, and V (14, 15). Fourth, it was also shown that cytosolic Bax plays a specific role in the steady state activity of Mfn2 as a regulator of mitochondrial fusion (16). Most importantly, Mfn2 seems to be critical for the function of the nervous system as point mutations in this molecule have been associated with Charcot Marie Tooth neuropathy type 2A (17).

Understanding the role of mitofusin 2 in response to acute neuronal injury is therefore crucial for development of novel therapeutic strategies. Because of the importance of Mfn2 in fusion as well as its importance in the nervous system, we

therefore asked whether Mfn2 is involved in the regulation of acute injury using primary cerebellar granule neurons. To examine the role of this GTPase in apoptosis signaling, we have constructed adenoviral vectors carrying both wild type and a hydrolysis-deficient mutant of Mfn2, Mfn2_{RasG12V} (18), and Mfn2_{RasG12V} lentivirus for long term transduction of these neurons. We asked: (a) whether Mfn2 could protect neurons against different mechanisms of injury and (b) whether this protection is by promoting mitochondrial fusion and shifting the morphological equilibrium or through an additional role for Mfn2 that is distinct from the stimulation of mitochondrial fusion.

2.4. EXPERIMENTAL PROCEDURES

Primary neuronal cultures and adenoviral construction - Primary Neuronal Cultures and Adenoviral Construction—Cerebellar granule neurons (CGNs) were cultured from CD1 mice at postnatal day 7 or 8 as described previously (19). Recombinant adenoviral vectors carrying ornithine carbamyl transferase (OCT), human mitofusin 2 (Mfn2), or its active mutant, Mfn2_{RasG12V}, expression cassettes were prepared using AdEasy system, as described previously (20). Mfn2 antisense adenovirus was a kind gift from Dr. Antonio Zorzano (14). Cells were infected at the time of plating with different multiplicity of infection (MOI) ranging from 25 to 150. 50 MOI was chosen based on the high efficiency and low toxicity. To measure the toxicity and efficiency of infection, Live/Dead assay (Molecular Probes, Eugene, OR) was performed 2 days postinfection. Three random fields were chosen for each group, and the images of cells in these fields were taken with a fluorescence microscope using the appropriate filters. Phase contrast micrographs of the same fields were taken with light microscopy, and the number of cells infected was

compared with the total number of cells in the field. To measure toxicity the number of infected dead cells was compared with the total number of infected cells in the field.

Cell viability assays - Cell death was measured by condensed nuclear morphology revealed by Hoechst staining. MTT or Live/Dead assay (Supplemental figure 1) was used to confirm the results of Hoechst staining, as described previously (18). In each replicate, three to five different fields were randomly chosen per treatment group. Representative samples were photographed using a Zeiss Axiovert 100 (Oberkochen, Germany) fluorescence microscope equipped with a QiCam Digital camera (QImaging Corporation, Burnaby, Canada) and Northern Eclipse software (Empix Imaging Inc. Mississauga, ON, Canada). Total number of cells in each field was counted. Cell death was expressed as a percentage of total cells.

DNA damage, K deprivation and reactive oxygen species (ROS) induced cell death - To model in vitro DNA damage induced cell death, CGNs were treated with 10 μ M camptothecin (Sigma-Aldrich, Oakville, ON, Canada) following 2 days in vitro (2 DIV). Hydrogen peroxide (H_2O_2) was used to model ROS induced cell death. CGNs were treated with H_2O_2 following 2 DIV for 5 minutes after which the medium was replaced with conditioned medium taken from the parallel cultures with no treatment. Due to unstable nature of H_2O_2 , the concentration used in each replicate was optimized prior to each treatment and 75-100 μ M was used to induce 50-70% cell death following 24 hours of treatment. To model K deprivation induced cell death, Neurons were transduced at the

time of plating with the concentrated lentiviruses and after 7 days in vitro the media containing 25 mM K was switched to low potassium media of 5 mM.

Cytochrome c release- Neurons were fixed and stained with cytochrome c and/or Tom20 and Hoechst following treatment with hydrogen peroxide or camptothecin treatment. For each replicate, a total of 100 neurons were counted from 3-5 different fields in each case a Z stack of the field was taken for analysis using a Ziess 510 Meta confocal microscope). A diffused cytochrome c staining or complete lack of staining was identified as release.

Time-Lapse Microscopy - CGNs were seeded on 4 well plates (Nalgene Nunc International, Rochester, NY) with attached glass coverslips coated with poly D Lysine (Fisher Scientific, Whitby, ON), and infected with the YFP-tagged Ornithine Carbamyl Transferase (OCT-YFP), a mitochondrial matrix protein, at the time of seeding. Following each treatment, neurons were imaged to track individual mitochondria in real time. The coverslips were mounted in a temperature controlled chamber (37°C) in regular growth media supplemented with 20 mM HEPES [pH 7.4], and visualized with an Olympus 100X oil immersion objective, numerical aperture 1.4, on an Olympus IX80 Laser scanning confocal microscope operated by FV1000 software v1.4a. The YFP was excited with 515 nm line of a multiple line Ar laser, the mitofluor Red was excited with the 543 nm line of He/Ne green laser and the Alexa 647 was excited with the 633 nm line of He/Ne red laser. All images shown demonstrate cells that are representative of

moderate infection efficiencies, and that have been obtained from at least three independent experiments.

Immunofluorescence - At each time point, neurons were fixed for 30 minutes with ice cold 4 % paraformaldehyde in 1X Phosphate Buffered Saline (1XPBS) and then rinsed twice with 1XPBS. Cells were permeabilized with 300 μ l of ice-cold 0.4% Triton-X in 1XPBS for 10 min. Cells were stained with the primary antibodies in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed 3 x 5 min with ice cold 1XPBS. Cells were incubated with the secondary antibodies to either TOM 20 (1: 250, a kind gift from Dr. Gordon Shore) (19), or cytochrome C (1:250; BD Biosciences, Franklin Lake, NJ) in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed for 5 minutes and stained with Hoechst for 5 minutes. Following Hoechst staining, neurons were washed with 1X PBS for 3 x 5 min and mounted. Representative samples were photographed using a Zeiss Axiovert 100 (Oberkochen, Germany) fluorescence microscope equipped with a QiCam Digital camera (QImaging Corporation, Burnaby, Canada) and Northern Eclipse software (Empix Imaging Inc. Mississauga, ON, Canada).

Mitochondrial length measurement - Whole cell images were acquired by exciting at 549 nm with the CY3 filter (Chroma Technology Corp., Rockingham, VT, USA). Mitochondrial length was measured by tracing the mitochondria using Northern Eclipse software. Mitochondrial length varied remarkably even in control neurons. For

comparison purposes mitochondria were classified into different categories with a length ranging from less than 0.5 μm , 0.5 to 1 μm , 1 to 2 μm , 2 - 3 μm , and greater than 3 μm .

Quantification and statistical analysis - For cell death studies, a minimum of 500 cells per field (three fields per replicate) was scored for each treatment at the indicated time points. For mitochondrial length measurements, a minimum of 500 mitochondria for each treatment (per replicate) was scored. The data represent the mean and standard deviation from three independent experiments ($n = 3$). n stands for each independent experiment. p values were obtained using two-way ANOVA and Tukey post hoc tests. A p value <0.05 was considered significant and was indicated on the graphs by an asterisk.

2.5. RESULTS

Mitochondrial Fragmentation Occurs Following DNA Damage-induced Neuronal Death

It has been suggested that mitochondria remodel following acute neuronal injury (23). We therefore asked whether mitochondria undergo fragmentation or remodeling following DNA damage-induced neuronal death. The DNA damage model induced by topoisomerase inhibitor, camptothecin, occurs physiologically following stroke or trauma and is believed to contribute to the extensive neuronal loss after acute injury (24). To model *in vitro* DNA damage-induced cell death, primary cerebellar granule neurons (CGNs) were treated with 10 μ M camptothecin. This concentration of camptothecin was shown to induce a slow cell death resulting in about 40% neuronal loss by 24 h (Fig. 2.1D). To track individual mitochondria in real time, we created adenovirus vectors containing the 32 amino acid targeting signal of ornithine carbamyl transferase fused to YFP (OCT-YFP) (25), and infected the CGN cultures at the time of seeding. Mitochondrial dynamics were documented within the first 16 h using fluorescence time lapse microscopy. We quantified the percentage and timing of mitochondrial fragmentation following treatment with camptothecin. CGN cultures were fixed and stained with anti-TOM20, a mitochondrial protein import receptor (22) and Hoechst to identify cell nuclei at different time points following treatment (Fig. 2.1B). Mitochondria in neurons exhibited variable length. The data were therefore binned into different length categories from less than 0.5 to greater than 3 μ m. Quantification of mitochondrial lengths showed that immediately following exposure to camptothecin, 96% of mitochondria had a length of greater than

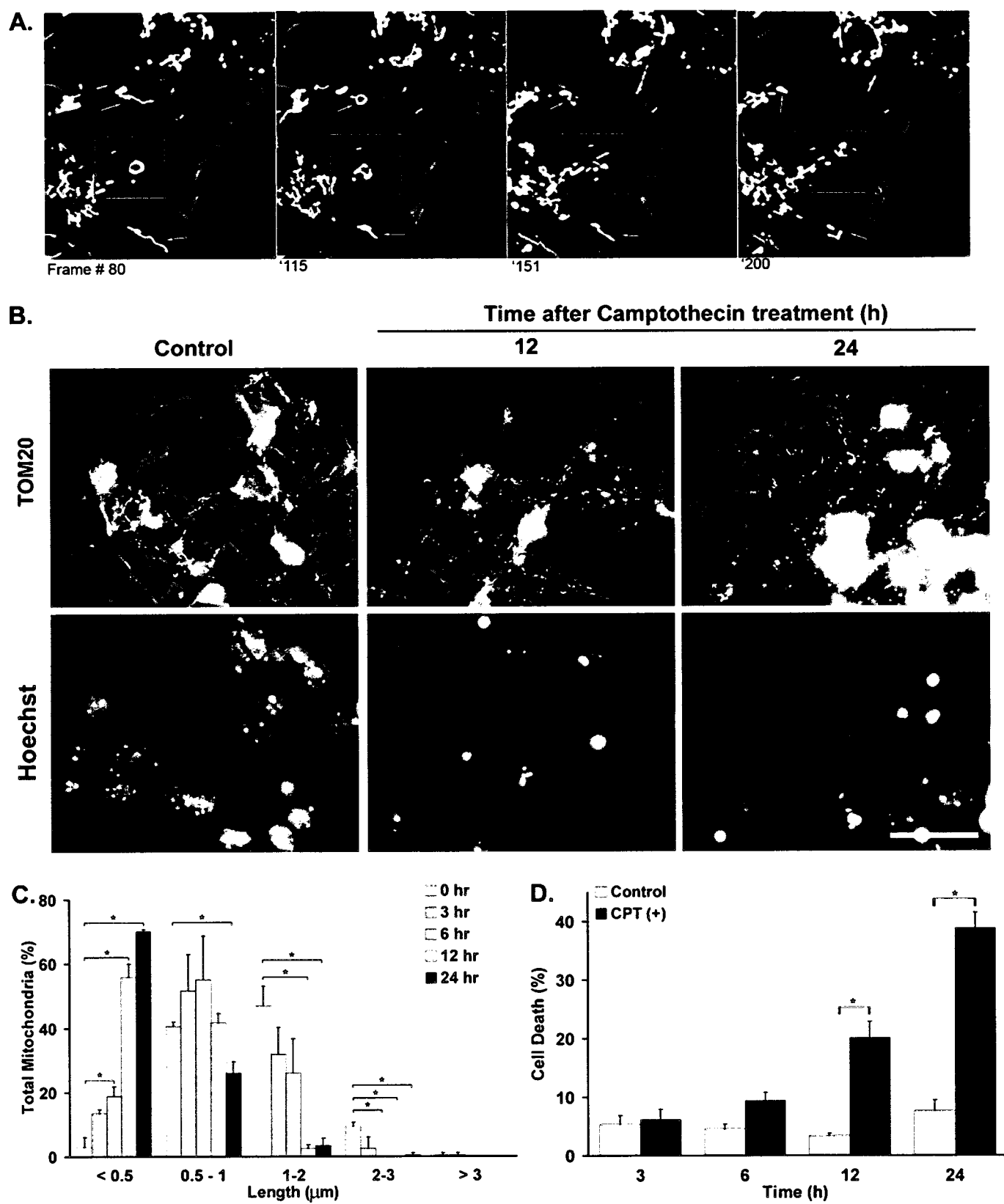


Figure 2.1

FIGURE 2.1. Mitochondrial fragmentation following DNA damage-induced cell death.

A, CGNs expressing YFP-tagged OCT-YFP were treated with 10 μM camptothecin. Individual mitochondria were tracked by exciting the YFP with the 515-nm line of a multiple line Ar laser. 200 frames (15 s per frames) were taken using confocal microscopy. (This figure represents still images for supplemental movie 1.) The *boxed area* indicates fragmentation of a swollen mitochondrion. *B*, CGNs were treated with 10 μM camptothecin at 2 DIV. Neurons were fixed and stained with antibody directed against Tom20 and nuclei stained with Hoechst at indicated time points following treatment. The panel shows representative images of mitochondria stained for Tom 20 at 0, 12, and 24 h. Hoechst images for nuclei corresponding to each field are presented. *C*, mitochondrial length was assessed at indicated time points following camptothecin treatment, and measurements were binned according to length. The length is classified based on the frequency at different lengths (less than 0.5, 0.5–1, 1–2, and greater than 3 μm). *D*, cell death was assessed at the indicated times by nuclear morphology revealed by Hoechst staining. *Bar*, 30 μm . *, $p < 0.05$.

0.5 μm , as in the control neurons (Fig. 2.1C). Of these $41 \pm 1\%$ ranged within 0.5–1 μm ; $47 \pm 4\%$ had a length of between 1 and 2 μm , and $9.3 \pm 0.9\%$ had a length of 2–3 μm . At 6 h following treatment with camptothecin, $20 \pm 2.5\%$ of the mitochondria were fragmented with a length of less than 0.5 μm . Following 12 h of treatment, however, there was a dramatic change in morphology where $56 \pm 3\%$ of mitochondria exhibited a length of less than 0.5 μm . The fragmentation was maximal by 24 h where $70.2 \pm 0.4\%$ of mitochondria exhibited a length of less than 0.5 μm . Only $3.4 \pm 1.7\%$ of mitochondria had a length of 2–3 μm at 24 h. These results demonstrate that mitochondrial fragmentation is initiated 3–6 h following exposure of neurons to camptothecin and there is a remarkable difference in the mitochondria pool by 12 h.

To confirm whether this change in morphology was because of mitochondrial membrane scission rather than organelle swelling, we performed a video analysis of mitochondria within cells treated with camptothecin. In many videos, mitochondria were observed to clearly divide into much smaller fragments upon 6 h of treatment with camptothecin. Therefore, although we did observe some mitochondrial swelling (Fig. 2.1A, *box*), we also observed mitochondrial fission (Fig. 2.1A and supplemental movie 1).

To ask whether mitochondrial fragmentation correlates with the cell death, the rate of cell death was evaluated by counting the percentage of cells exhibiting pyknotic nuclei. Pyknotic nuclei indicative of apoptosis were observed following 12 h of treatment (Fig. 2.1D). Cell death increased to $20 \pm 3\%$ at 12 h and was maximal at 24 h ($39 \pm 2.6\%$) within the given time frame. Based on these results, we conclude that mitochondrial

fragmentation is initiated 6–9 h prior to the degradation of the nucleus following DNA damage-induced cell death.

Mitochondrial Fragmentation Is an Early Event Following Oxidative Stress—In many types of acute neuronal injury such as stroke, a primary cause of death is the exposure to ROS, which initiates a complex signaling cascade (26). We next asked whether mitochondrial fragmentation also occurs following oxidative stress. CGN cultures were infected with OCT-YFP at the time of seeding and treated with H₂O₂ for 5 min after which the medium was replaced by conditioned media. Time lapse microscopy studies revealed that mitochondria undergo fragmentation within 1 h following treatment (Fig. 2.2A and supplemental movie 2). Mitochondria were also documented to transition from rod-like to spherical following fragmentation. This kind of mitochondrial remodeling following fission was indeed a common event in this mode of cell death (Fig. 2.2B and supplemental movie 3).

Interestingly exposure of neurons to hydrogen peroxide for longer than 5 min resulted directly in mitochondrial remodeling within first 20 min (Fig. 2.2C and supplemental movie 4). This concentration was toxic to cells as 100% of neurons were dead following first 2 h of treatment. Interestingly, unlike treatment with camptothecin where the fragmented mitochondria remained highly motile, the motility of fragmented mitochondria was substantially attenuated upon treatment with hydrogen peroxide (Fig. 2.2D and supplemental movies 1 and 5). To quantify the timing and percentage fragmentation of mitochondria and its correlation to cell death, mitochondrial and nuclear

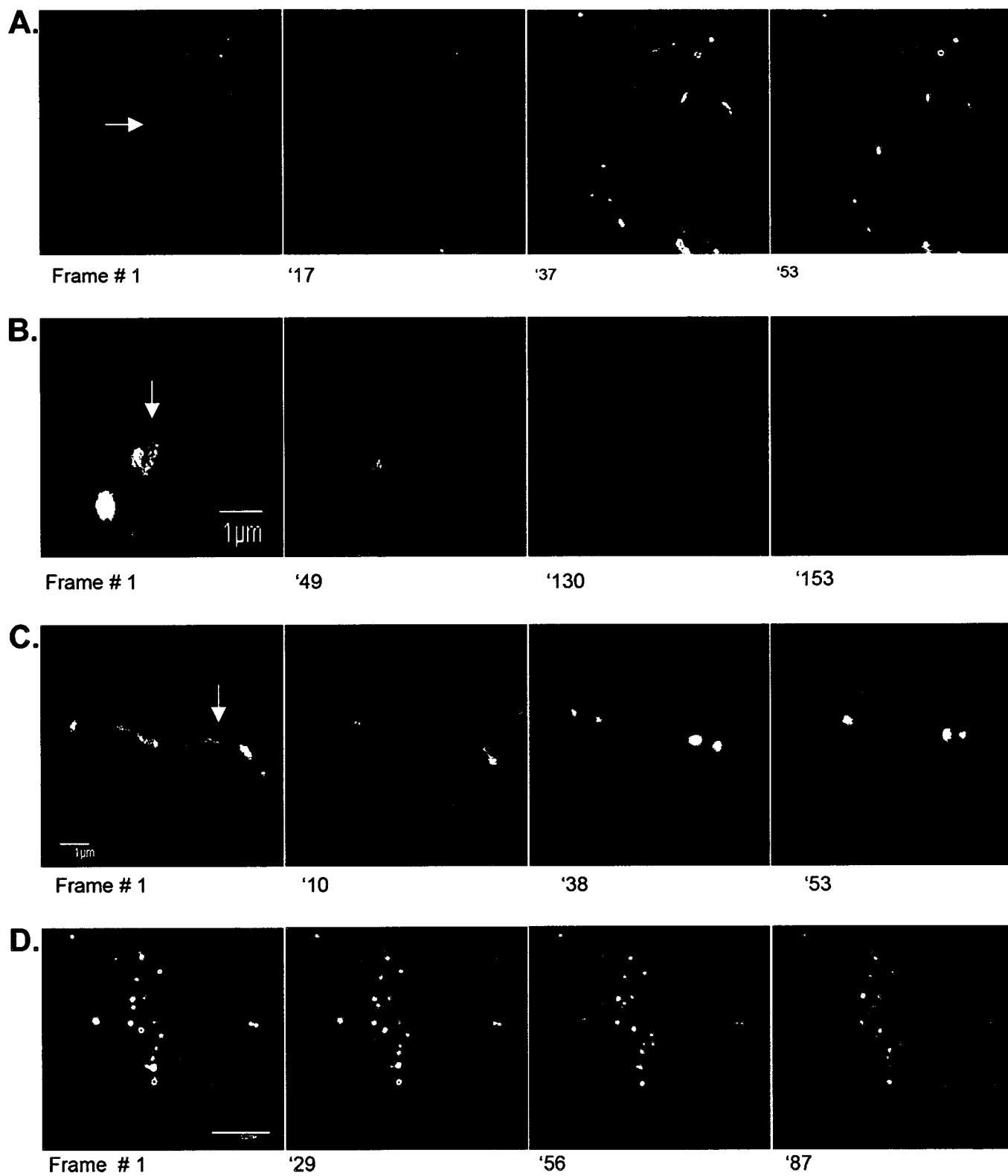


Figure 2.2

FIGURE 2.2. Mitochondrial dynamics following oxidative stress.

CGNs expressing OCT-YFP were treated with 100 μM H_2O_2 for 5 min after which the media was switched to conditioned media (*panels A, B, and D*). Neurons were treated with 100 μM H_2O_2 for 20 min without switching to conditioned media (*panel C*). Individual mitochondria were tracked by exciting the YFP at 515 nm. *A*, 200 frames (15 s per frame) were taken at 1 h following treatment. Confocal images represent still figures for supplemental movie 2. *B*, 200 frames (15 s per frame) were taken following treatment (supplemental movie 3). *C*, 60 frames were taken following 20 min of treatment with 100 μM of H_2O_2 . Confocal images represent still figures for supplemental movie 4. *D*, 100 frames were taken following treatment. Representative images are still figures corresponding to supplemental movie 5. $n > 10$.

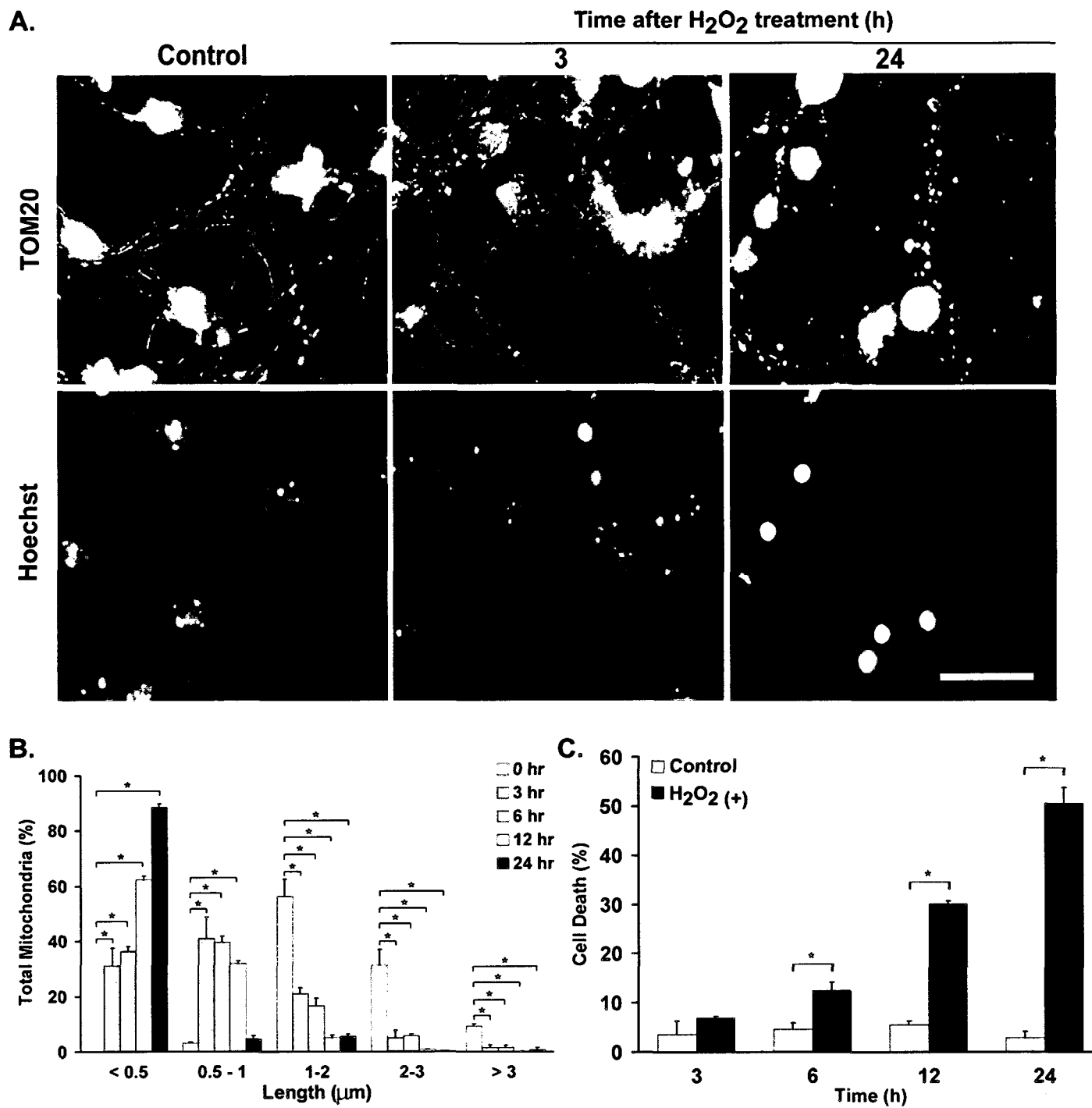


Figure 2.3

FIGURE 2.3. Mitochondrial fragmentation following oxidative stress.

CGNs were treated with 100 μM H_2O_2 at 2 DIV. Neurons were stained as described in the legend to Fig. 1. *A*, panel shows representative images of mitochondria at each time point stained for Tom20 and Hoechst. *B*, mitochondrial length was assessed at the indicated time points following H_2O_2 treatment and measurements were binned according to length. The length is classified based on the frequency at different lengths (less than 0.5, 0.5–1, 1–2, and greater than 3 μm). *C*, cell death was assessed at the indicated times by nuclear morphology revealed by Hoechst stain. $n = 3$, *, $p < 0.05$; *bar*, 30 μm .

morphology was evaluated (Fig. 2.3A). Immediately following exposure to ROS, greater than 94% of mitochondria had a length of greater than 0.5 μm (Fig. 2.3B). Neurons treated with hydrogen peroxide exhibited signs of mitochondrial fragmentation as early as 3 h where $31 \pm 6\%$ of mitochondria had a length of less than 0.5 μm . Mitochondrial fragmentation continued to increase to $62.3 \pm 1.4\%$ and $88.5 \pm 1.2\%$ at 12 and 24 h, respectively.

To ask whether the onset of mitochondrial fragmentation correlates with cell death, apoptosis was examined by Hoechst to detect pyknotic nuclei (Fig. 2.3C). The results of cell death studies were confirmed using colorimetric MTT survival assay (data not shown). At 12 h following treatment, $30 \pm 2.5\%$ of neurons exhibited pyknotic nuclei whereas cell death was maximal by 24 h when $50 \pm 2\%$ of neurons exhibited pyknotic nuclei. These results show that mitochondrial fragmentation was detected 3 h after treatment and 9 h before the apoptotic nuclei were considerably detected. These findings suggest that mitochondrial fragmentation may serve as an early apoptotic signaling event in this mode of injury.

Together, our results demonstrate that mitochondrial fragmentation is an early common event following acute injury in CGNs. We therefore asked whether preventing the fragmentation of the mitochondria by activation of the mitochondrial fusion machinery could prevent cell death induced by DNA damage and oxidative stress.

Increased Activation of Mfn2 Blocks Mitochondrial Fragmentation and Protects Neurons against Acute Injury—To examine whether activating mitochondrial fusion could protect neurons against cell death, we created adenovirus vectors containing the CFP-tagged

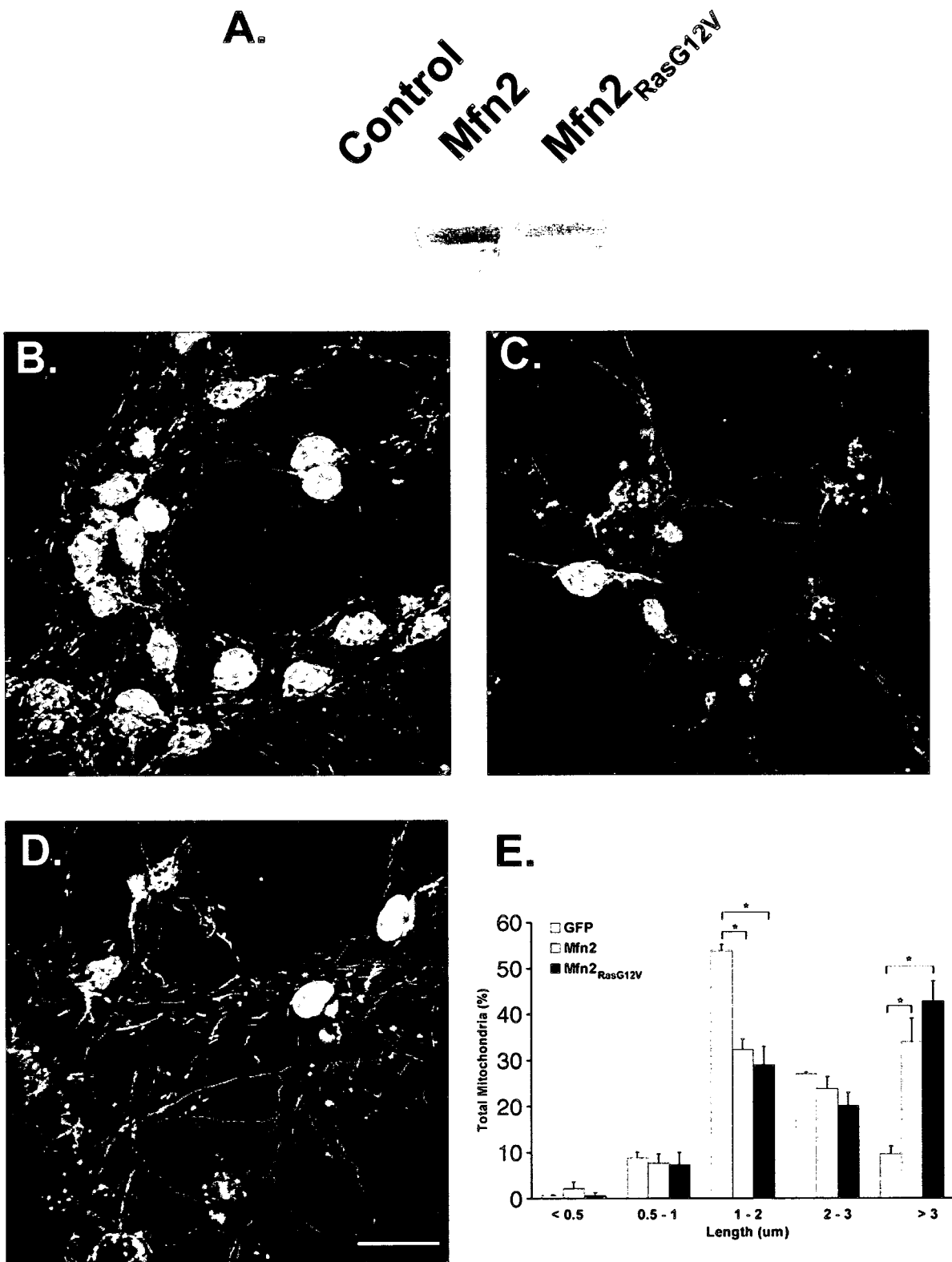


Figure 2.4

FIGURE 2.4. Mfn2 expression induces mitochondria fusion in CGNs.

CGNs were infected at the time of plating with recombinant adenoviral vectors containing an expression cassette for Mfn2 or Mfn2_{RasG12V} at 50 MOI. Whole cell lysates were analyzed in parallel with the control (no virus) by Western blot using an antibody against FP (A). CGNs were infected at the time of plating with recombinant adenoviral vectors containing an expression cassette for Mfn2 (C) or Mfn2_{RasG12V} (D) or GFP control (B) at 50 MOI. E, neurons expressing the indicated constructs were fixed and stained with TOM 20 to assess changes in mitochondrial morphology following increased expression of Mfn2 and Mfn2_{RasG12V}. Mitochondrial length of the indicated group is classified based on the frequency at different lengths (less than 0.5, 0.5–1, 1–2 and greater than 3 μm). $n = 3$; *, $p < 0.05$; *mag bar*, 20 μm ; *cyan*, nuclei; *green*, mitochondria.

wild-type Mfn2 and the hydrolysis-deficient, constitutively active mutant Mfn2_{RasG12V} (18). Primary neurons were infected 48 h prior to exposure to oxidative stress or DNA damage. We first examined whether these proteins could affect the mitochondrial morphology in untreated neurons. Cells were infected in parallel with adenovirus vectors carrying Mfn2, Mfn2_{RasG12V}, or GFP control, and the mitochondrial morphology was evaluated 48-h later (Fig. 2.4). To confirm protein expression a Western blot analysis was performed with neurons infected with Mfn2 or Mfn2_{RasG12V} (Fig. 2.4A). In unchallenged neurons (Fig. 2.4, *B* and *E*) the majority of mitochondria had an average length between 1 and 2 μm ($53.8 \pm 1.3\%$); however, increased expression of Mfn2:CFP (Fig. 2.4C) or Mfn2_{RasG12V}: CFP (Fig. 2.4D) resulted in a significant increase in the length of the mitochondria. The majority of mitochondria were greater than 3 μm as a result of increased levels of Mfn2 ($33.97 \pm 5\%$) and Mfn2_{RasG12V}:CFP ($42.9 \pm 4.2\%$) expression (Fig. 2.4E). These results demonstrate that enhanced activation of Mfn2 results in increased mitochondrial length.

We next asked whether activation of Mfn2 could prevent mitochondrial fragmentation and ultimately protect neurons against death induced by neuronal injury. To test whether activation of Mfn2 could protect neurons against DNA damage, parallel cultures were exposed to 10 μM camptothecin. The mitochondrial morphology and apoptosis were evaluated following 24 h (Fig. 2.5A). Mitochondrial fragmentation was dramatically inhibited with both Mfn2:CFP or Mfn2_{RasG12V}:CFP expression (Fig. 2.5A). Following 24 h of treatment, GFP-infected neurons showed only $9.6 \pm 1.1\%$ of mitochondria measuring greater than 3 μm , whereas the expression of Mfn2:CFP and Mfn2_{RasG12V}:CFP resulted in $33.4 \pm 3.2\%$ and $42.17 \pm 7\%$ of mitochondria being greater

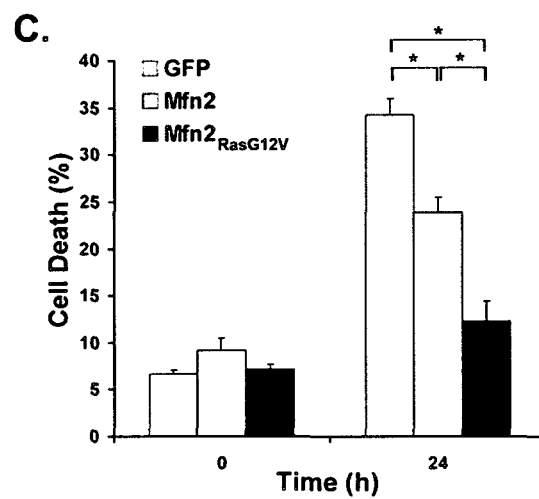
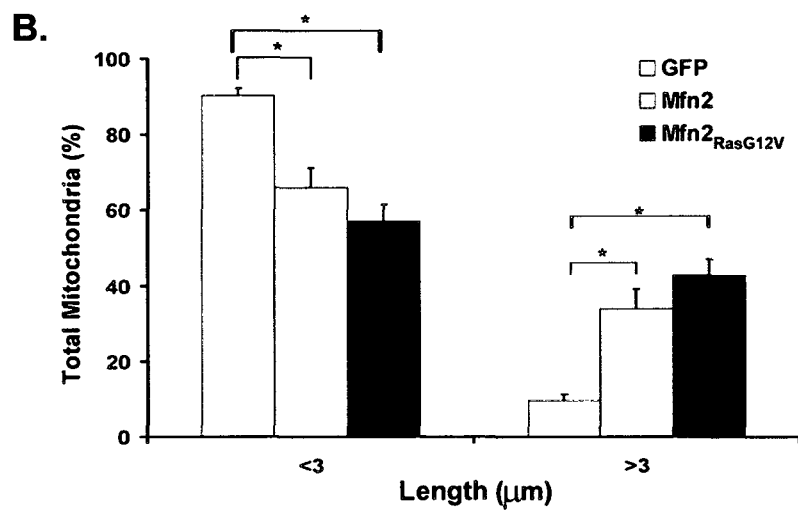
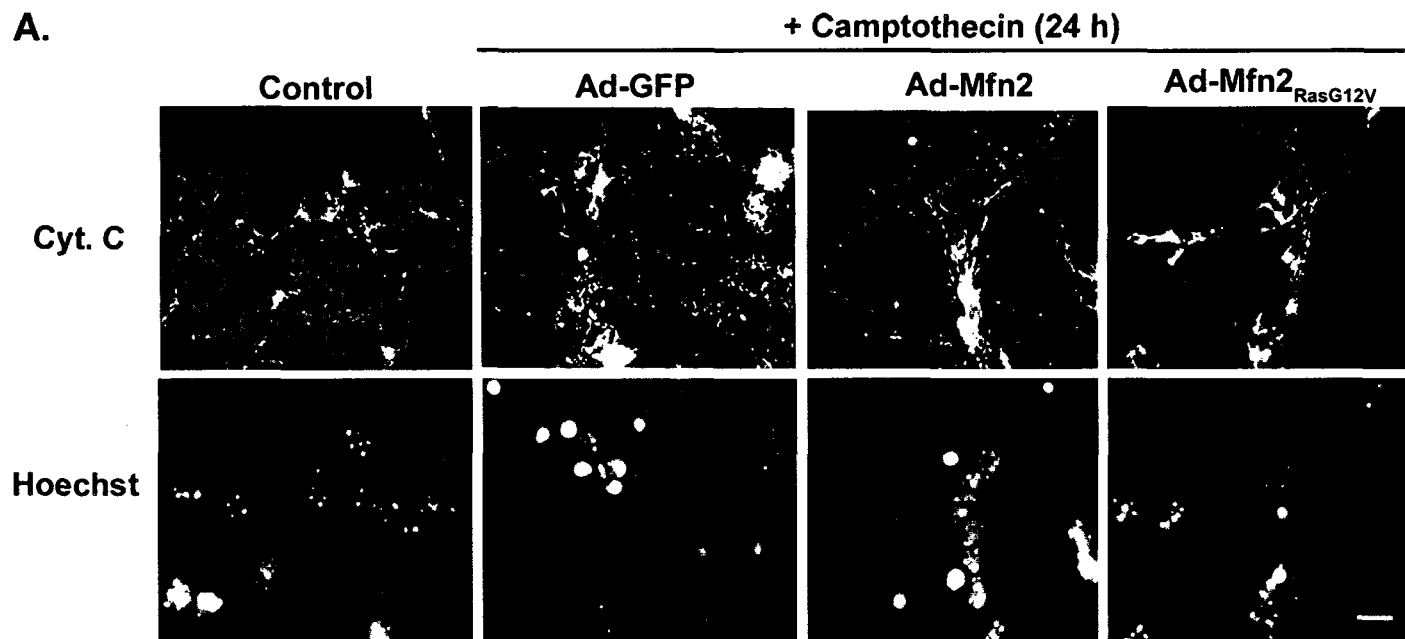


Figure 2.5

FIGURE 2.5. Increased activity of Mfn2 maintains the mitochondrial structure and protects cells against DNA damage.

CGNs were infected at the time of plating with Ad-Mfn2, Mfn2_{RasG12V}, or GFP control at 50 MOI and were treated with camptothecin (10 μM). At indicated time points, cells were fixed, and mitochondria were stained with an antibody against cytochrome *c*. Nuclei were stained with Hoechst. *A*, panel contains representative fields of mitochondrial structure and nuclear morphology 24 h following treatment with camptothecin. *B*, mitochondrial length as determined at 24 h. The length is classified as described previously. *C*, cell death was assessed at 24 h following treatment by nuclear morphology revealed by Hoechst stain. $n = 3$; *, $p < 0.05$; *bar*, 20 μm .

than 3 μm . Interestingly, the group of mitochondria measuring greater than 3 μm had a widely varied length distribution, with some single mitochondria spanning long projections and measuring greater than 30 μm . These results reveal that expression of Mfn2 or constitutive activation by expression of Mfn2_{RasG12V} could equally prevent the breakdown of the mitochondria typically seen following DNA damage. Most importantly, increasing the levels of Mfn2 resulted in increased protection against cell death induced by DNA damage (Fig. 2.5C). Following 24 h of treatment, $34 \pm 1.6\%$ of neurons exhibited pyknotic nuclei in the GFP control, and this was reduced to $23.9 \pm 1.6\%$ in the Mfn2 cultures. Increased activation of Mfn2 through delivery of Mfn2_{RasG12V}:CFP was significantly more protective than the wild type counterpart as it reduced cell death to $12.3 \pm 2.1\%$ (Fig. 2.5C). These results demonstrate that whereas Mfn2 and Mfn2_{RasG12V} result in equal fusion of mitochondria, the GTP bound form of Mfn2_{RasG12V} shows a 2-fold increase in protection of these neurons against DNA damage.

We then examined whether activation of Mfn2 could protect neurons against injury induced by ROS. Neuronal cultures were infected in parallel with adenovirus-expressing GFP control, Mfn2:CFP, and Mfn2_{RasG12V}:CFP. After 48 h, cells were exposed to H_2O_2 , and mitochondrial morphology and cell death were evaluated as described above. Increased expression of Mfn2:CFP or enhanced activation of Mfn2 by delivery of Mfn2_{RasG12V}:CFP protected neurons against the ROS-induced fragmentation, and resulted in significantly increased mitochondrial lengths (Fig. 2.6A). At 24 h following treatment with hydrogen peroxide, only $3.09 \pm 1.81\%$ of mitochondria had a length of greater than 3 μm in cells expressing the GFP control (Fig. 2.6B). This number increased to $30.65 \pm 5.7\%$ and $35.05 \pm 7.9\%$ in the parallel cultures expressing Mfn2:CFP & Mfn2_{RasG12V}:CFP.

FIGURE 2.6. Increased activity of Mfn2 maintains mitochondrial structure and protects cells against ROS-mediated injury.

CGNs were infected as described in the legend to Fig. 5, and the neurons were treated with H_2O_2 ($75\ \mu\text{M}$). At indicated time points, cells were fixed and stained with Hoechst for nuclei and cytochrome *c* antibody for mitochondria. *A*, panel shows representative fields for the structure of mitochondria and corresponding Hoechst at 24 h. *B*, mitochondrial length as determined at 24 h. *C*, cell death was assessed at 24 h following treatment by nuclear morphology revealed by Hoechst stain. $n = 3$; *, $p < 0.05$; bar, 20 μm .

respectively (Fig. 2.6B). More importantly, whereas expression of wild type Mfn2 resulted in an increased survival with an intermediate $47.8 \pm 3.2\%$ of cells remaining alive, Mfn2_{RasG12V} led to a dramatic 2–3-fold increase in survival, with $68 \pm 4.77\%$ of cells expressing Mfn2_{RasG12V}:CFP remaining viable relative to only $24.59 \pm 1.62\%$ in GFP-expressing controls (Fig. 2.6C). These data demonstrate that increased levels of Mfn2 prevent the breakdown of the mitochondria in response to injury and maintain the mitochondrial integrity in neurons. Most importantly, stabilization of the GTP-bound form of Mfn2 provides additional protection against death induced by ROS and DNA damage. These results highlight a novel therapeutic target to maintain neuronal survival after acute injury.

*Mfn2 Protects Neurons against Injury by Attenuating Cytochrome *c* Release*

Cytochrome *c* protein, a critical component of the electron transport chain, is normally localized to the mitochondria intermembrane space where it is sequestered within the cristae. Permeabilization of the outer mitochondria membrane results in partial release of the accessible cytochrome *c* into the cytosol; however the release of the majority of mitochondrial cytochrome *c* pool demands structural remodeling of mitochondria cristae (27, 28). We therefore asked whether mitofusin 2 regulates release of cytochrome *c* following different cell death stimuli. 24 h following treatment of the neurons with camptothecin, the neurons were fixed and stained with an antibody against cytochrome *c*, Tom 20, and/or Hoechst. $61.85 \pm 1.31\%$ of neurons had their cytochrome *c* released from the mitochondria in the LacZ control group. The cytochrome *c* release was decreased to $43.29 \pm 4.6\%$ in the wild type mitofusin group and to $22.32 \pm 1.77\%$ in the

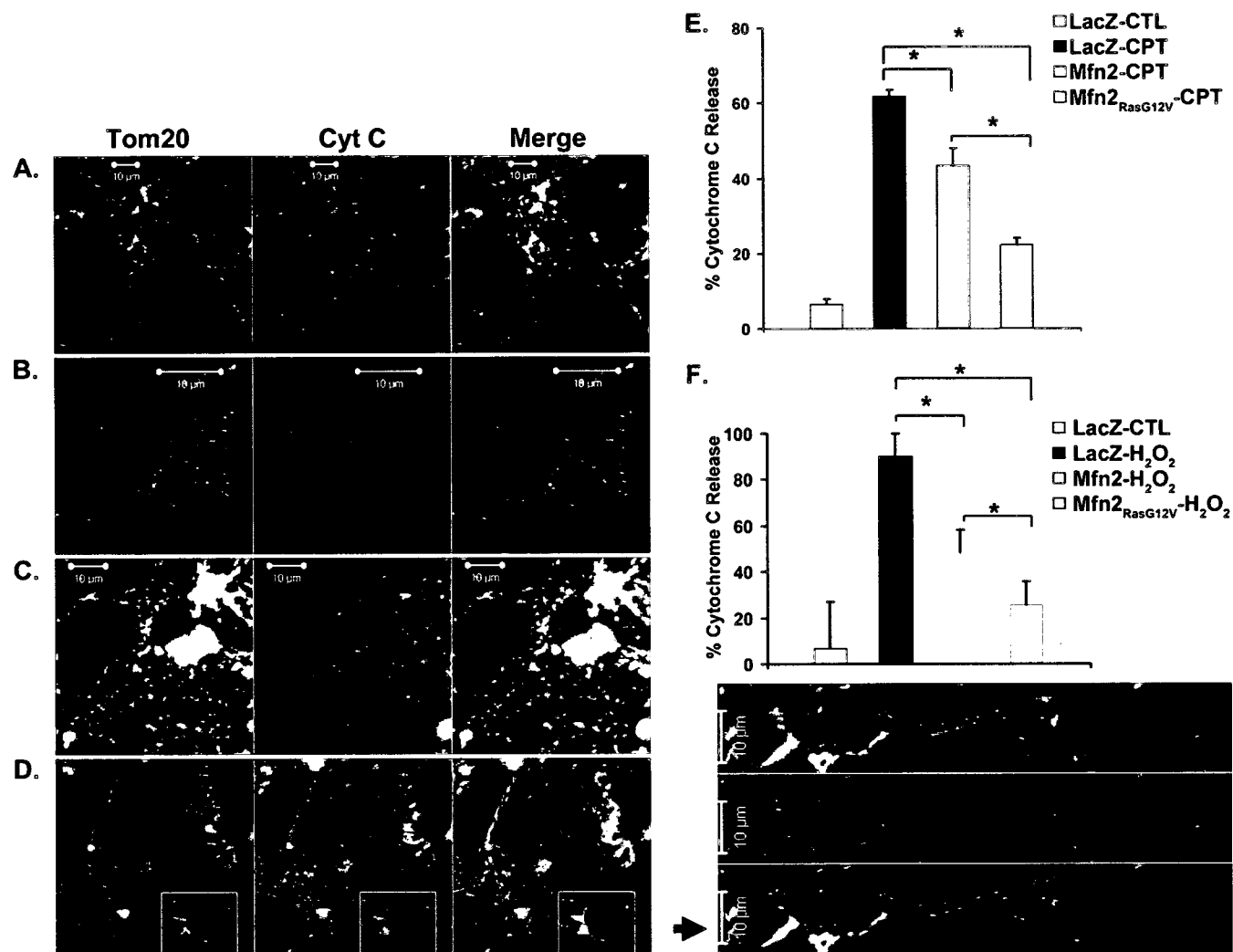


Figure 2.7

FIGURE 2.7. Mfn2 attenuates cytochrome *c* release following DNA damage and ROS-mediated injury.

CGNs were infected as described and treated with either H₂O₂ (75 μ M) or camptothecin (10 μ M). The cells were fixed and stained with cytochrome *c* and Tom 20 following 24 h of treatment with camptothecin or hydrogen peroxide. Z stack sections of different fields were taken for each replicate. *A*, colocalization of Tom 20 and cytochrome *c* in the control neurons. *B*, representative field demonstrating release of cytochrome *c* from mitochondria following treatment with camptothecin and *C*, following treatment with hydrogen peroxide. *D*, cytochrome *C* and Tom 20 colocalization in the neurons infected with Mfn2_{RasG12V} following treatment with hydrogen peroxide. *E*, percentage of cytochrome *c* release following camptothecin treatment in CTL, Mfn2, and Mfn2_{RasG12V} group. *F*, quantification of cytochrome *c* release following treatment with hydrogen peroxide in CTL, Mfn2, and Mfn2_{RasG12V} group. $n = 3$; *, $p < 0.05$.

Mfn2_{RasG12V} group (Fig. 2.7E). Similarly, following treatment with hydrogen peroxide, Mfn2 and Mfn2_{RasG12V} attenuated release of cytochrome *c* to $47.8 \pm 2.21\%$ and $26.42 \pm 0.98\%$, respectively when compared with control at $84.6 \pm 12.7\%$ (Fig. 2.7F). Our data not only indicate that activation of mitofusin 2 results in attenuation of the cytochrome *c* release following both DNA damage and ROS, but it further supports the distinction in protective response between wild type mitofusin 2 and the activated Mfn2_{RasG12V} mutant. These findings also have identified Mfn2:GTP as an inhibitor of cell death upstream of cytochrome *c* release, which positions the function of Mfn2 within the apoptotic cascade in primary neuronal models of cell death.

Transduction of Neurons by a Mfn2_{RasG12V} Lentivirus Protects Neurons against Potassium Deprivation-induced Apoptosis

Cell excitability is a critical determinant of neuronal survival during brain development (29). K⁺ channels set both the resting membrane potential and the duration of the action potential. Opening of these channels can influence neuronal death or neuronal survival (30). Low K⁺ exposure of granule neurons initiates a complex set of proapoptotic, metabolic, and signal transduction mechanisms that include up-regulation of c-Jun target genes and inhibition of glycolysis (31).

Also, it has been recently demonstrated that upon K⁺ deprivation, CGN exhibit an immediate reduction in mitochondrial respiration, a decrease in ATP turnover which correlates with decreased calcium concentration (32) and depletion of NF κ B(33). Because cerebellar granule neurons yield a classic model for depolarization-induced apoptosis, we asked whether mitofusin 2 protects against this mode of cell death. We constructed a

lentivirus for the Mfn2_{RasG12V} to transduce CGN. The media containing 25 mM K⁺ was changed to the media of 5 mM K⁺ following 7 DIV and following 24 h in the low potassium media, the percentage of cell death was evaluated using the "Live/dead" assay. The percentage cell death declined from 74.06% in the control group to 49.1% in the Mfn2_{RasG12V} group. Following correction for the basal cell death (15% in CTL to 22% in Mfn2_{RasG12V} group) Mfn2_{RasG12V} counts for a greater than 50% protection against K⁺ deprivation-mediated apoptosis (Fig. 2.8).

Mfn2 Loss of Function Induces Cell Death in Cerebellar Granule Neurons

To further support the antiapoptotic role of mitofusin 2 in primary neuronal culture, we induced Mfn repression by antisense adenoviral expression, previously described (14) (Fig. 2.9). Following 48 h of infection of CGN (MOI 50), neuronal survival was assessed by live/dead assay. Our results show that Mfn2-knocked-down neurons exhibit a significant $33 \pm 3.0\%$ cell death, when compared with LacZ CTL at $9 \pm 0.9\%$ in the primary neurons even in the absence of any cell death stimuli.

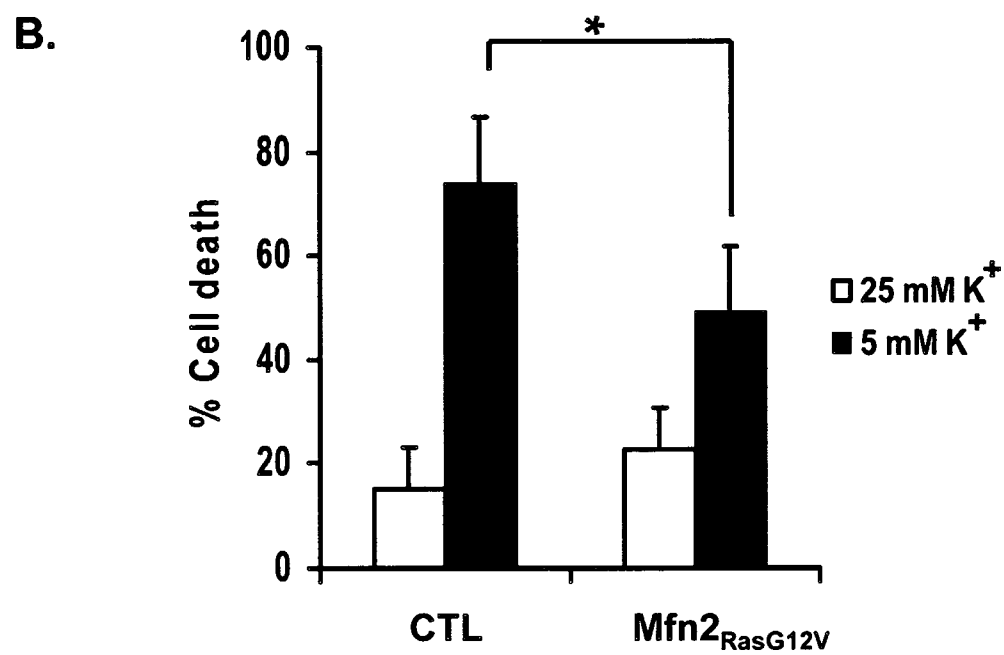
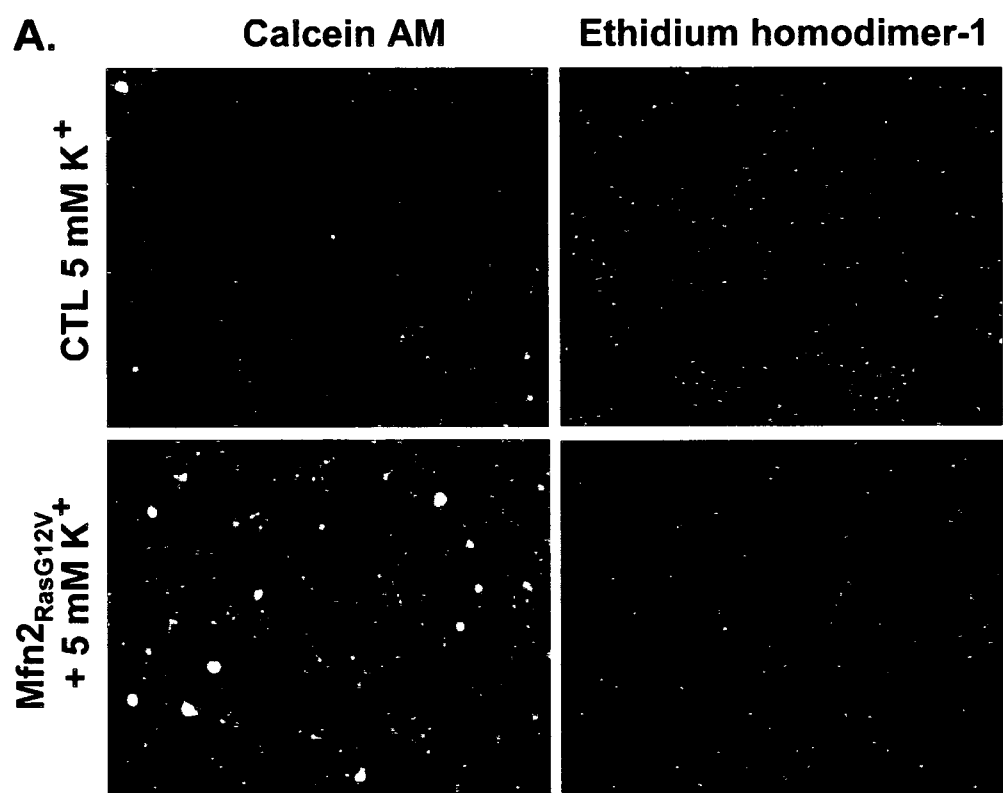


Figure 2.8

FIGURE 2.8. Transduction of neurons by Mfn2_{RasG12V} protects against K⁺ deprivation-mediated cell death.

CGNs were transduced with Mfn2_{RasG12V} concentrated lentivirus at the time of seeding (1.5 MOI). Following 7 days *in vitro* the media with 25 mM K⁺ was completely switched to the media containing 5 mM K⁺. At 24 h, the rate of cell death was assessed with the live/dead assay. *A*, CTL at 5 mM K⁺ (*top panel*) and Mfn2_{RasG12V} at 5 mM K⁺ (*lower panel*); *B*, assessment of cell death by live/dead assay following 24 h of K deprivation. $n = 3$; *, $p < 0.05$.

2.6. DISCUSSION

The results of our studies support a number of conclusions: first we show that mitochondrial fragmentation occurs as an early event in response to injury in cerebellar granule neurons. The extent of mitochondrial fragmentation, however, is variable and depends on the mode of neuronal injury and the severity of the death stimuli. Second, expression of Mfn2 prevents mitochondrial fragmentation in response to oxidative stress and DNA damage-induced neuronal death. Third, we show that in addition to stimulating the fusion machinery, Mfn2 protects neurons against different modes of neuronal injury including DNA damage, oxidative stress, and K deprivation-induced apoptosis. Most importantly, we demonstrate that while the wild type Mfn2 and the constitutively activated mutant Mfn2_{RasG12V} function equally to promote fusion and lengthening of mitochondria, neuronal protection against acute injury is much more effective in the GTPase hydrolysis-deficient Mfn2 mutant *versus* the wild type Mfn2. Furthermore, mitofusin 2 exerts its protective effect at an early stage upstream of cytochrome *c* release. Finally, down-regulation of mitofusin 2 induces cell death in the absence of any apoptotic stimuli. Taken together, these findings implicate an anti-apoptotic role for Mfn2 during death models representative of acute neuronal injury and neuronal development.

Mfn2 has been proposed to function along with its homologue Mfn1 as a direct tethering/fusion component (8, 10). Our results indicate that the increased activation of mitochondrial fusion by expression of Mfn2 or Mfn2_{RasG12V} could cause a dramatic increase in the mitochondrial lengths to greater than 30 μm . This neuronal response

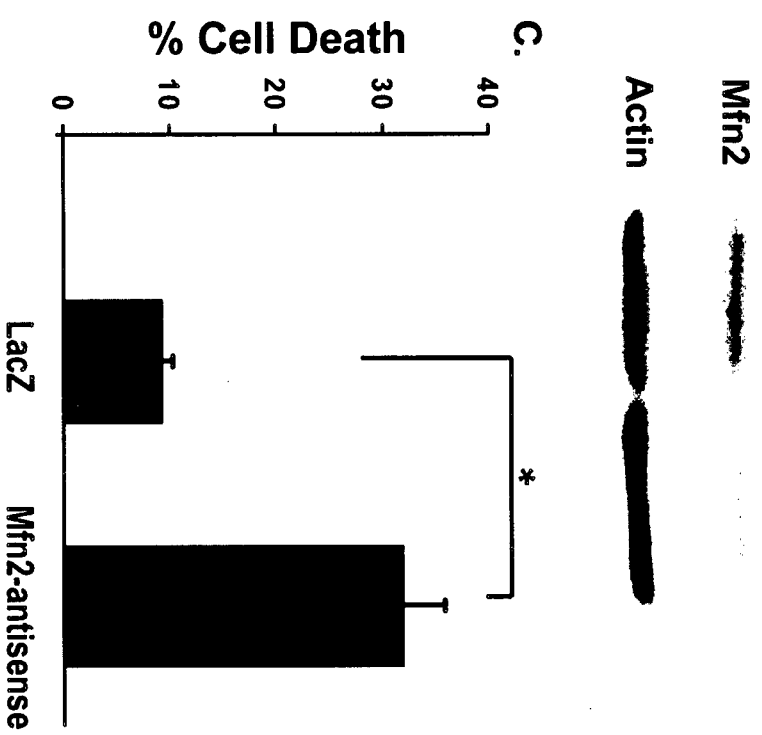


Figure 2.9

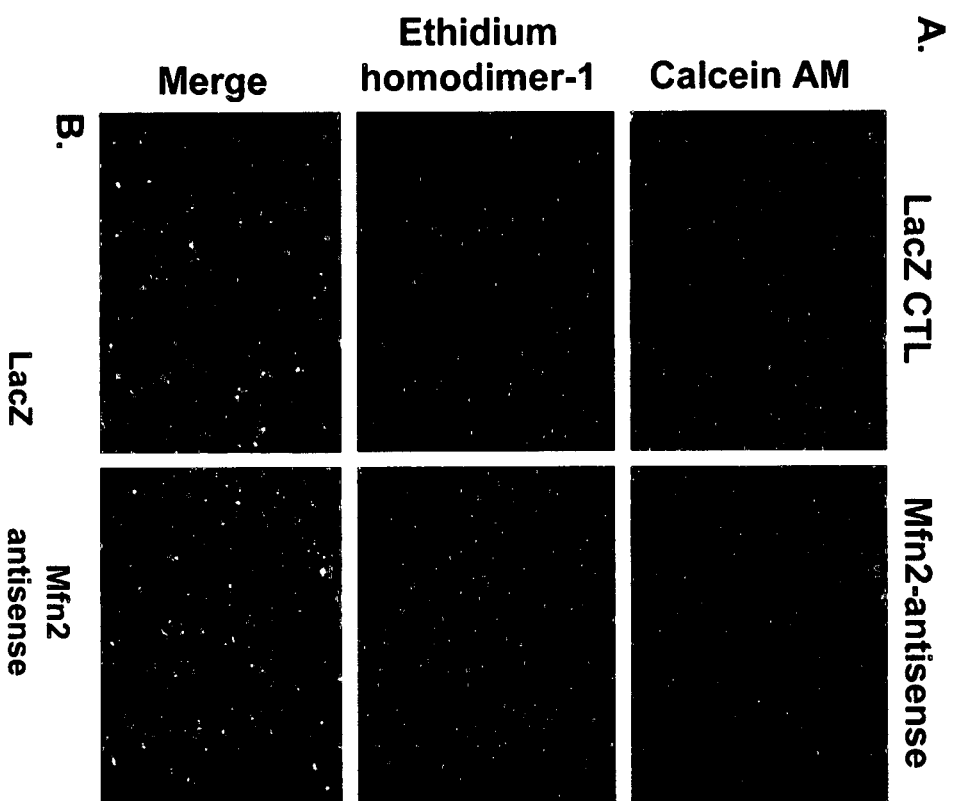


FIGURE 2.9. Mfn2 loss of function induces cell death in the absence of any cell death stimuli.

CGN were infected with an Mfn2-antisense adenovirus at the time of plating at 50 MOI. Following 48 h, neuronal survival was evaluated by live/dead assay. *A*, live/dead assay to show representative fields of Mfn2-antisense and LacZ CTL group at 48 h. *B*, Western blot demonstrating repression of Mfn2. *C*, a significant 33% increase in cell death is evident following Mfn2 loss of function compared with CTL (*, $p < 0.05$, $n = 3$).

exhibiting a dramatic lengthening of the mitochondria is unlike that previously found in other cell types where the ectopic expression of Mfn2 resulted in mitochondria fusion within a non-motile perinuclear cluster (18). Neurons may therefore be unique in their ability to respond to these factors and may express a distinct repertoire of proteins that regulate mitochondrial fusion relative to other cell types.

A key question is whether the longer tubular mitochondria are more supportive of survival than the short fragmented mitochondria or alternatively; do molecules involved in the fusion machinery interact with cell death signaling? Previous studies have demonstrated that inducing fusion by overexpression of Mfn1 or a dominant negative mutant of Drp1 protects against nitric oxide-mediated cell death (34). Unlike Mfn1, which interacts with Opa1 to induce fusion, Mfn2 has been associated with apoptotic signaling proteins (16, 18). Interestingly, we show here that expression of both the wild type Mfn2 and the constitutively active mutant, Mfn2_{RasG12V}, had similar effects on mitochondrial lengthening; however, the hydrolysis-deficient mutant exhibited a more profound protection against cell death. These distinct biological responses suggest that the protection from death may not be due only to the increased fusion because both the wild type and mutant Mfn2 result in similar increases in mitochondrial length. Instead, the data suggest that the nucleotide state of Mfn2 may regulate other interactions on the mitochondrial membrane that are critical for the cell death.

Our data supporting an additional role for Mfn2 beyond the activation of fusion is consistent with recent findings that demonstrate interactions with the Bcl family proteins. First Mfn2 is colocalized in punctate with Bax and Drp1 at sites of future fission and

affects mitochondrial recruitment of Bax or Drp1 during cell death, indicating a spatial relationship between fusion and fission during cell death (13). Second, it was shown that cytosolic Bax plays a specific role in the steady state activity of Mfn2 as a regulator of mitochondrial fusion (16). In that study, the mitochondria within the Bax/Bak double knockout (DKO) cells demonstrated a reduced rate of mitochondrial fusion. Mfn2 is normally found in a punctate pattern (18); however, in Bax/Bak DKO cells, the protein circumscribed the outer membrane (16). The introduction of Bax into the DKO cells resulted in a stable shift of Mfn2 within the outer mitochondrial membrane into foci, which rescued the rates of fusion. Interestingly, the GTP-bound form of Mfn2 did not respond to Bax/Bak expression and retained its highly mobile, even distribution along the outer membrane regardless of Bax/Bak expression levels (16). Given that this mutant was not affected by Bax/Bak expression, it is possible that Mfn2_{RasG12V} is also resistant to Bax/Bak-induced changes on the membrane during apoptotic stimuli. This resistance of Mfn2_{RasG12V} to assemble into Bax-dependent foci may interfere with the efficient assembly of proapoptotic complexes required for the progression of cell death. This would at least partially explain the increased protectivity of the activated mutant to multiple Bax-dependent apoptotic stimuli that we have observed in primary neurons.

Finally, Mfn2 can interact directly with Ced9 or BclXl in HEK293 cells further suggesting a mechanism for protective cross-talk with antiapoptotic Bcl family proteins (3). Because Mfn2 protein levels have not yet been shown to be reduced during apoptosis, the nucleotide state of Mfn2 could be required to mediate cross talk with the apoptotic

machinery. We envision a model whereby the GTP-bound form of Mfn2 may interact with the Bcl-2 family of proteins, remaining circumscribed along the outer membrane, functioning in a protective manner and protect against cell death. In contrast, the GDP-bound form would be susceptible to modulation by Bax to allow foci formation and assembly of the death machinery on the outer membrane. Future studies will be required to determine how this activity is regulated in the context of the apoptosis signaling cascade.

Finally, there is growing evidence to support the idea that the machineries that govern mitochondrial fusion are linked to the metabolic processes within the organelle. For example, Mfn2 has been shown to modulate metabolism through function of complex 1, IV, and V (14, 15). Consistent with this idea, down-regulation of fusion proteins (Mfn1 and Mfn2) led to fragmented mitochondria with reduced oxygen consumption and electrochemical potential (35). This suggests that mitochondrial fusion is likely to be a central player in relating mitochondrial dynamics to mitochondrial metabolism, and could also be a mechanism for modulating cell death during neuronal injury.

In conclusion, we show that mitochondria undergo extensive fragmentation in acute neuronal injury and that activating the mitochondrial fusion machinery can protect neurons against injury induced cell death. These results demonstrate the importance of mitochondrial dynamics in acute injuries such as trauma and stroke. Demonstrating that the control of the nucleotide state of Mfn2 can dramatically affect the outcome of cell death suggests that Mfn2 may serve as an accessible therapeutic target for the treatment of these human diseases. Future work to investigate the factors that control the nucleotide

state of Mfn2 and to delineate the interaction with the apoptotic machinery should further elucidate these mechanisms.

2.7. REFERENCES

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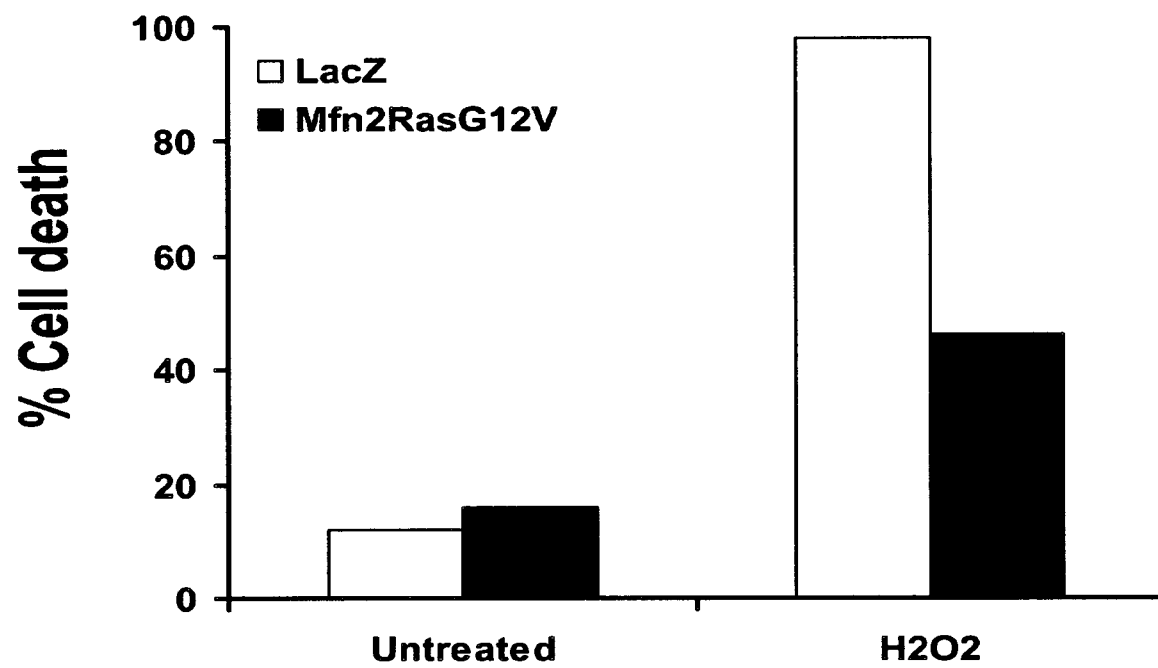
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2.8. FOOTNOTES

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Supplemental figure 2.1

Supplemental Material

1) Supplemental figure: Confirmation of Hoechst data by Live Dead Assay.

Neurons were infected with Mfn2 RasG12V or Lac Z adenoviruses and treated with H₂O₂ as described previously. Cell survival was evaluated with Live/Dead assay. (n = 2

2) Supplemental Movies are found on:

<http://www.jbc.org/cgi/content/full/M703812200/DC1>

CHAPTER 3: Regulation of mitochondrial dynamics during excitotoxic cell death

(submitted *J cell Biol.*

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Jahani-Asl A, Xu W, Pilon Larose K, Park DS, McBride HM, Slack RS

3.1. STATEMENT OF AUTHOR CONTRIBUTION

I performed 95 % of the experimental work and all the statistical analyses. Experimental design and interpretation of the results was a joint effort between me and Dr. Slack with valuable input from Dr. Park. I wrote the manuscript and prepared the figures with advice from Dr. Slack. William Xu was a dedicated honors student who worked on this project with me and contributed to the characterization of mitochondrial length following NMDA treatment in control primary neurons. Karine Pilon Larose contributed to the analysis of the Opal oligomers by performing crosslinking on isolated mitochondria and Western blot analysis. Dr. McBride advised us in the mitochondrial fusion assays (both technical and data analysis) as well as EM analysis. Dr. Park provided the reagents for the calpastatin studies as well as advice in the design of these experiments.

Regulation of mitochondrial dynamics during excitotoxic cell death

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Running Title: Mitochondrial dynamic in excitotoxicity

Key Words: Mitochondria, Excitotoxicity, Opa1, Calpain

3.2. ABSTRACT

Overactivation of glutamate receptors occurs following acute neuronal injury and is accompanied by excessive calcium influx. Presently, little is known regarding how glutamate receptor activation causes loss of mitochondrial integrity and function. Here, we report that treatment of neurons with NMDA results in defects in mitochondrial morphology. Overactivation of NMDA receptors (NMDAR) results in a disassembly of Opa1 oligomerization, an inner membrane GTPase essential for the maintenance of cristae structure and mitochondrial fusion. While activation of the outermembrane fusion GTPase, Mfn2, provides a partial protection, upregulation of Opa1 restores Opa1 oligomerization and protect neurons against excitotoxic injury. We show that calpain activation is a key trigger of Opa1 deoligomerization as inhibition of calpain activity rescues Opa1 oligomerization, mitochondrial morphology defects and neuronal survival. Opa1 is essential for neuroprotection by calpain inhibition as calpastatin fails to protect against excitotoxicity following Opa1 knockdown. Our results define a common pathway whereby mitochondrial dysfunction after excitotoxicity results in loss of Opa1 oligomerization through activation of calpain, an impairment in fusion rate and breakdown of mitochondrial network. Highly relevant to a broad range of neurological diseases, mitochondrial integrity can be preserved by driving Opa1 oligomerization through upregulation of Opa1 to maintain mitochondrial structure and function.

3.3. INTRODUCTION

Excitotoxicity is a distinct mode of neuronal death which is attributed to increased presynaptic glutamate release. Disturbance of extracellular glutamate levels acting on NMDA receptors (NMDAR) is the major cause of cell death following acute neuronal injury such as stroke and trauma [reviewed in (Arundine and Tymianski, 2004)]. Overactivation of NMDAR is also implicated in neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases [Reviewed in (Hardingham and Bading, 2003)]. Increased calcium influx through NMDAR induces neuronal death by modulating the activity of calcium dependent effector proteins such as calpain, cytosolic phospholipase A2, calcium/calmodulin protein kinase II (CamKII), and endonuclease such as DNase II.

Mitochondria play a central role in directing cell death signalling generated at the NMDAR. First, the accumulation of cytoplasmic calcium results in subsequent accumulation of calcium in the mitochondria. Inhibiting the mitochondrial calcium uptake has been previously shown to reduce calcium mediated excitotoxicity (Stout 1998). Blocking the mitochondrial calcium overload through manipulation of mitochondrial membrane potential has been shown to block neuronal death following NMDAR overactivation (Seo et al 1999). Second, excitotoxic pathway converges on the mitochondria to induce release of apoptosis inducing factor 1 (AIF1) and its translocation to the nuclei to cause chromatin condensation and DNA fragmentation (Cheung et al., 2006; Cheung et al., 2005; Cregan et al., 2004; Cregan et al., 2002; Yuan et al., 2003). In

recent studies, Calpain I (μ -Calpain), a calcium activated protease with mitochondria targeting sequence, was shown to regulate AIF truncation and release from the mitochondria (Badugu et al., 2008; Cao et al., 2007; Norberg et al., 2008; Polster et al., 2005).

Recent studies suggest a link between components of mitochondrial dynamics and calcium signalling. In mammals, Optic atrophy 1 (Opa1) and mitofusins 2 (Mfn2) are two major GTPases identified to regulate mitochondrial fusion and dynamin-related protein 1 (Drp1) is the key protein which regulates mitochondrial fission [reviewed in (Knott et al., 2008)]. The regulation and function of Drp1 is tightly regulated by Ca^{2+} /calmodulin-dependent protein kinase I α (CaMKI α) and calcineurin (Cereghetti et al., 2008; Cribbs and Strack, 2007; Han et al., 2008). An increase in mitochondrial calcium influx results in mitochondrial fragmentation (Breckenridge et al., 2003; Cereghetti et al., 2008; Hom et al., 2007) and this fragmentation can be in part rescued by down-regulation of Drp1 activity (Breckenridge et al., 2003; Hom et al., 2007). Also, the BH3 only protein, BIK, acts on the ER facilitating calcium release leading to mitochondrial uptake results in cristae remodeling and cytochrome c mobilization (Germain et al., 2005).

Our knowledge of how mitochondrial fusion components Mfn2 and Opa1 respond to calcium transients is limited. Mfn2 was recently reported to bridge ER and mitochondria in cell lines and facilitate mitochondrial calcium influx from ER stores (de Brito and Scorrano, 2008). Components of the mitochondrial fusion machinery play important roles in the nervous system. For example mutations in the Mfn2 results in

Charcot Marie Tooth neuropathy type II and mutations in Opa1 result in autosomal dominant Optic atrophy. We have previously shown that Mfn2 rescues mitochondrial morphology defects and protects neurons against Bax dependent mechanisms of neuronal injury (Jahani-Asl et al., 2007). Excitotoxicity exhibits a partial apoptotic-necrotic like cell death which does not require Bax/Bak mediated signalling (Cheung et al., 2005; Dargusch et al., 2001; Stout et al., 1998). To determine the role of key fusion proteins in response to calcium deregulation in a physiologically relevant model such as excitotoxicity is essential for our understanding of these events and will lay down the foundation for development of novel neurotherapeutic strategies.

In the present study, we have taken a combination of loss and gain of function approach and real time imaging to investigate the mechanisms by which excitotoxicity in neurons results in loss of mitochondrial integrity and survival. Our results demonstrate that excitotoxic injury results in a dramatic mitochondrial phenotype resulting from an impairment of in mitochondrial fusion and mitochondria swelling. Importantly, components of mitochondrial fusion machineries, Opa1 and Mfn2, could rescue mitochondrial morphology defects and promote neuronal survival following NMDA induced toxicity. We identify Opa1 as a key regulator of mitochondrial integrity the activity of which is modulated by calpain activation. Finally, we show that Opa1 is required for neuroprotection by calpain inhibition because calpastatin fails to protect neurons against excitotoxicity in the absence of Opa1. Our study for the first time provides a strong link between component of inner mitochondrial membrane fusion and deregulation of calcium signaling following excitotoxic neuronal injury.

3.4. RESULTS

Mitochondrial morphology defects following excitotoxic injury

Excitotoxicity is a key mechanism of cell death following acute neuronal injury that involves deregulation of intracellular calcium. To model excitotoxicity, we used the agonist, NMDA, to activate NMDA receptors in cultured primary neurons. NMDA receptors are ionotropic receptors for glutamate and their overactivation is implicated as the primary cause of brain damage following stroke and trauma. We first established a frame work to study mitochondrial morphology switches following excitotoxicity. Primary neurons at 7 days in vitro (DIV) were treated with 100 μ M NMDA and 10 μ M Glycine for 1hr, followed by return to conditioned media. This concentration of NMDA results in 60 to 70% neuronal death at 24 hours (**Figure 3.1A**). To establish a time frame for mitochondrial morphology switches relative to the time course of cell death induced by NMDA, mitochondrial and nuclear morphology were analyzed at 0, 3, 8, 12 and 24 hrs. Neurons were co-stained with an antibody against *cytochrome c* and Hoechst to reveal mitochondrial and nuclear morphology, respectively (**Figure 3.1B**). A minimum of 500 mitochondria were scored from each independent experiments (n=3) and mitochondrial length was measured using Axiovision software. To perform a comparative analysis at different time points, mitochondria length was binned into different categories of <0.5, 0.5-1, 1-2, 2-3, and > 3 μ m. In control neurons at 7 DIV, $42.82 \pm 3.52\%$ of mitochondria exhibit a length of 1-2 μ m (**Figure 3.1C, Table 1**, n= 3). In contrast, NMDA- treated neurons exhibited a shift in the mitochondrial pool in each binned category such that most mitochondria had a length less than 0.5 μ m following 24 hrs of treatment ($64.5 \pm 0.68\%$ of mitochondria have a length of < 0.5 μ m compared to

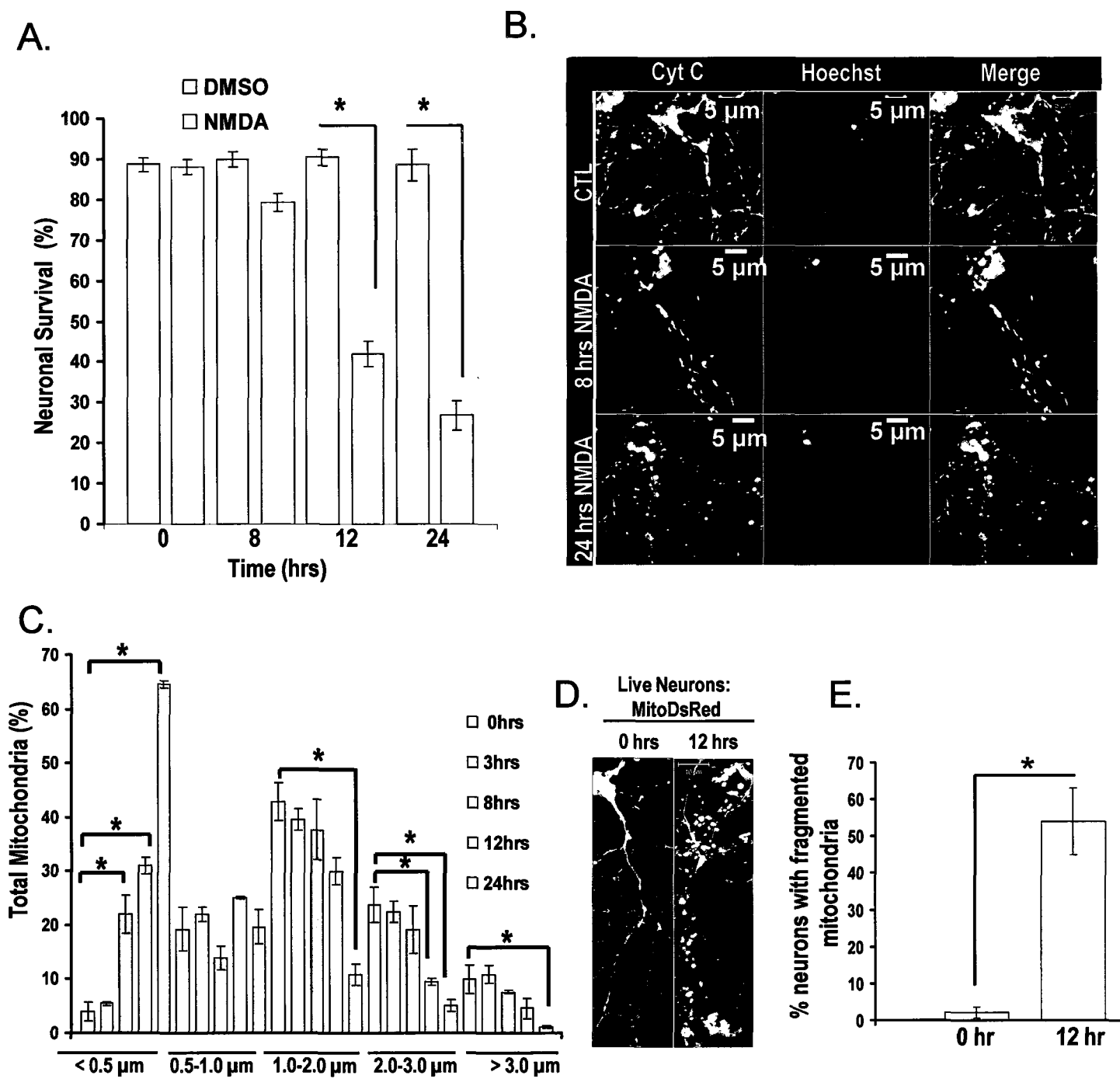


Figure 3.1

Figure 3.1. Mitochondrial Morphology Defects Following NMDA Induced Neuronal Death

Primary neurons were treated with 100 μ M NMDA and 10 μ M Glycine for 1 hrs followed by switching to condition media. **(A)**. Neuronal survival was analyzed at different time points. **(B)**. Mitochondrial and nuclear morphology was assessed by staining with an antibody against cytochrome c and Hoechst. Representative panel demonstrate mitochondrial morphology switches at 8 and 24 hrs. **(C)**. Mitochondrial length was binned into different length categories of <0.5, 0.5-1, 1-2, 2-3, and > 3.0 μ m. Panel demonstrate quantification of mitochondrial length (expressed as percentage) following NMDA induced neuronal death. **(D)**. Mitochondrial morphology in the absence and presence of NMDA was assessed in live neurons targeted with MitoDs Red. **(E)**. Percentage of live neurons exhibiting fragmented mitochondrial at 0 and 12 hrs is plotted.

* demonstrate $p < 0.05$, Three independent experiment (n =3).

Bin	<u>0h</u>	<u>3h</u>	<u>8h</u>	<u>12h</u>	<u>24h</u>
< 0.5	3.99 ±1.79	5.39±.33	21.9±3.52	30.9±1.47	64.5±0.68
0.5 - 1	19.17±3.99	21.94±1.24	13.84±2.23	25±0.30	19.57±3.105
1 - 2	42.83±3.52	39.54±2.04	37.56±5.59	29.87±2.54	10.7±2.03
2 - 3	23.65±3.52	22.44±1.97	19.10±4.46	9.46±0.74	5.02±1.069
> 3	9.86±2.57	10.8±1.62	7.56±0.36	4.55±1.87	1.11±0.171

Table 3.1

Table 3.1. Quantification of mitochondrial length following treatment with NMDA.

Neuronal mitochondria exhibit variable length. For comparative analysis mitochondrial length was binned into different length categories of <0.5, 0.5-1, 1-2, 2-3, and > 3.0 μm at different time points (0, 8, 12, and 24 hrs) following treatment with NMDA. This table depicts percentage of total mitochondria at each binned category and the shift in the mitochondrial length in the absence and presence of NMDA.

3.9 ± 1.79% at 0 hrs). A detailed time course is shown in Table 3.1. This indicates that mitochondria undergo dramatic fragmentation within 24hrs following excitotoxicity (**Figure 3.1C**, n=3, p< 0.005).

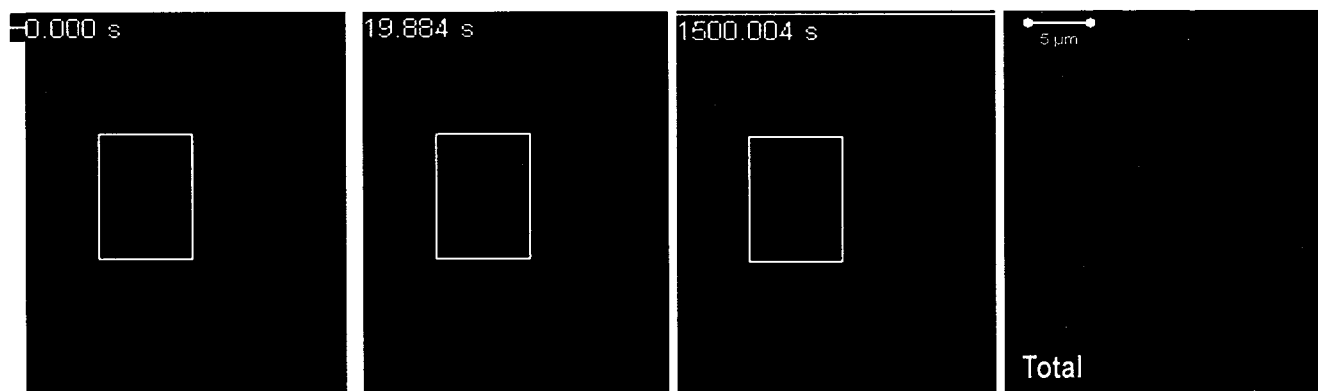
To confirm the results obtained by immunostaining, we also analyzed mitochondrial morphology by live imaging. Primary neurons were seeded on 4 well plates with attached glass coverslips coated with poly D Lysine and were transduced with MOI 2 of MitoDsRed lentivirus at 1 DIV. At 7 DIV, the coverslip was mounted in a temperature controlled chamber (37°C) in regular growth media supplemented with 20 mM HEPES (pH 7.4), and visualized with an LSM-510-Confocal-Laser-Scanning-Microscope using a 63X oil immersion objective (numerical aperture 1.4, **Figure 3.1D**). Mitochondria were visualized by exciting the MitoDsRed with 594 nm line of a multiple line Ar laser. 100 neurons were scored at 0 and 12 hrs following treatment with NMDA. The percentage of live neurons exhibiting fragmented mitochondria increased from 2 ± 1.53% at 0 hr to 54 ± 9.07 % at 12 hrs (P <0.004, n =3, **Figure 3.1E**). Together, these results demonstrate that mitochondria become significantly fragmented following NMDA induced neuronal injury.

Excitotoxicity results in a defect in mitochondrial fusion

We next asked if mitochondrial fragmentation resulting from overactivation of NMDA receptors resulted from a defect in mitochondrial fusion. To measure the rate of fusion in a quantitative manner we employed a mitochondrial fusion assay involving

photoactivable GFP (Zunino et al., 2007). A lentivirus was constructed carrying the photoactivable GFP (PAGFP) fused to the 32-amino-acid-long matrix-targeting signal of ornithine carbamyl transferase (OCT:PAGFP). Neurons were transduced with MOI 2 of OCT: PAGFP at 1 DIV. At 7 DIV, the GFP signal was activated in a small region (aimed at 2-4 mitochondria) within the axonal processes of each neuron using a laser at 405 nm (**Figure 3.2A**). The spread of the signal throughout the axonal processes was then captured by obtaining images of the neurons every 5 minutes for a total of 25 minutes. Following this, all of the PAGFP within the targeted neuron (including soma and axons in the given field) was activated at 405 nm. By creating a mask that scored one binary signal per voxel, we calculated the percent of the voxels containing a PAGFP signal at each time point within the neuron relative to the total signal upon complete illumination of the mitochondria. The increase in the area over the 25-minute period was plotted (**Figure 3.2B**). A minimum of 18 assays were performed in the absence and presence of NMDA (**Figure 3.2B**). Our data show that mitochondria of control neurons shared their signal with 2.34 ± 0.52 % of inactivated mitochondria (18 assays, Figure 2B). Following 6-8 hrs of treatment with NMDA, not only was there no significant increase in the percentage fusion over 25 minutes, but there was a decrease in the area that was initially photoactivated (-0.037 ± 0.19 , 19 assays, **Figure 3.2B**). These results indicate that: a) overactivation of NMDA receptors results in a complete arrest in mitochondrial fusion, and b) there is a decrease in the area initially occupied by the mitochondrial pool indicative of mitochondrial swelling.

A.



B.

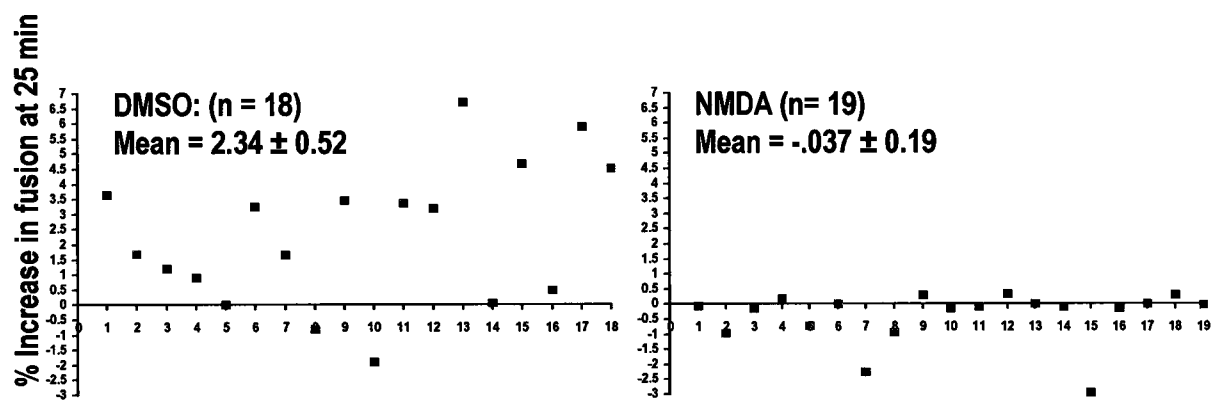


Figure 3.2

Figure 3.2. Mitochondrial fusion rates are attenuated following NMDA induced cell death.

(A) Neurons were transduced with a lentivirus for OCT:PAGFP at 1 DIV. At 7 DIV, the GFP signal was activated in a small region (aimed at 2-4 mitochondria) within the axonal processes of each neuron using a laser at 405 nm (Boxed area). The spread of the signal throughout the axonal processes was captured by obtaining images of the neurons every 5 minutes for a total of 25 minutes. At the end of the assay, all of the PAGFP within the targeted neuron was activated at 405 nm (Total). (B). The increase in the area containing mitochondria pool over the 25-minute period was plotted. 18 assays (n = 18) and 19 assays (n = 19) were performed in the absence and presence of NMDA, respectively.

Mitochondria undergo remodelling following overactivation of NMDA receptors

To determine whether mitochondria undergo swelling following NMDA induced excitotoxicity, we performed time lapse imaging of individual mitochondria in real time. Neurons were seeded on glass coverslips and transduced with a MitoDsRed lentivector at 1 DIV. The coverslip was mounted in a temperature controlled chamber and visualized at 7 DIV, as described above. Time lapse imaging revealed that, at steady state (prior to treatment), the mitochondria of 7 DIV primary neurons constantly undergo fission and fusion. A representation of these events is presented in **Figure 3.3A** (and supplemental movie 1), where two fission (**Figure 3.3A and B**) and two fusion (**Figure 3.3A and C**) events are captured simultaneously within 30 minutes in the given field. Following treatment with NMDA, there was a switch in mitochondrial dynamics whereby mitochondria undergo swelling and turn from tubular structures into round spheres following treatment with NMDA (**Figure 3.3D**, supplemental movie 2). Electron microscopy (EM) confirms mitochondrial morphology defects within the inner mitochondrial membrane and loss of the cristae structure following neuronal exposure to elevated levels of NMDA (**Figure 3.4**). Following 8 hrs of treatment with NMDA, mitochondrial cristae were widened and the overall mitochondrial morphology was significantly affected (**Figure 3.4B**) when compared to control (**Figure 3.4A**). Post 8 hrs of treatment, mitochondria exhibited aberrant morphology in which the cristae looked dilated (**Figure 3.4C**, 8 hrs NMDA).

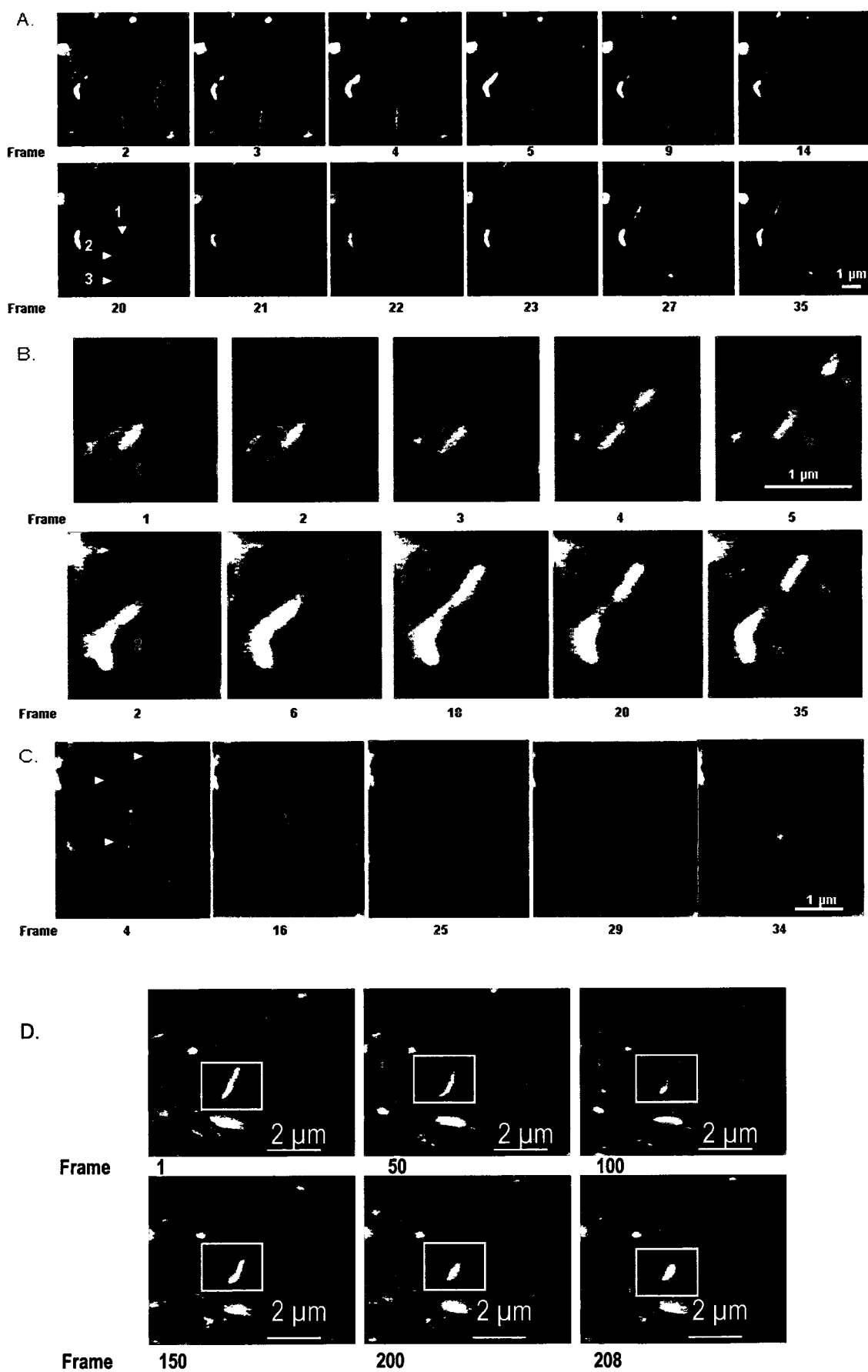


Figure 3.3

Figure 3.3 Mitochondria Dynamics in 7 DIV neurons in the absence and presence of NMDA

Primary neurons were seeded on coverslips and transduced with MOI 2 of a MitoDsRed lentivirus. Time Lapse Imaging was performed at 7 DIV, by mounting the slips in a temperature controlled chamber (37°C) in regular growth media supplemented with 20 mM HEPES (pH 7.4). Mitochondria were visualized in time lapse by exciting the MitoDsRed with 594 nm line of a multiple line Ar laser using LSM-510-Confocal-Laser-Scanning-Microscope (63X oil immersion objective, numerical aperture 1.4). Images were captured every 30 seconds. The frame number is indicated beneath each panel. **(A)** Demonstration of two fission (red arrow) and two fusion (yellow arrow) events captured within the same field (and supplemental movie 1). **(B)** Inset showing the fission events (numbered 1 and 2). **(C)** Inset shows the two fusion event (3 red arrows point at the 3 mitochondria that come together to fuse). **(D)** Mitochondria were visualized following 8 hrs of treatment with NMDA. Panel represents the Boxed mitochondrion (Boxed) that switches from a tubular morphology into a swollen round morphology (and supplemental movie 2).

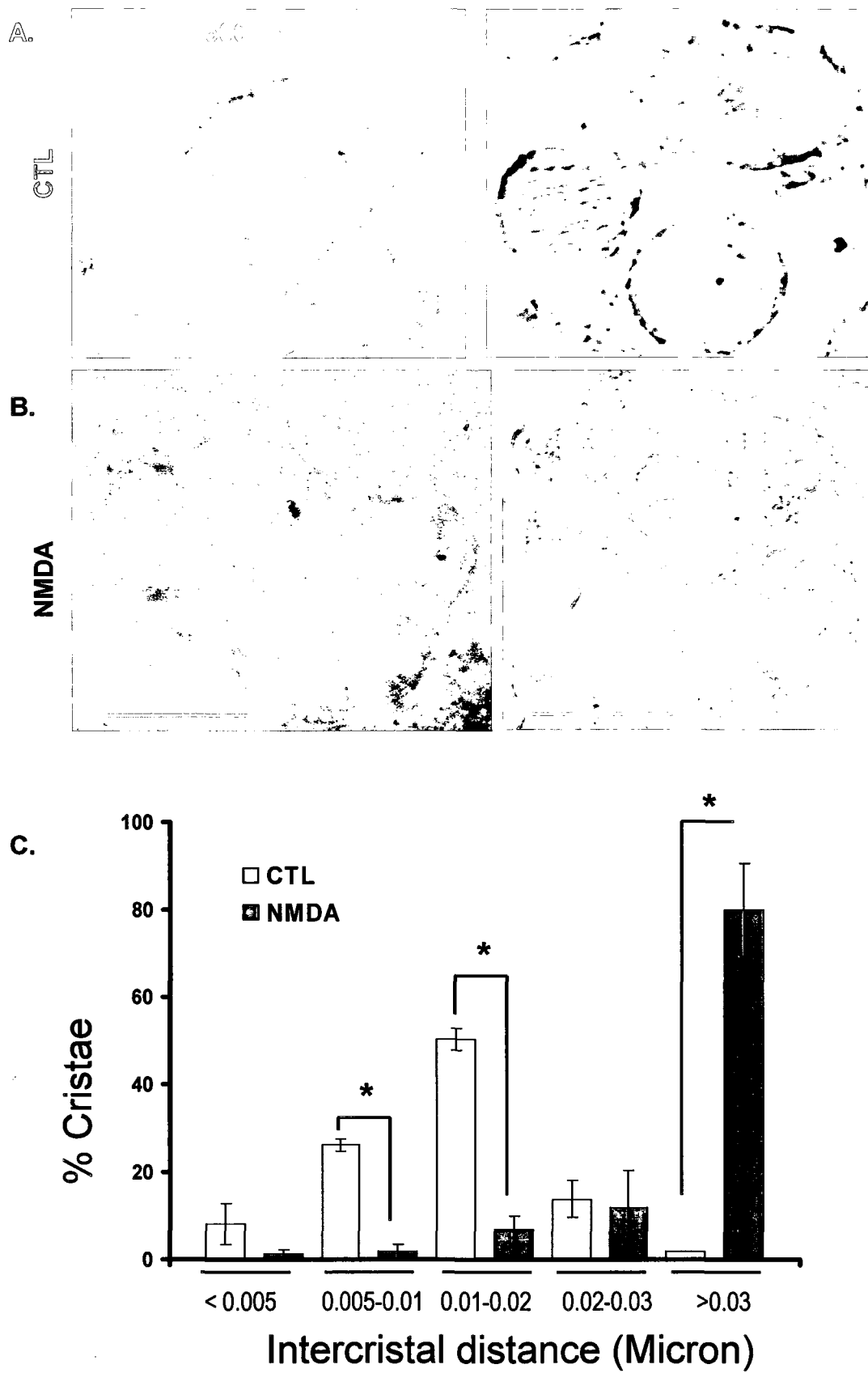


Figure 3.4

Figure 3.4. Mitochondrial morphology defects following NMDAR overactivation as depicted by EM

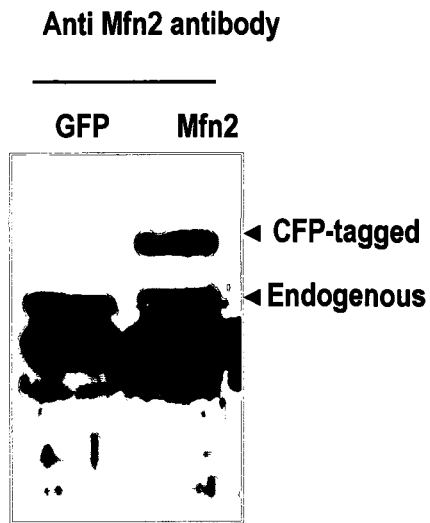
Neurons were plated on glass coverslips and treated with NMDA at different time point as described in Figure 1. Neurons were fixed with 4% glutaraldehyde and subjected to EM analysis. (A, B) Panel demonstrate mitochondrial morphology in the absence and presence of NMDA. (C) Intercristal distance was measured for mitochondria in the NMDA and CTL group and the difference was plotted as percentage of cristae in each length category.

Consistent with results obtained from fusion assays, these studies confirm that mitochondria cease fusion and undergo swelling following overactivation of NMDA receptors.

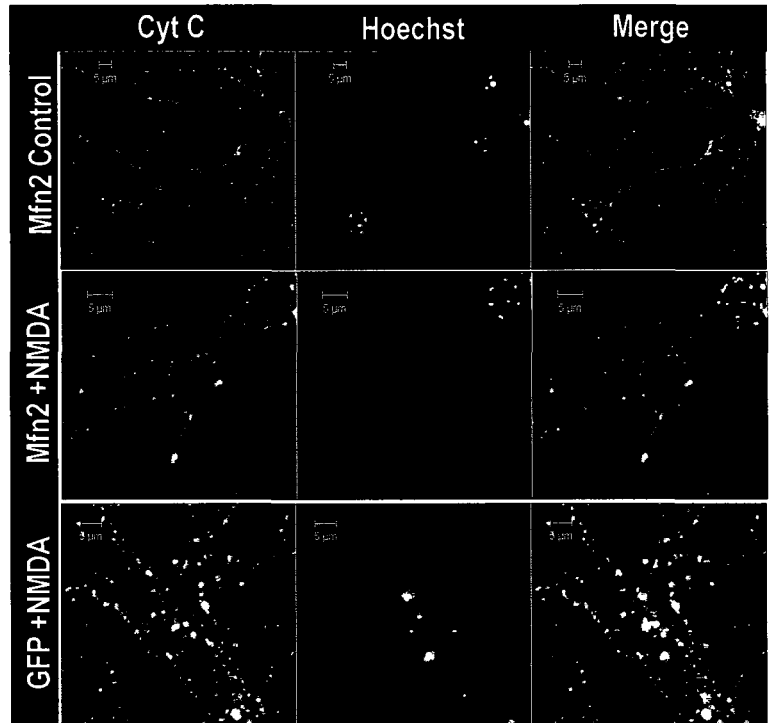
Role of the mitochondrial fusion components, Opa1 and Mfn2 in the NMDA induced excitotoxicity

Since our results reveal a defect in mitochondrial fusion in neurons exposed to NMDA, we asked if driving mitochondrial fusion by expressing fusion proteins could rescue mitochondrial morphology and thereby promote neuronal survival. We first asked whether the outer membrane GTPase, Mitofusin 2, could rescue mitochondrial morphology and cell death following exposure to NMDA. Primary neurons were infected with an adenovirus carrying an expression cassette for Mfn2 or GFP control at 5 DIV and then treated with NMDA at 7 DIV. The upregulation of Mfn2 was confirmed by Western blot (**Figure 3.5A**). To analyze mitochondrial morphology, neurons were stained with an antibody against *cytochrome c*, and 500 mitochondria were scored as above in each independent experiment (**Figure 3.5B**, $n = 3$). In untreated neurons, expression of Mfn2 resulted in a mitochondrial pool in which 35.76 ± 4.93 % had a length of greater than 3 μm relative to 6.93 ± 7.78 % of cells expressing GFP only (**Figure 3.5 C**). Mfn2 therefore induces elongation of mitochondria in primary neurons at 7 DIV ($n = 3$, $p < 0.03$). Following treatment with NMDA, mitochondrial fragmentation was evident in the GFP group with 52.69 ± 6.20 % having a length less than 0.5 μm . This fragmented phenotype following NMDA treatment was significantly attenuated in the neurons

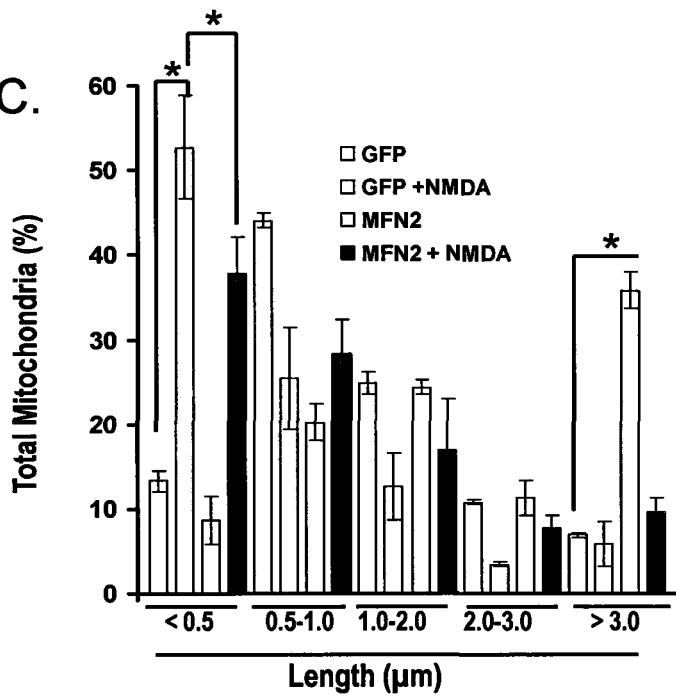
A.



B.



C.



D.

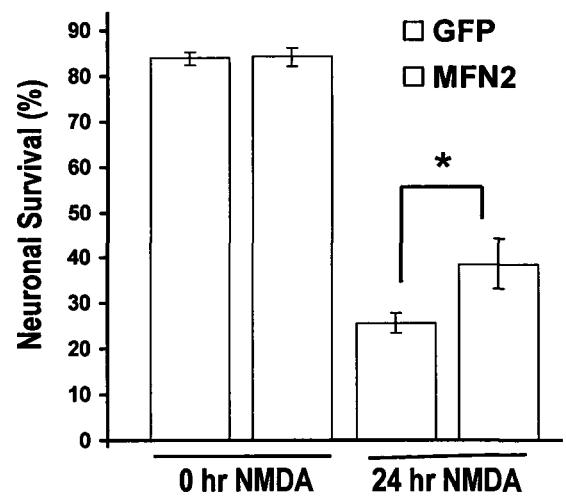


Figure 3.5

Figure 3.5. Mfn2 partially Rescues Mitochondrial morphology defects and protects neurons against excitotoxicity.

Primary neurons were infected with adenoviruses for Mfn2 or GFP control at 5 DIV. **(A)** Western blot depicts the expression of Mfn2. **(B)** Neurons were treated with NMDA at 7 DIV and stained with an antibody against *cytochrome c* and Hoechst. Mitochondrial morphology was analyzed in the infected neurons expressing either Mfn2 or GFP control. **(C)** Mitochondrial length was binned into different categories and quantified as described in figure 1. **(D)** Live neurons were scored in each group by scoring the live infected neurons remaining in the entire well following 24 hrs of treatment with NMDA. * demonstrate $p < 0.05$. 3 independent experiments (n =3)

expressing Mfn2 where 37.86 ± 4.25 % had mitochondria shorter than $0.5\mu\text{M}$ (**Figure 3.5C**, $p < 0.05$). To ask if upregulation of Mfn2 could restore neuronal survival, we scored the total number of live infected neurons remaining in the well following 24 hrs of treatment with NMDA. In the Mfn2 group $38.8 \pm 5.44\%$ of neurons were scored alive compared to 25.9 ± 2.26 % in the GFP treated neurons (**Figure 3.5D**, $p < 0.05$, $n = 3$). These results indicate that upregulation of Mfn2 could only provide a modest rescue of neuronal survival following NMDA toxicity. More importantly, these results point to other factors that may also be involved in this process. Given that we have found significant mitochondrial swelling and deformation of cristae structure, we asked if Opa1 function might be disrupted following NMDA exposure and be the key defect underlying loss of mitochondrial integrity.

Not only is Opa1 an important component of the mitochondrial fusion machinery(Chan, 2006), Opa1 is also essential for maintaining inner membrane structure (Frezza et al., 2006; Herlan et al., 2003). Opa1 is anchored to the inner membrane and proteolytically processed to produce functional long and short isoforms in the inter mitochondrial membrane space. A combination of both short and long isoforms is required to induce Opa1 oligomerization which is essential for maintaining mitochondrial cristae junctions (Frezza et al., 2006). To determine whether mitochondrial defects following excitotoxicity may be due to a perturbation of Opa1 oligomerization we examined the oligomerization of Opa1 following exposure to NMDA. We reasoned that if Opa1 is essential for maintaining cristae structure, then loss of Opa1 oligomers maybe a key contributor to mitochondrial swelling and fragmentation after excitotoxicity. In

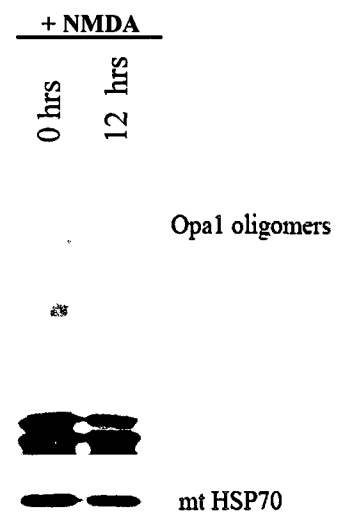
healthy cells, Opa1 forms oligomers between 200-240 kDa (**Figure 3.6**). Following 6 hrs of exposure to NMDA, levels of Opa1 oligomers was unaffected (**Figure 3.6**), consistent with the results obtained by EM analysis (data not shown) in which there was no change in cristae morphology. The levels of Opa1 oligomers were clearly reduced in a time dependent manner. Consistent with the loss of Opa1 oligomerization, electron microscopy revealed the deformation of cristae and loss of tight cristae junctions in cells exposed to NMDA. These findings demonstrate that excitotoxicity leads to the loss of Opa1 oligomers and suggest that disintegration of Opa1 oligomers may be a pivotal event in the onset of mitochondrial morphology defects and dysfunction.

To test the hypothesis that loss of Opa1 oligomerization is the underlying defect leading to mitochondrial dysfunction after excitotoxicity we asked if increasing Opa1 expression could preserve mitochondrial integrity and neuronal survival. To test this hypothesis, primary neurons were infected with adenovirus vectors carrying Opa1 or GFP at 5 DIV and then treated with NMDA at 7 DIV. Neurons were fixed following 24 hrs of treatment and stained with an antibody against MAP2 and Hoechst. The number of live infected neurons in the entire well was counted by identifying the cells that were: a) positive for MAP2, b) contained the fluorescence tag (Opa1), and c) had intact nuclei in the corresponding wells (**Figure 3.6D**). Following treatment with NMDA, 22.5 ± 2.49 % of GFP treated cells survived, whereas more than twice the cells survived in the Opa1 group ($51.98 \pm 4.17\%$). These results show that upregulation of Opa1 provides significant protection against NMDA induced excitotoxic cell death ($p < 0.02$, $n = 3$).

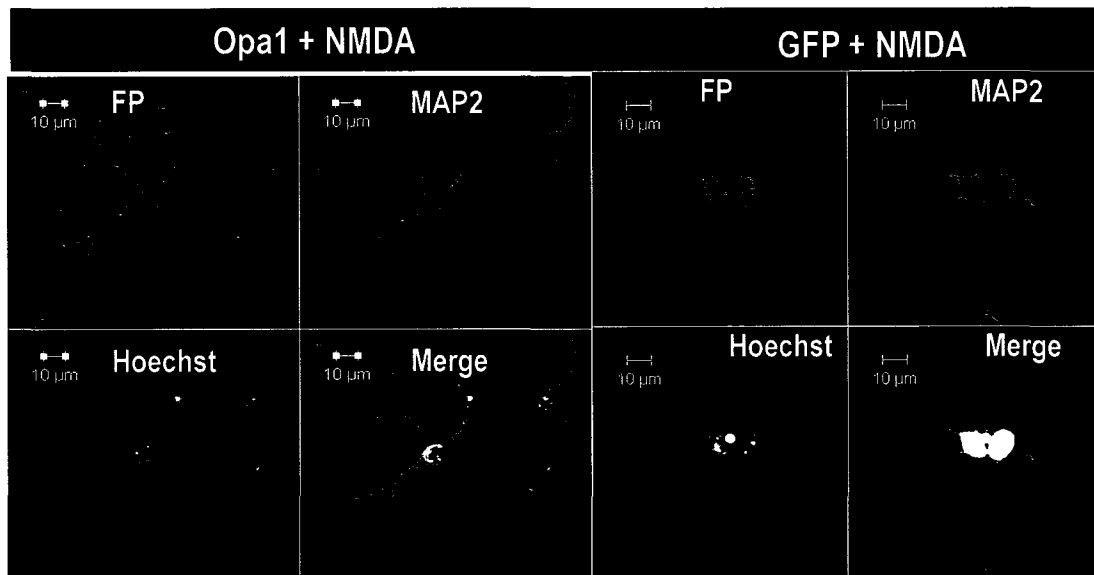
A.



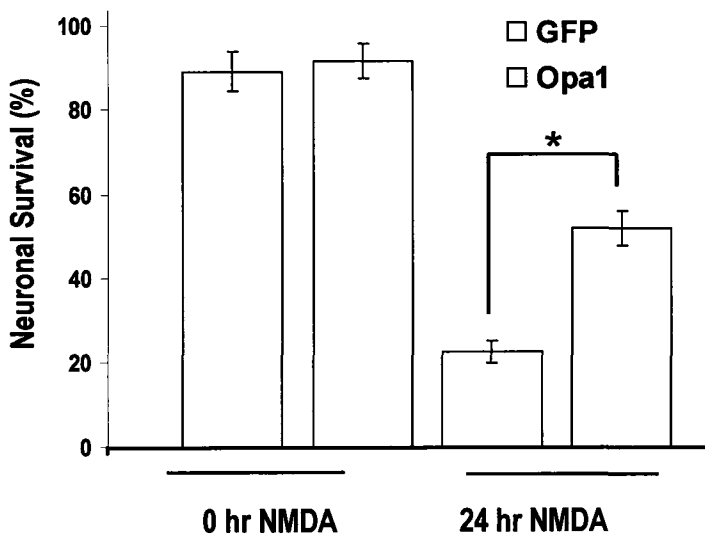
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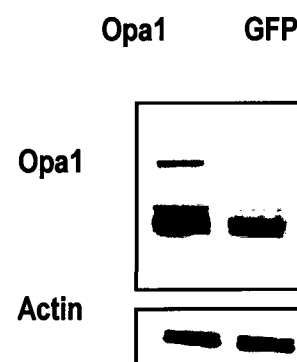


Figure 3.6

Figure 3.6. Opa1 promotes neuronal survival following NMDA induced neuronal death

(A-B). Primary neurons were treated with NMDA at different time points. Analysis of Opa1 oligomers was performed on isolated mitochondria of those neurons. Following subcellular fractionations, mitochondrial fractions were cross-linked prior to analysis of samples by SDS-PAGE. Membranes were blotted for an antibody against Opa1 and standardized by an antibody against mitochondrial HSP70. **(C).** Neurons were infected with an adenovirus for Opa1 or GFP control at 5 DIV and treated with NMDA at 7 DIV. Neurons were stained with Hoechst and antibodies against MAP2 and FP. Panel represent the live infected neurons in GFP and Opa1 groups. **(D)** Neuronal survival was assessed by counting the neurons positive for FP and MAP2 in the entire well following 24 hrs of treatment with NMDA. * demonstrate $p < 0.05$. 3 Independent experiments ($n = 3$). **(E)** Western blot demonstrate Opa1 expression.

We then asked whether upregulation of Opa1 could preserve mitochondrial morphology following excitotoxicity. Following NMDA exposure, cultures expressing GFP alone exhibited 45.5 ± 0.99 % of neurons with mitochondrial lengths less than $0.5 \mu\text{m}$ (**Figure 3.7 A, B**). In contrast, neurons expressing elevated levels of Opa1 had only 15.4 ± 3.75 % fragmented mitochondria with lengths less than $0.5 \mu\text{m}$ (**Figure 3.7B**). This indicates that Opa1 significantly rescues the mitochondrial morphology defect caused by overactivation of NMDA receptors ($p < 0.005$, $n = 3$, Figure 6A, B). These results demonstrate that Opa1 upregulation may protect against NMDA induced excitotoxicity by restoring mitochondrial architecture and ultimately mitochondrial function.

The results of our studies identify a mechanism implicating the disruption of Opa1 function in the onset of excitotoxic damage and point to the importance of the inner mitochondrial membrane dynamics in regulating the onset of cell death following in this mode of injury. These results also raise key questions regarding the mechanisms by which Opa1 oligomerization is controlled during neuronal injury. Identifying the mechanisms that trigger the disintegration of Opa1 oligomers may open new strategies to preserve mitochondrial integrity following injury.

Opa1 oligomerization is negatively regulated by calpain activation

As calpain activation through calcium influx is a major signaling pathway contributing to cell death following excitotoxicity, we asked whether blocking calpain activity might preserve Opa1 oligomerization and mitochondrial function. One of the calcium activated cysteine proteases that cleaves structural proteins and contributes to the

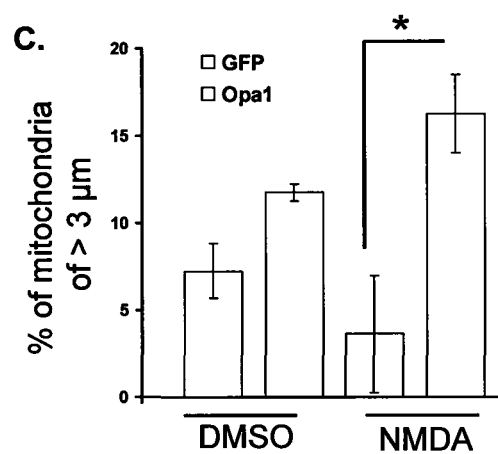
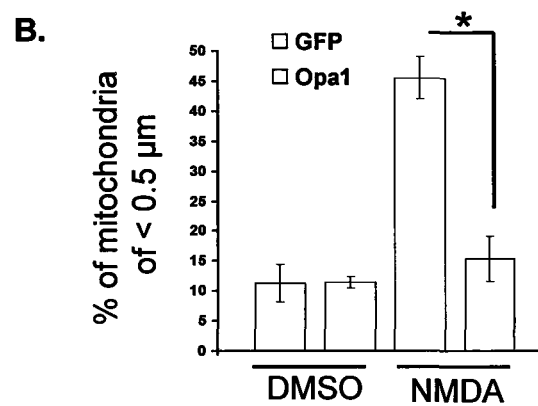
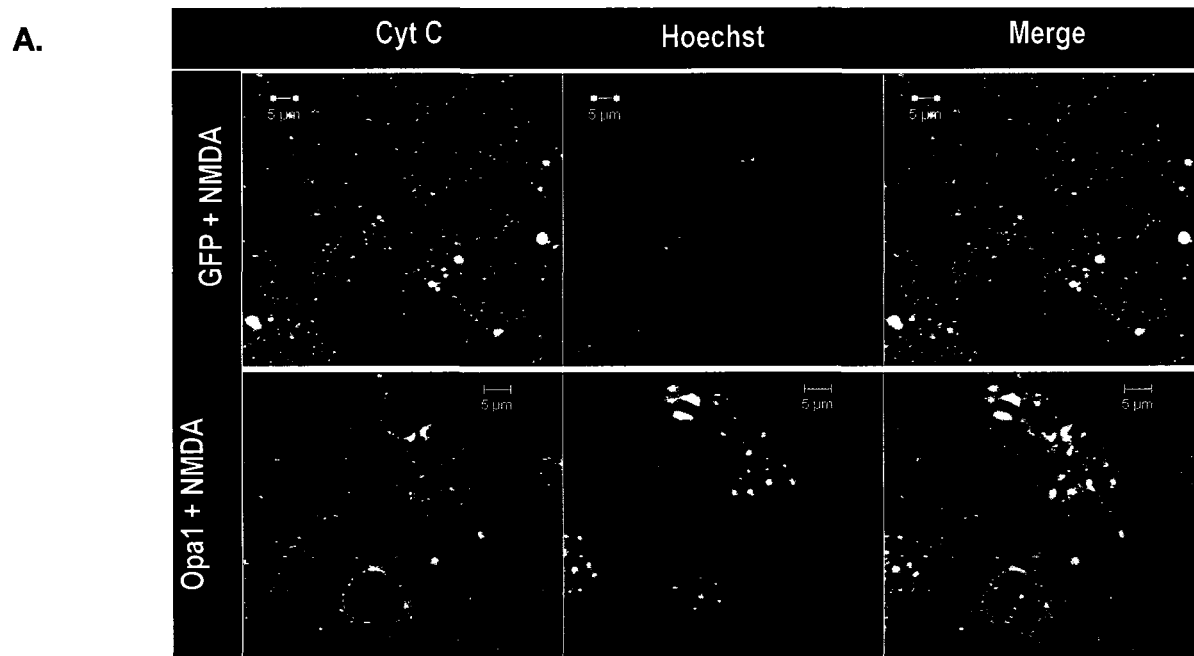


Figure 3.7

Figure 3.7. Opa1 rescues mitochondrial morphology defects following NMDA induced neuronal death.

(A). Neurons were infected and treated as described in figure 5. Mitochondrial morphology was evaluated by staining for cytochrome c antibody. (B) Percentage of fragmented mitochondria with a length of less than 0.5 μm was plotted. (C) Percentage of elongated mitochondria with a length of greater than 3 μm was plotted.

* demonstrate $p < 0.05$. Three independent experiments ($n = 3$)

release of AIF from the mitochondria in the nervous system is μ -calpain (Badugu et al., 2008; Cao et al., 2007; Norberg et al., 2008; Polster et al., 2005). μ -calpain contains an N-terminal mitochondrial targeting sequence and it is found in the innermembrane space fractions (Polster et al., 2005). We hypothesized that calpain activation might lead to the breakdown of Opa1 oligomers during excitotoxicity. To test this possibility, we asked if inhibiting calpain activity could rescue Opa1 oligomerization and mitochondrial morphology defects following excitotoxicity. An adenovirus expressing calpastatin, the endogenous inhibitor of calpain, was constructed. Primary neurons were infected with either calpastatin or GFP control at 5 DIV, treated with NMDA at 7 DIV, and analyzed for mitochondrial morphology at 8 DIV (**Figure 3.8A and B**). Staining with a *cytochrome c* antibody (**Figure 3.8A**) revealed that in the NMDA treated neurons expressing GFP, 52.69 ± 6.19 % of mitochondria exhibited a fragmented phenotype with a length of less than 0.5 μ m. In neurons expressing calpastatin, this number was reduced to 24.77 ± 2.50 % (**Figure 3.8B**). Calpastatin significantly rescued mitochondrial morphology defects induced by NMDA ($p < 0.006$, $n = 3$). Primary neurons expressing calpastatin also exhibited a significant rescue of neuronal death such that 76.02 ± 6.61 % of neurons were scored alive compared to 39.41 ± 4.31 in the GFP treated group ($p < 0.001$, $n = 3$, **Figure 3.8D**). To ask if calpastatin preserved Opa1 oligomerization, protein was extracted from GFP and calpastatin treated neurons following NMDA treatment, crosslinked and blotted for the detection of Opa1. Our results revealed that following NMDA treatment, Opa1 oligomers can be maintained in the presence of calpastatin (**Figure 3.8E**) or a pharmacological inhibitor of calpains (**Figure 3.8F**). These results suggest that calpains mediate the disintegration of Opa1 oligomers and the subsequent

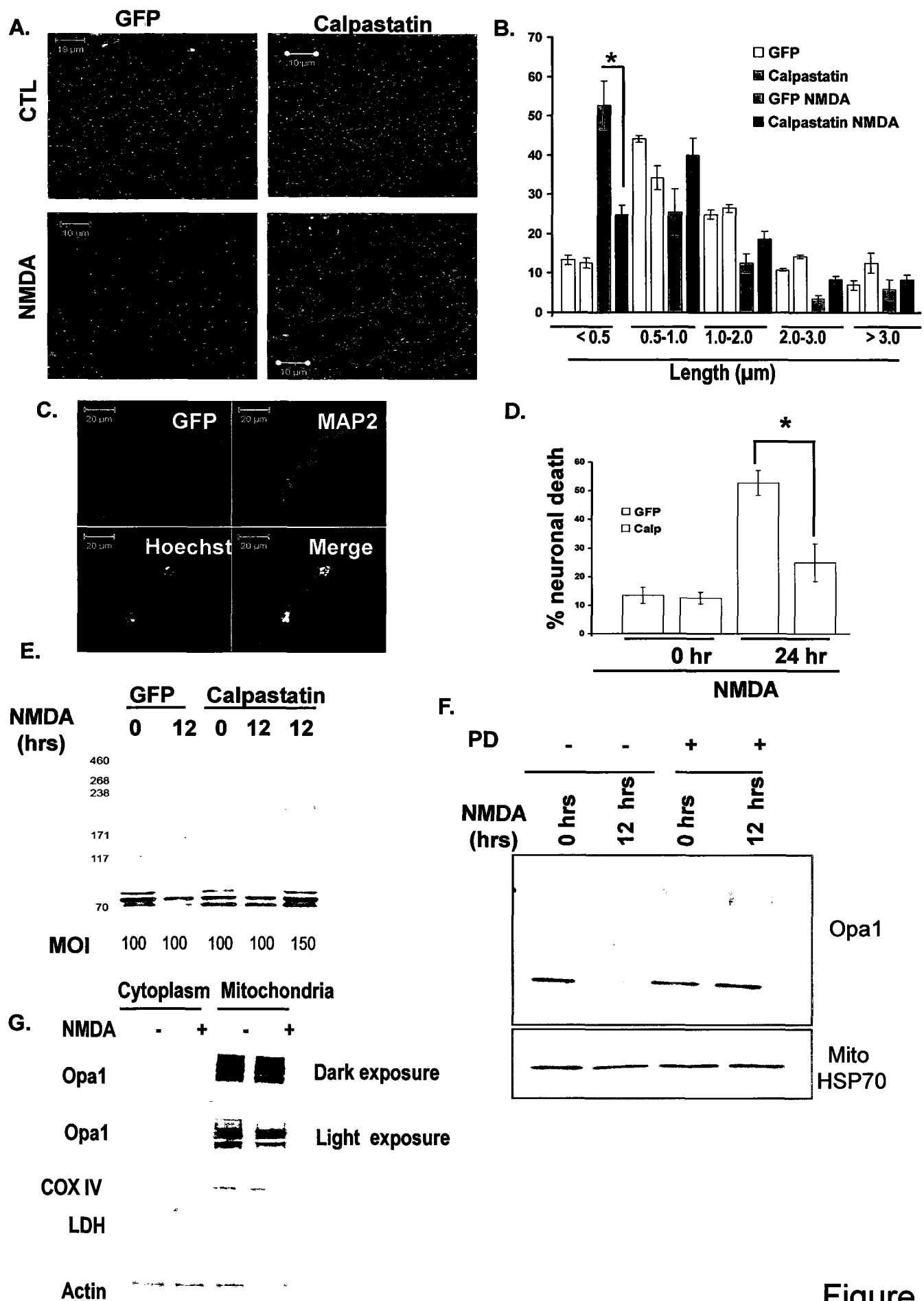


Figure 3.8

Figure 3.8. Calpastatin rescues mitochondrial morphology defects, Opa1 deoligomerization and neuronal death following excitotoxic injury.

(A) Neurons were infected with adenoviruses for calpastatin or GFP control at 5 DIV and treated with NMDA at 7 DIV. Mitochondrial morphology was assessed by staining with an antibody against cytochrome c. (B) Quantification of mitochondrial morphology in the absence and presence of NMDA. (C) Neurons were stained with Hoechst and an antibody against MAP2 to reveal live infected neurons. Panel represent the calpastatin infected neuron (D) The graph demonstrates percentage of neuronal death in calpastatin and GFP overexpressing neurons (n = 3). (E). Opa1 oligomers were analyzed in neurons expressing elevated calpastatin or GFP control in the absence and presence of NMDA, as described in Figure 6. (F) Opa1 oligomers were analyzed in neurons pretreated with pharmacological inhibitor of calpain (PD), followed by treatment with NMDA at 0 and 12 hours. (G) Neurons were subjected to subcellular fractionation to compare Opa1 release between NMDA treated and non treated control. Cox IV and LDH were used as mitochondrial and cytoplasmic controls, respectively.

disruption of mitochondrial architecture and function. These studies place calpains upstream of Opa1 in the calcium signaling pathway modulating mitochondrial integrity following excitotoxic injury.

To determine whether Opa1 indeed functions on the same pathway as calpain activation, we asked if simultaneous activation of both Opa1 and calpastatin in primary neurons could provide synergistic protection against cell death. If Opa1 and calpastatin function by the same mechanism, then no further protection would be expected. Alternatively, if they function by distinct pathways one would predict that protection would be enhanced by adding both Opa1 and calpastatin. Neurons were infected with calpastatin and either of Opa1, or GFP and compared to control neurons infected with LacZ and GFP. Our results indicate that there was no significant increase in protection against NMDA induced toxicity between neurons expressing both calpastatin and Opa1 (68.87 ± 6.87) versus neurons expressing calpastatin alone (70.9 ± 3.58 , **Figure 3.9C**). This suggests that calpastatin and Opa1 are acting on a common pathway modulating mitochondrial inner membrane structure in response to calcium influx following excitotoxic injury.

To further define the molecular mechanism by which Opa1 and calpain interact we asked if Opa1 is required for neuroprotection mediated by calpastatin-mediated inhibition of calpain. To answer this question, mitochondrial morphology and neuronal survival were compared in the neurons expressing calpastatin in the absence and presence of Opa1. An adenovirus for ShOpa1 was used to knock down Opa1. Mitochondrial

morphology was assessed in Opa1 knockdown neurons in the absence and presence of NMDA (**Figure 3.9 A & B**). $52.5 \pm 3.5\%$ of mitochondria exhibited a length of less than $0.5 \mu\text{m}$ in the ShOpa1 group in the non treated group (**Figure 3.9A**). These results indicate dramatic fragmentation of mitochondria in Opa1 knockdown neurons in the absence of any cell death stimuli. Interestingly, ectopic expression of calpastatin failed to efficiently rescue the mitochondrial defects (**Figure 3.9A & B**) when Opa1 was absent, indicating that Opa1 is required for calpastatin-mediated protection. These results suggest that the control of Opa1 oligomerization is a downstream target for calpains in the regulation of mitochondrial integrity during NMDA-mediated cell death. Similarly, neurons expressing calpastatin and GFP exhibited $70.9 \pm 3.58\%$ survival rate while those expressing both calpastatin and ShOpa1 exhibited a significant reduction in survival at $51.8 \pm 3.64 \%$ (**Figure 3.9D**). Together, our results demonstrate that the control of Opa1 activity is a critical downstream target of the calcium signaling cascade, and that activation of calpains following NMDA-mediated calcium influx results in neuronal loss, in part, through modulation of Opa1 function and disturbance in the inner mitochondrial membrane dynamics.

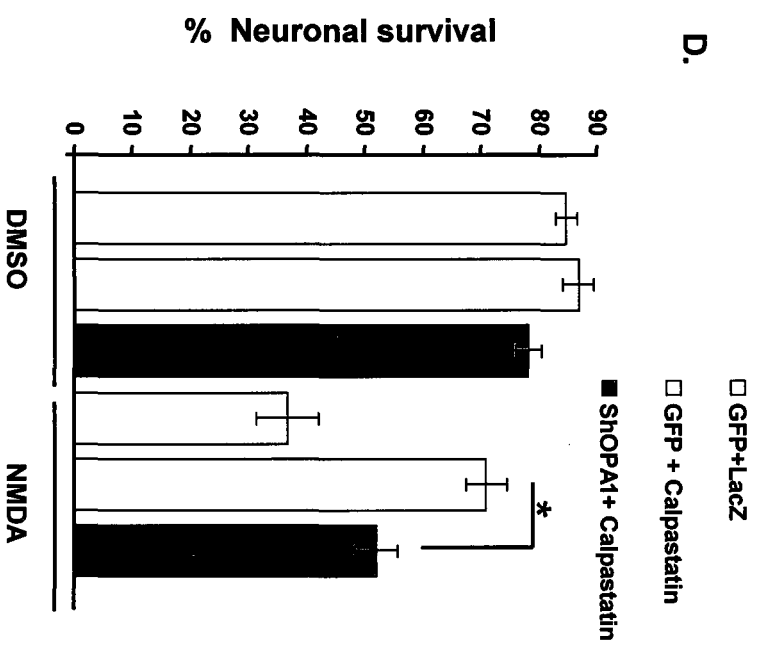
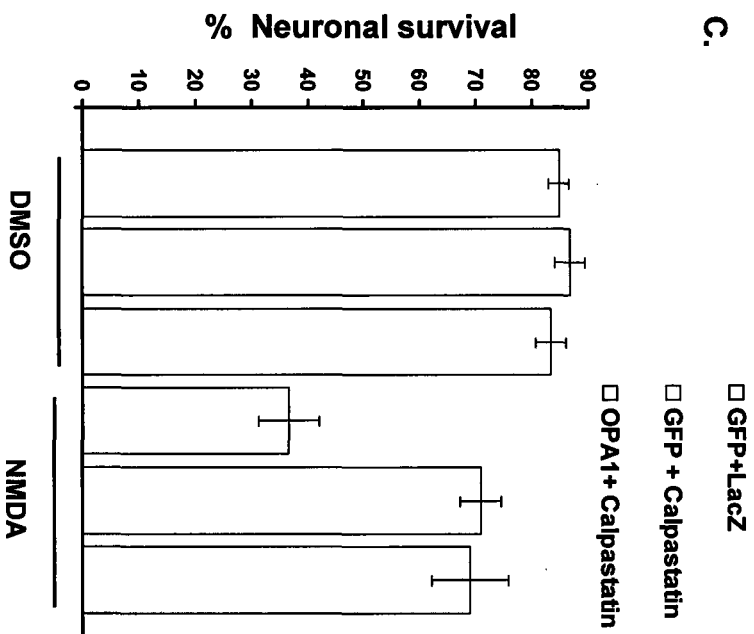


Figure 3.9

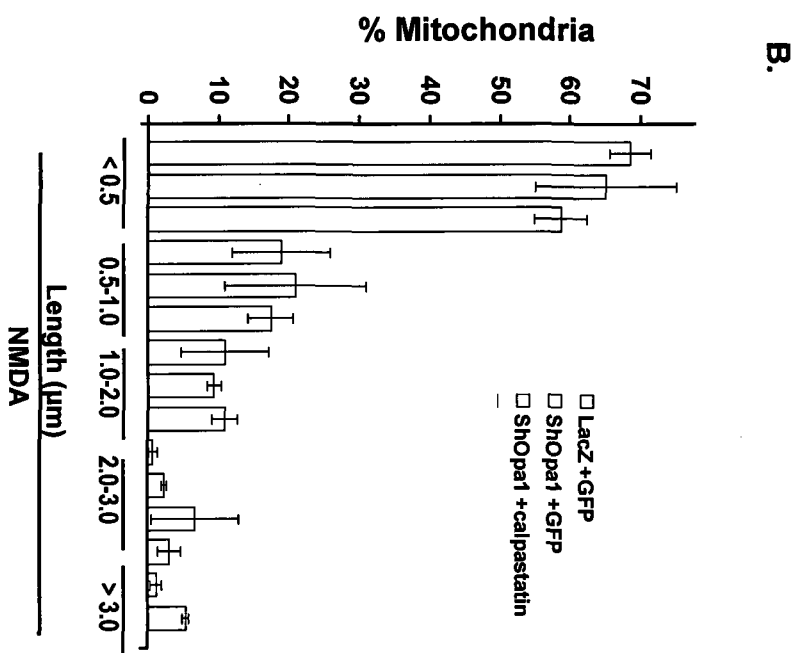
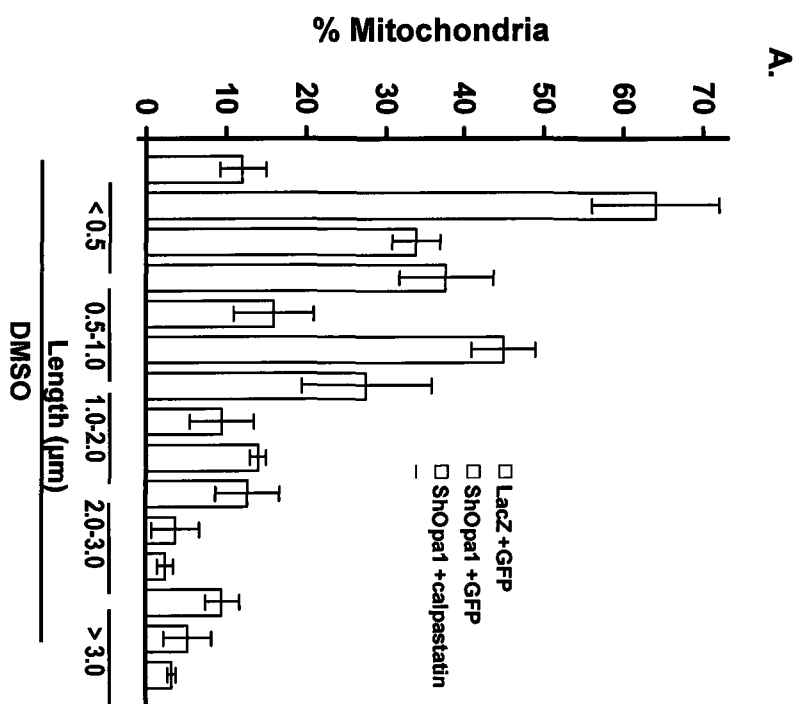


Figure 3.9. Opa1 confers its function downstream of calpain activation

(A) Mitochondrial morphology was assessed in Opa1 knock down neurons in the absence and presence of calpastatin as described in figure 1. (B) Mitochondrial morphology was assessed in neurons expressing ShOpa1 and either of calpastatin or GFP and was compared to control neurons expressing LacZ and GFP following treatment with NMDA (C) Neuronal survival was plotted in neurons expressing calpastatin alone (calpastatin + GFP) or calpastatin and Opa1 (calpastatin + Opa1) at 0 and 24 hrs following treatment with NMDA. The results were compared with control neurons expressing LacZ + GFP. (D) Neuronal survival was plotted in neurons expressing calpastatin alone (calpastatin + GFP) or calpastatin and ShOpa1 (calpastatin + ShOpa1) at 0 and 24 hrs following treatment with NMDA. The results were compared with control neurons expressing LacZ + GFP.

* demonstrate $p < 0.05$. 3 Independent experiments (n = 3)

3.5. DISCUSSION

The results of these studies have culminated in a number of key findings: First, mitochondrial morphology defects are identified as key early events in the progression of excitotoxic neuronal injury. Second, defects in the fusion machinery and an aberrant inner mitochondrial dynamics are identified as two major causes of mitochondrial dysfunction. Third, we show that mitochondrial remodeling as a result of loss of Opa1 oligomers are hallmarks of excitotoxic injury. Activation of Opa1 promotes neuronal survival by maintaining the cristae structure. Importantly, inhibition of calcium activated calpain proteases restores Opa1 oligomers, mitochondrial morphology and neuronal survival. Our findings are the first to identify Opa1 as a key regulator of neuronal fate following calcium deregulation and these studies underscore the potential therapeutic value of Opa1 in a broad range of neurological diseases.

Mitochondria were previously shown to undergo dramatic fragmentation in response to cell death stimuli that induces classical apoptotic cell death. Mitochondrial fission were identified as an early event in these modes of injury and ectopic expression of the fusion proteins, Mfn1 and Mfn2, or down-regulation of key fission protein, Drp1, were shown to rescue mitochondrial morphology defects and neuronal death (Barsoum et al., 2006; Jahani-Asl et al., 2007). Excitotoxicity is associated with increased calcium influx and activation of calcium activated effector proteins. The role of mitochondrial dynamics in excitotoxic injury remains largely unknown. Although, our knowledge on the significance of fusion machinery in regulation of calcium signaling is limited, recent

studies demonstrate that the function of Drp1 is regulated by calcium activated kinases and phosphatases (Cereghetti et al., 2008; Cribbs and Strack, 2007; Han et al., 2008). In addition, Mfn2 was reported to bridge mitochondria and ER and to regulate the calcium transients between these two organelles (de Brito and Scorrano, 2008). These studies suggest a strong link between components of mitochondrial dynamics and calcium signaling. We therefore aimed to decipher the role of key regulators of mitochondrial fusion machinery, Mfn2 and Opa1, in the context of excitotoxicity and as relevant to neuronal fate.

Mitochondrial morphology switches following NMDA induced neuronal death was examined. NMDA receptors which are highly permeable to calcium ions can be fully activated following membrane depolarization. Mitochondria switch from tubular rod shape morphology into fragmented round circles following NMDAR activation. In addition, results with fusion assay indicate that mitochondrial fusion rate is significantly blocked following 8 hrs of treatment with NMDA. These data for the first time shows that the defects in the rate of mitochondrial fusion are major contributor of mitochondrial defects following excitotoxicity. Mitochondrial fusion requires initial physical proximity which maybe mediated by cytoskeletal elements followed by mitofusin's mediated docking and tethering. Whether the fusion defects following excitotoxicity could be ascribed to functional defects in key components of mitochondrial fusion or to defects in cytoskeletal tracks and motor proteins responsible for mitochondrial transport requires further investigation. Although rate of mitochondrial fusion seems to play a significant role in mitochondrial dysfunction, other possibilities can not be excluded. For example,

Drp1 mediated fission could be in part accountable for the dramatic fragmented phenotype observed following excitotoxicity. This hypothesis is supported by studies in cell lines where an increase in mitochondrial calcium influx results in mitochondrial fragmentation (Breckenridge et al., 2003; Cereghetti et al., 2008; Hom et al., 2007), in part rescued by down-regulation of Drp1 (Hom et al., 2007). A recent study identified a subfamily of Ras GTPases (Miro GTPases) in control of mitochondrial fission and fusion (Macaskill et al., 2009; Wang and Schwarz, 2009). Miro GTPases that are localized on outer mitochondrial membrane contain two EF-hand calcium binding domains and regulate mitochondrial dynamics in response to calcium oscillations (Saotome et al 2008). These results identify a delicate mechanism through which mitochondrial adaptor proteins and cytoskeletal elements can profoundly impact mitochondrial fission and fusion events.

In addition, the EM studies and many videos of mitochondria taken following NMDAR overactivation demonstrate that mitochondria undergo swelling. This data suggests possibility of a Drp1 independent fragmentation. In support of this, the BH3 only protein, BIK, acting on ER which facilitate calcium release from ER and uptake into mitochondria results in cristae remodeling and cytochrome c mobilization independent of fission activity of Drp1 (Germain et al., 2005).

In many types of acute neuronal injury such as stroke, a primary cause of death is the exposure to reactive oxygen species, and generation of ROS is one of the pathways by which neurons die following excitotoxicity. For example, reactive oxygen species are

generated at the mitochondria level as a result of ionic imbalance, defects in oxidative phosphorylation and ATP depletion. Also, high levels of nitric oxide are generated through PSD95-mediated activation of neuronal nitric oxide synthase [reviewed in (Arundine and Tymianski, 2004)]. Nitric oxide serves as a substrate for the production of highly reactive peroxynitrites which can ultimately cause DNA damage and PARP over-activation. We have previously shown that ectopic expression of wild type mitofusin 2 promoted neuronal survival following treatment of primary neurons with hydrogen peroxide (Jahani-asl et al 2007). We, therefore, examined whether Mfn2 can rescue neuronal death following NMDA induced neuronal death. Our results show that Mitofusin 2 provides mild protection against NMDA induced neuronal death. This may be due to existence of regulatory mechanisms that induce neuronal death independently or upstream of ROS generation. In a recent study Mfn2 was reported to bridge ER and mitochondria and facilitate mitochondrial calcium influx from ER stores (de Brito et al 2008). It is therefore possible that Mfn2 may have a dual function in excitotoxic injury where it contributes to calcium mediated toxicity through ER pathway while rescuing the toxic effect of ROS production.

Importantly, our results show a critical role for Opa1 in the regulation of neuronal survival following NMDA toxicity. Opa1 confers two fold protections and almost completely rescues mitochondrial morphology defects. Our results indicate that Opa1 confers its protection through a mechanism beyond inducing mitochondrial fusion. We have also shown that Opa1 oligomers that are essential regulators of cristae morphology are lost following excitotoxicity. A recent report showed that activation of glutamate

receptors results in release of Opa1 from mitochondria in ischemic rat retina (Ju et al 2008). Whether Opa1 release results in loss of Opa1 oligomers or Opa1 release is a consequence of loss of oligomers requires further investigation.

More importantly, we have identified a novel signaling pathway that puts Opa1 downstream of calpain activation. Calcium accumulation in the mitochondria can result in neuronal death in a number of different ways. These include ATP depletion due to collapse of mitochondrial oxidative phosphorylation, dysfunction of ATP dependent calcium pumps, generation of reactive oxygen species, and an increase in mitochondrial membrane permeability which can lead to further release of apoptosis inducing factor1 (AIF). Importantly, release of AIF from the mitochondria was recently ascribed to activation of Calpain I (μ -calpain). Calpain I has been shown to be targeted to mitochondria via its N terminus mitochondrial targeting sequence (Badugu et al 2008). In addition, An atypical calpain, calpain 10, was recently localized to the mitochondrial matrix (Arrington et al., 2006). Overexpression of this calpain resulted in mitochondrial swelling and dysfunction through the cleavage of Complex I subunits and activation of MPT. Our data show that while inhibition of calpain activity rescues neuronal death and mitochondrial morphology defects; this effect is significantly attenuated upon down-regulation of Opa1. Calpain activity has also been reported to be required for truncation and release of AIF from isolated mitochondria in vitro (Polester et al 2005) and in vivo model of ischemia (Cao et al 2007). In addition, an increase in intracellular calcium levels was reported to be essential for both activation of mitochondrial calpains and AIF release following Bax dependent mechanisms of cell death (Norberg et al 2008). Whether

calpain directly target Opa1 or whether it modulates Opa1 activity through AIF remains to be studied. Finally, recent studies has revealed an essential role for the ATP synthase complex in cristae biogenesis (Amutha et al., 2004; Paumard et al., 2002). Dimerization of ATP synthase is required for normal cristae morphology perhaps due to induction of membrane curvature (Minauro-Sanmiguel et al., 2005). The yeast homologue of Opa1, Mgm1, is required for oligomerization of ATP synthase and therefore it regulates ATP production. Subcellular localization studies have demonstrated Mgm1/Opa1 in association with cristae (Olichon et al., 2002). Although Mgm1 and Opa1 are reported to be different, it is possible that disruption of ATP synthase through loss of Opa1 leads to cristae remodeling.

In conclusion, our studies identify a link between Opa1, a key component of mitochondrial dynamics and NMDA induced toxicity. We propose a model in which overactivation of NMDAR followed by calcium influx results in loss of Opa1 oligomers and deformation of cristae junctions downstream of calpain activation. This can explain the mitochondrial morphology defects, disruption of respiratory chain complex and ATP depletion following excitotoxicity. Since Opa1 belongs to family of large GTPases and GTPases are highly accessible to therapeutic modulation, Opa1 is a promising target for the study of novel pharmaceutical agents to rescue neuronal death following acute neuronal injury.

3.6. EXPERIMENTAL PROCEDURES

Virus construction- Cerebellar granule neurons (CGNs) were cultured from CD1 mice at postnatal day 7 or 8 as described previously (Fortin et al., 2001). Recombinant adenoviral vectors carrying human mitofusin 2 and Opa1 expression cassettes were prepared using AdEasy system, as described previously (He et al., 1998). Lentiviruses vectors carrying photoactivable GFP Ornithine Carbamyl Transferase (PA-OCT-GFP), and MitoDsRed, were prepared using ViraPower Lentiviral Expression System (Invitrogen Cat no. K4990-0). For studies with lentiviruses, neurons were transduced with MOI 2-3 at 1 day in Vitro (DIV) and for studies with adenoviruses, neurons were infected at 5 DIV.

NMDA treatment, mitochondrial length measurements and cell viability assays – To model excitotoxicity, neurons were treated with 100 μ M NMDA and 10 μ M Glycine at 7 DIV (1hrs, 100 μ M in the 4 well plates and 150 μ M in the 96 well plates), after which they were switched to conditioned media from parallel cultures. The cells were first stained with antibodies against MAP2, fluorescence tag (FP) and Hoechst. The number of live infected neurons in the entire well was counted by identifying the cells that were a) positive for MAP2, b) contained the fluorescence tag, and c) had intact nuclei in the corresponding wells.

Time-Lapse Imaging and Mitochondrial fusion assay - CGNs were seeded on 4 well plates (Nalgene Nunc International, Rochester, NY) with attached glass coverslips coated with poly D Lysine (Fisher Scientific, Whitby, ON), and transduced with the

photoactivable GFP tagged to Ornithine Carbamyl Transferase (PA-GFP-OCT) and/or MitoDsRed lentiviruses, at 1 DIV. The coverslip was mounted in a temperature controlled chamber (37°C) in regular growth media supplemented with 20 mM HEPES [pH 7.4], and visualized with an LSM-510-Confocal-Laser-Scanning-Microscope (Axiovert 200), with a 63X oil immersion objective, numerical aperture 1.4. For time lapse studies the MitoDsRed was excited with 594 nm line of a multiple line Ar laser. For fusion assay, the PA-GFP-OCT construct was photoactivated with 405 laser (3 scan, 50-60% intensity) and the spreading of the signal was imaged every 5 minutes using a 488 nm line for a total of 25 minutes. The fusion rate was expressed as percentage of the area with pixel intensity at 25 minutes over that of 0 minutes (0 min = the signal detected right after photoactivation).

Analysis of Opa1 Oligomers

Mitochondria were isolated from CGNs after treatment with NMDA as described previously (Germain et al. 2002). Isolated mitochondria were resuspended in isotonic buffer with 1mM EDC (Pierce) and incubated for 30 minutes. Crosslinking was quenched by adding β -mercaptoethanol, followed by centrifugation at 10 000rpm for 10 minutes. The mitochondrial pellet was resuspended in 1X LDS Running buffer (Invitrogen) and samples were analyzed on a 3-8% Tris-Acetate gradient gel (Invitrogen) followed by western blotting for opa1 (1:1000, BD bioscience) and mitochondrial Hsp70 (1:7000, ABR Bioreagents).

Immunofluorescence - At each time point, neurons were fixed for 30 minutes with ice cold 4 % paraformaldehyde in 1X Phosphate Buffered Saline (1XPBS) and then rinsed twice with 1XPBS. Cells were permeabilized with 300 µl of ice-cold 0.4% Triton-X in 1XPBS for 10 min. Cells were stained with the primary antibodies in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed 3 x 5 min with ice cold 1XPBS. Cells were incubated with the secondary antibodies in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed for 5 minutes and stained with Hoechst for 5 minutes. Following Hoechst staining, neurons were washed with 1X PBS for 3 x 5 min and mounted. Representative samples were photographed using a Zeiss 510 Meta (Oberkochen, Germany) confocal microscope (Oberkochen, Germany). The mitofluor Red was excited with the 543 nm line of He/Ne laser, the Alexa 647 was excited with the 633 nm line of He/Ne red laser, the Hoechst was excited with the 405 nm and the GFP was excited with the 488 nm line of He/Ne green laser. All images shown demonstrate cells that are representative of moderate infection efficiencies, and that have been obtained from at least three independent experiments.

EM analysis- Neurons were seeded on glass coverslips and treated with NMDA at different time points. Neurons were fixed with 4% glutaraldehyde and subjected to EM analysis as previously described (Cheung et al., 2006).

Quantification and statistical analysis The data represent the mean and standard deviation from three independent experiments (n = 3). n stands for each independent experiment. p values were obtained using two-way ANOVA and student t tests. A p value <0.05 was considered significant and was indicated on the graphs by an asterisk.

3.7. Acknowledgment

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3.8. List of Abbreviations

Opa1, Optic Atrophy 1; Mfn2, Mitofusin 2; Drp1, Dynamin related protein 1; NMDA, N-methyl-D-aspartic acid; NMDAR, NMDA receptors; ER, Endoplasmic reticulum; AIF1, Apoptosis inducing factor 1; Cyt c, Cytochrome c; PSD95, Post synaptic density protein 95; GFP, Green fluorescence protein; YFP, Yellow fluorescence protein; OCT, Ornithine carbamyl transferase; PAGFP, photoactivable GFP; Sh Opa1, Short hairpin Opa1; ROS, reactive oxygen species; EM, electron microscopy; Calp, Calpastatin; DIV, Days in vitro.

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CHAPTER 4

Cdk5 Regulates Dynamin related protein 1 (Drp1) and Mitochondrial Fission in Postmitotic Neurons

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(In Preparation)

4.1. STATEMENT OF AUTHOR CONTRIBUTION

I performed 95 % of the experimental work and all the statistical analyses. Experimental design and interpretation of the results was a joint effort between myself, Dr. Slack and Dr. Park. I wrote the manuscript myself with input from Dr. Slack. Dr. Rashidian assisted me with *in vitro* kinase assays in which radioactive ATP was used. Dr. Irrcher contributed to *in vivo* studies for this work which is still in progress (not included in this chapter). Dr. Ishihara kindly provided the antibody for phosphorylated Drp1 which he generated in his own laboratory.

4.2. ABSTRACT

In neurons, mitochondrial fission is required to regulate multiple processes such as synaptic activity and neuronal death. Dynamin related protein 1 (Drp1) cycles between cytosol and mitochondria to regulate mitochondrial fission. Initial recruitment of Drp1 on the mitochondrial outer membrane is a rate limiting step and is tightly regulated by Drp1's post translational modifications. Here, we identify a neuronal intrinsic mechanism for the regulation of Drp1 mediated fission in post mitotic neurons. First, we show that Drp1 is a target of Cyclin dependent kinase 5 (Cdk5), an important player in synaptic plasticity, neuronal development and neuronal death. Second, using Drp1 wild type and mutant constructs, we have identified that Cdk5 phosphorylates Drp1 at a conserved serine residue (Ser585 in rat/ 616 in human). Ectopic expression of a cytoplasmic Cdk5 significantly results in mitochondrial fragmentation in primary neurons suggesting that Cdk5 tightly regulates mitochondrial morphology. In contrast, cdk5 ablated neurons exhibit an elongated mitochondrial morphology. Finally, using imaging and biochemical techniques, we demonstrate that Cdk5 induces the recruitment of Drp1 from the cytoplasm to the mitochondria. These studies identify an important mechanism through which Cdk5 may regulate mitochondrial morphology at steady state and suggests a mechanism by which Cdk5 may affect neuronal death through the regulation of mitochondrial network.

4.3. INTRODUCTION

Dynamin related protein 1 (Drp1) is a cytoplasmic protein belonging to the family of large GTPase. Drp1 is a key regulator of mitochondrial fission in different organisms ranging from yeast to higher eukaryotes. The extent of Drp1-mediated-mitochondrial fission determines mitochondrial morphology and movement and is tightly linked to the physiological states of the cell. For example, expression of a dominant negative Drp1 (Drp1-K38A) in primary neurons has been shown to reduce the content of dendritic mitochondria and result in loss of synapses and spines (Li et al., 2004). While Drp1 is required during synaptogenesis to regulate neuronal development and synaptic strength, excessive Drp1 mediated mitochondrial fission is linked to neuronal death (Barsoum et al., 2006). Neuronal mitochondrial undergo early dramatic fragmentation in response to DNA damage and oxidative stress (Jahani-Asl et al., 2007). Importantly, down regulation of Drp1 confers neuroprotection against nitric oxide induced neuronal loss (Barsoum et al., 2006). Regulation of Drp1 activity is therefore a critical event in the nervous system and the extent of Drp1 activity seems to determine the boundaries of life or death in neurons.

Upon mitochondrial fission, Drp1 is recruited from the cytosol to the mitochondria at the site of scission. It is proposed that similar to dynamin, Drp1 forms a ring like structure on the mitochondrial surface where the energy from its GTP hydrolysis drives membrane constriction (Soubannier and McBride, 2009). Drp1 recruitment is tightly regulated by post translational modifications. For example, Drp1 is shown to be

sumoylated by small ubiquitin like modifier (SUMO) protein (Harder et al., 2004). Upon its sumoylation, Drp1 becomes stably associated with the mitochondrial membranes (Wasiak et al., 2007; Zunino et al., 2007). Importantly, multiple kinases have been recently identified to regulate Drp1 phosphorylation at various sites (Cereghetti et al., 2008; Chang and Blackstone, 2007b; Cribbs and Strack, 2007; Han et al., 2008; Taguchi et al., 2007). Drp1 phosphorylation status was shown to have a profound impact on overall mitochondrial integrity and cell fate. For example, during mitosis, Drp1 is phosphorylated by cyclin dependent kinase 1 (Cdk1) at a conserved serine residue (Ser585 rat/Ser616 human) (Taguchi et al., 2007). Although this phosphorylation is suggested to contribute to mitochondrial segregation in cycling cells, the mechanism that links Drp1 phosphorylation to mitochondrial fission at this site remains to be studied. Interestingly, in post mitotic neurons Drp1 has been identified to be phosphorylated by Ca^{++} /Calmodulin-dependent protein kinase I α (CamKI α) (Han et al., 2008). CamKI α -mediated Drp1 phosphorylation induced mitochondrial fragmentation in response to calcium influx associated with neuronal activity.

Cdk5 plays an important role in the regulation of synaptic plasticity under steady state and during neuronal death in response to stress [reviewed in (Zhang and Herrup, 2008)]. Steady state mitochondrial fission is required for increasing synapse number and strength (Li et al., 2004) whereas injury induced mitochondrial fission is required to induce neuronal loss (Barsoum et al., 2006). These studies suggest a functional interaction between Cdk5 and mitochondrial fission. In addition, cyclin dependent kinase 5 (cdk5) was reported to regulate neuronal death and mitochondrial morphology (Meuer

et al., 2007; Sung et al., 2008). Overexpression of p25/Cdk5 complex was shown to induce mitochondrial fragmentation and cell death (Meuer et al., 2007; Sung et al., 2008). The mechanism by which Cdk5 induced mitochondrial fission is presently unknown.

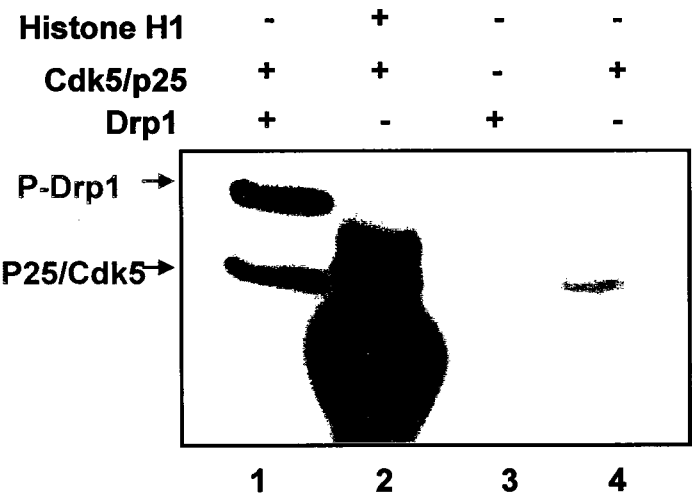
In order to identify neuronal mechanisms that regulate Drp1 activity, we tested the hypothesis that cdk5 is a direct regulator of mitochondrial shape and function. We identified that Cdk5 is a regulator of Drp1 activity through its phosphorylation at Ser585 (in rat, Ser616 in human). Importantly, increased expression of cytoplasmic Cdk5 induced dramatic mitochondrial fragmentation while expression of a dominant negative Cdk5 mutant (DnCdk5) resulted in mitochondrial elongation. Loss and gain of function studies indicated that Cdk5 is a key regulator of Drp1 recruitment from the cytoplasm to the mitochondria. In summary the results of our study identifies a novel mechanism by which Cdk5 may regulate mitochondrial morphology in postmitotic neurons.

4.4. Results

Drp1 is a direct target of cyclin dependent kinase 5 (Cdk5)

Similar to other cyclin dependent kinases, Cdk5 alone shows no kinase activity and its association with a regulatory substrate is required for its activation. Of the non cyclin proteins that are identified to target and activate Cdk5 are p35 (in physiological state) and its cleavage product p25 (in response to neuronal injury)[reviewed in (Dhavan and Tsai, 2001)]. To test whether Drp1 is a direct target of Cdk5, *in vitro* kinase assays were carried out using recombinant Drp1 protein and Cdk5/p25 active complex. 2 µg of recombinant Drp1 protein was incubated with 0.2 µg of Cdk5/p25 complex in the presence of 10 µCi of radiolabeled ATP ($[\gamma\text{-}^{32}\text{P}]$ ATP, 30° C, 30 minutes, **Figure 4.1A**). Histone H1 was used as a positive control (lane 2, Figure 1.A). Our results clearly demonstrated that Cdk5/p25 complex directly targets Drp1 and results in its phosphorylation. In the absence of Cdk5 active complex (lane 3) no band appeared for the wild type (wt) recombinant Drp1 while in the presence of active Cdk5 complex (lane 1) two bands appeared with one of them shifted upwards, indicative of phosphorylation (Arrow labeled P-Drp1 in Figure 1A). The lower band is phosphorylated Cdk5/p25 which also appeared when Cdk5/p25 was incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP in the absence of substrate (lane 4). We next asked whether the Cdk5/p35 complex which is active in steady state in post mitotic neurons could also target and phosphorylate Drp1. To this end the *in vitro* kinase assays were performed in the presence of Cdk5/p35 in comparison with Cdk5/p25 (**Figure 4.1B**). Our results show that both complexes result in phosphorylation of Drp1 *in vitro* (Lane 5 and 6, Figure 4.1B).

A.



B.

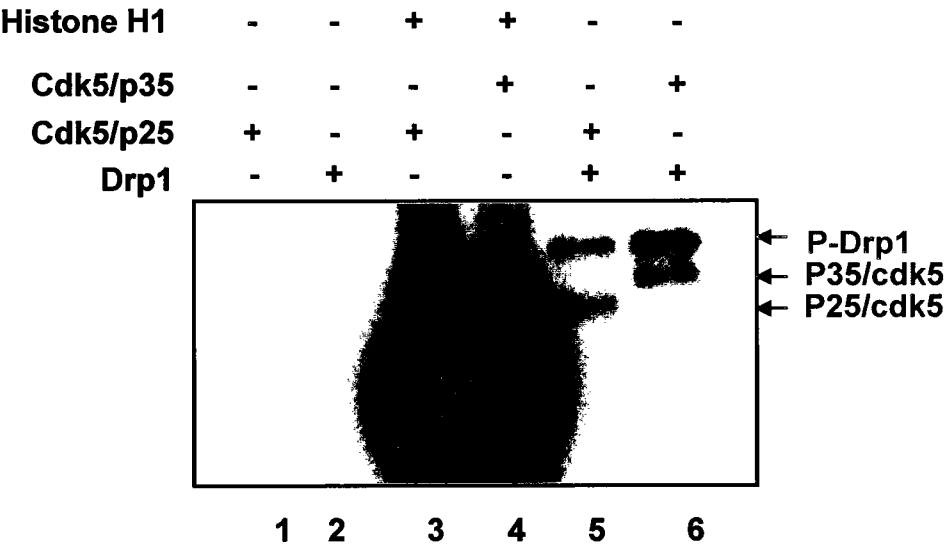


Figure 4.1

Figure 4.1. In vitro kinase assay identifies Drp1 as a substrate of Cdk5

Wild type recombinant rat Drp1 was incubated with radiolabeled ATP ($[\gamma\text{-}^{32}\text{P}]$ ATP, in kinase assay buffer, 30° C, 30 minutes) in the absence and presence of either of Cdk5/p25 active complex (Panel A) or Cdk5/p35 and /p25 active complex (Panel B). Histone H1 was used as a positive control. Drp1 alone was used as negative control. Red arrow illustrates a band shift demonstrating Drp1 phosphorylation (labeled P-Drp1). Blue arrow illustrates phosphorylated Cdk5.

Cdk5 phosphorylates Drp1 at a conserved Serine residue

We next sought to identify the phosphorylation site(s) within Drp1 protein that is/are targeted by Cdk5 active complexes. Cdk5 is a proline-directed kinase that phosphorylates serine and threonines on the consensus sites (S/T)PX(K/H/R). A proline residue at position +1 is required and a basic amino acid at position 3 is preferred (Beaudette et al., 1993). Drp1 has multiple recognition sites for Cdk5 (**Figure 4.2**). Of these sites, Ser585 was previously shown to be phosphorylated by Cyclin B1/Cdk1. Since, Cdk5 and Cdk1 have similar substrate specificity, we first asked whether Ser585 (Rat, Ser616 human, **Figure 4.2**) is targeted. Recombinant wild type rat Drp1 and S585A mutant Drp1 (in which Ser was mutated to Ala) were incubated in the presence of Cdk5/p35 (**Figure 4.3A**) and Cdk5/p25 complexes (**Figure 4.3B**) (Kinase assay buffer, 30° C, 30 minutes). Following analysis by autoradiography, the wild type recombinant Drp1 revealed presence of two bands in the presence of active Cdk5 (Lane 6 Figure 3A and Lane 8 of Figure 3B) . In the mutant recombinant Drp1 (S585A), however, the upwardly shifted band, which is indicative of phosphorylation, completely disappeared (Lane 7 Figure 3A and lane 6 figure 3B). These results identify S585 as the only Cdk5 recognition site. To assure that substrate saturation did not contribute to these differences, the *in vitro* kinase assays were carried out with 4 µg of the mutant Drp1 as opposed to 2 µg in the wt group (See CBB stain, **Figure 4.3**).

```

      20          40          60          80          100
DRP1a_Mu : HEALIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGVVTRRPLILQLVHVS EDKRRTTGEEN-----PATUNNKHNLKRGVEAE : 101
DRP1b_MS : HEALIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGVVTRRPLILQLVHVS EDKRRTTGEEN-----GVEAE : 99
DLPI-fat : HEALIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGVVTRRPLILQLVHVS EDKRRTTGEENKFSQSNPATUNNKHNLKRGVEAE : 107
DRP1-1-hu : HEALIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGIVTRRPLILQLVHVS EDKRRTTGEEN-----GVEAE : 99
DRP1-2-hu : HEALIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGIVTRRPLILQLVHVS EDKRRTTGEEN-----GVEAE : 99
DRP1-3-hu : HEALIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGIVTRRPLILQLVHVS EDKRRTTGEEN-----GVEAE : 99
      HRAIPVINKLQDVNTVCGADIQLPQIVVVGTSQSSKSSVLESVGRDLLPRGTGVVTRRPLILQLVHVS EDKRRTTGEEN GVEAE

      120          140          160          180          200
DRP1a_Mu : EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE : 200
DRP1b_MS : EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE : 195
DLPI-fat : EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE : 214
DRP1-1-hu : EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE : 195
DRP1-2-hu : EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE : 195
DRP1-3-hu : EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE : 195
      EWGKFLHTKXKLYTDFDEIRQEIENETERISGNHKGVSPEPIHLKVFSPNVNLTLDVLPQMTKVPVGDQPKDIELQIRELILRFISNPNSIILAVTAANTOMATSE

      220          240          260          280          300          320
DRP1a_Mu : ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP : 315
DRP1b_MS : ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP : 302
DLPI-fat : ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP : 321
DRP1-1-hu : ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP : 302
DRP1-2-hu : ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP : 302
DRP1-3-hu : ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP : 302
      ALKISREVDPPGRRRLAVITKLDLMDAGTDAMDVLHGRVIVPKLGIIGVNRSGOLDINNNKSVTDSIRDEYAFLOKKYPSLANRNGTKYLARTLNRLLMHHIROCLP

      340          360          380          400          420
DRP1a_Mu : ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP : 422
DRP1b_MS : ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP : 409
DLPI-fat : ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP : 420
DRP1-1-hu : ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP : 409
DRP1-2-hu : ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP : 409
DRP1-3-hu : ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP : 409
      ELKTRINVLAAQYQSLNLSYGEVDDKSATLLQLITKFATEYCNITECTAKYIETSELCCGARIYI FHETFGRTLESVDPLGGLNTIDILTAIRNATGPRPALEVP

      440          460          480          500          520
DRP1a_Mu : EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR : 529
DRP1b_MS : EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR : 516
DLPI-fat : EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR : 535
DRP1-1-hu : EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR : 516
DRP1-2-hu : EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR : 516
DRP1-3-hu : EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR : 516
      EVSFELLVKRQIKRLEEPSLRCELVHEEMQRIIQHCNSYSTQELLRPKLHDAIVEVVTCLLRKRLPVTNEMVHNLVAIELAYINTKHPDFAACGLMNNHIEBQR

      540          560          580          600          620          640
DRP1a_Mu : RNRLARELPSAERSDK-----VSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV : 599
DRP1b_MS : RNRLARELPSAERSDK-----VSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV : 586
DLPI-fat : RNRLARELPSAERSDK-----VSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV : 605
DRP1-1-hu : RNRLARELPSAERSDKSSKVPFALAPASQEPSPASASADGKLQDSRRKSTKNVSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV : 623
DRP1-2-hu : RNRLARELPSAERSDK-----LIQDSRRKSTKNVSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV : 597
DRP1-3-hu : RNRLARELPSAERSDK-----VSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV : 586
      RNRLARELPSAERSDK VSSAGGGIGDQOEPTTGNWRGHLKTSKAEELLAEBSKSIIPIMPASPOKGHAV

      660          680          700          720          740
DRP1a_Mu : NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI : 706
DRP1b_MS : NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI : 693
DLPI-fat : NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI : 712
DRP1-1-hu : NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI : 730
DRP1-2-hu : NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI : 704
DRP1-3-hu : NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI : 693
      NLLDVPVPVARKLSAREQRDCEVIERLISYFLIVRKNIQDSVPKAVMHFLVNHVKDTLOSELVGOLYKSSLLDOLLTESEDMAQRKKEAADMLKALOGASQIIAEI

      *
DRP1a_Mu : RETHLW : 712
DRP1b_MS : RETHLW : 699
DLPI-fat : RETHLW : 716
DRP1-1-hu : RETHLW : 736
DRP1-2-hu : RETHLW : 710
DRP1-3-hu : RETHLW : 699
      RETHLW

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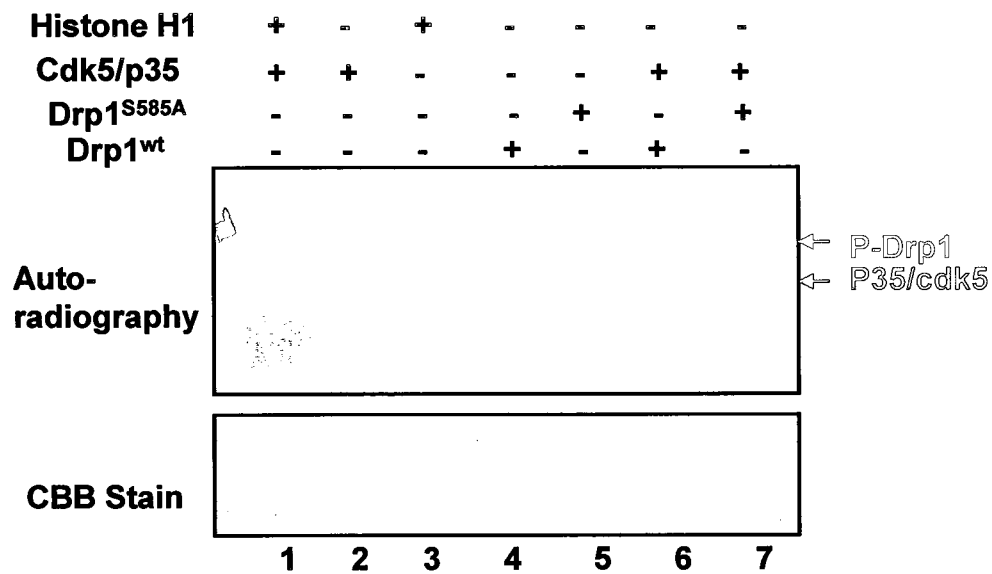
IPIMPASPOKGHAV

Figure 4.2

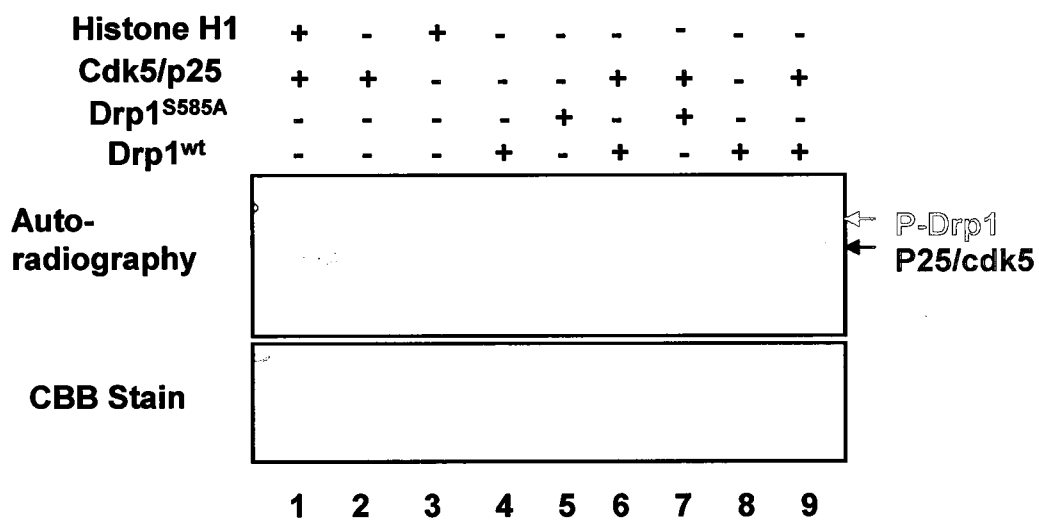
Figure 4.2. Sequence alignment of Drp1 isoforms

Drp1 protein sequences from different species were retrieved from the National Center for Biotechnology Information (NCBI) database and multiple sequence alignment was carried out using ClustalX, a windows interface of ClustalW (Thompson et al. 1994).

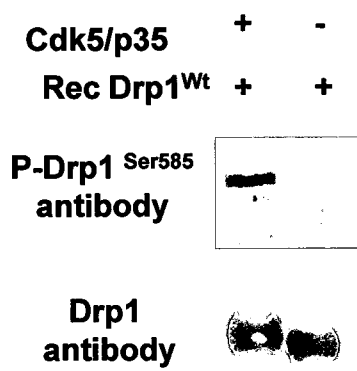
A.



B.



C.



D.

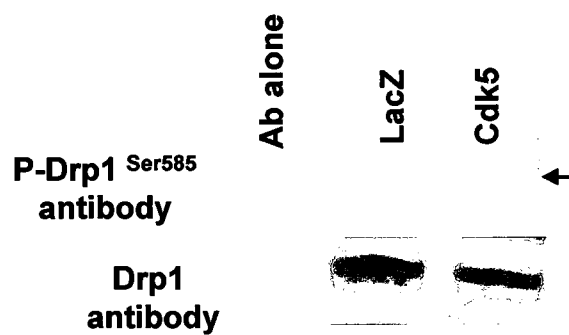


Figure 4.3

Figure 4.3. Cdk5 phosphorylates Drp1 on a conserved serine residue (Ser585) in vitro and in primary neurons.

In vitro kinase assays were performed on wild type recombinant Drp1 and a mutant Drp1 in which Ser585 was mutated to alanine (Panel A and B). The kinase assays were carried out as outlined in Figure 1. The CBB stain is included to illustrate substrate concentration. *In vitro* kinase assays were repeated and the results were analyzed by Western blot using a specific antibody that recognized Drp1 phosphorylation at Ser 585 (P-Drp1 Ser585 antibody, Panel C). Cerebellar granule neurons infected with a cytoplasmic Cdk5-NES or LacZ control were assessed for Ser585 phosphorylation using an antibody against phosphorylated Drp1^{Ser585} (Panel D). To standardize the Western blots were reprobbed with an antibody against Drp1 to depict total Drp1 that was loaded in each lane.

To further confirm that Ser585 is targeted by Cdk5, we aimed at detecting the phosphorylated band by Western blot analysis using a specific Drp1 antibody which is only capable of recognizing phosphorylated Drp1 at Ser585 (Taguchi et al., 2007). *In vitro* kinase assays were carried out as mentioned above except that [γ -³²P] ATP was replaced by 100 μ M of cold ATP (**Figure 4.3.C**). Phosphorylation of recombinant wild type Drp1 was detected by this antibody in the presence of Cdk5. Importantly the band was completely absent in the absence of Cdk5. The blots were re-probed with an antibody against Drp1 to depict the total Drp1 in each lane (**Figure 4.3C**). Together these results confirm that Drp1 is phosphorylated by Cdk5 at the conserved Ser585.

We next asked if the Ser585 is phosphorylated endogenously in post mitotic neurons. To answer this question, primary granule neurons were seeded and infected at the time of plating with adenoviruses vectors carrying expression cassettes for either a cytoplasmic Cdk5 [i.e. Cdk5 tagged to Nuclear Exclusion Signal (NES; Cdk5-NES)] or LacZ control. 3 days *in vitro* (DIV) neurons were harvested, subjected to immunoprecipitation (IP) with a Drp1 antibody, and analyzed by Western blot using phosphorylated Drp1 at Ser585 (p-Drp1^{Ser585}) antibody. The membranes were next re-probed with a Drp1 antibody to detect the total amount of Drp1 in each lane. Our results demonstrated that Cdk5 phosphorylates Drp1 in steady state in the absence of any cell death stimuli (**Figure 4.3D**). p-Drp1^{Ser585} antibody detected a band in the lac Z control group. This band was significantly intensified upon overactivation of Cdk5 (n= 2). Since the in house antibody is not a very strong one, presently these experiments will be repeated using higher number of neurons as a substrate for the IP studies.

Cdk5 regulates mitochondrial morphology in postmitotic neurons

To assess the physiological relevance of Cdk5 mediated Drp1 phosphorylation, mitochondrial morphology was next evaluated in neurons expressing Cdk5 constructs. Neurons were infected with adenoviruses carrying either of a wild type Cdk5-NES (wtCdk5-NES), a dominant negative mutant form of Cdk5-NES (DnCdk5-NES) or a GFP control, at the time of plating. Neurons were fixed and stained with an antibody against *cytochrome c* to assess mitochondrial morphology. Analysis of mitochondrial length depicted the involvement of Cdk5 in the regulation of mitochondrial morphology (**Table 4.1**). In primary neurons, mitochondria exhibited variable lengths. For comparative analysis, mitochondrial length was therefore binned into different length categories of < 0.5, 0.5-1.0, 1.0-2.0, 2.0-3.0 and > 3 μm . In the Cdk5 activated neurons, mitochondrial length was significantly shifted from elongated to more fragmented (**Figure 4.4**). $33.34 \pm 5.9\%$ of mitochondria exhibited a length of less than 0.5 μm (fragmented) in the Cdk5 group compared with control GFP at 13.95 ± 1.15 ($p < 0.001$, $n = 3$, **Figure 4.4**, **Table 4.1**). In contrast, mitochondria in the Cdk5 ablated neurons exhibited elongated length compared to control. $26.97 \pm 1.7\%$ of mitochondria maintained a length of greater than 3 μm (elongated) compared to GFP control at $8.9 \pm 0.97\%$. Similarly the pool of mitochondria in the length categories of 2-3 and 1-2 μm was significantly greater in the DnCdk5 group compared to control (**Table 4.1**, **Figure 4.5**). In contrast percentage of fragmented mitochondria (mitochondria exhibiting a length of < 0.5 μm) was significantly attenuated in the cdk5 ablated neurons compared with GFP control (**Table 4.1**). Together these studies demonstrate that Cdk5 regulates mitochondrial length in post mitotic neurons in the steady state.

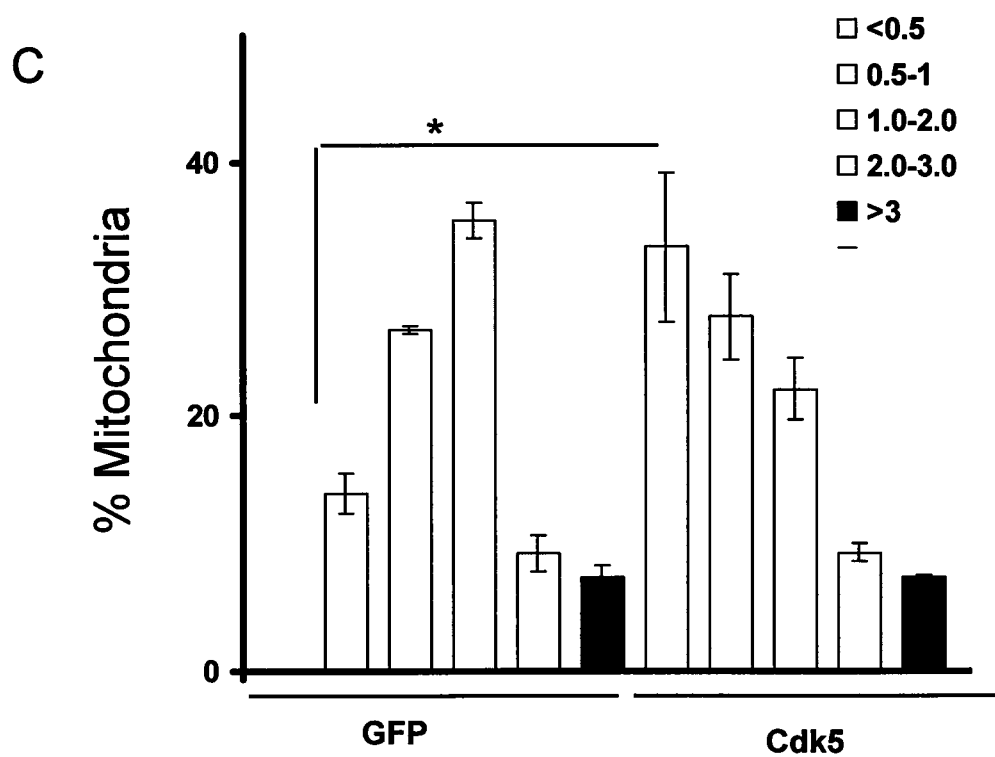
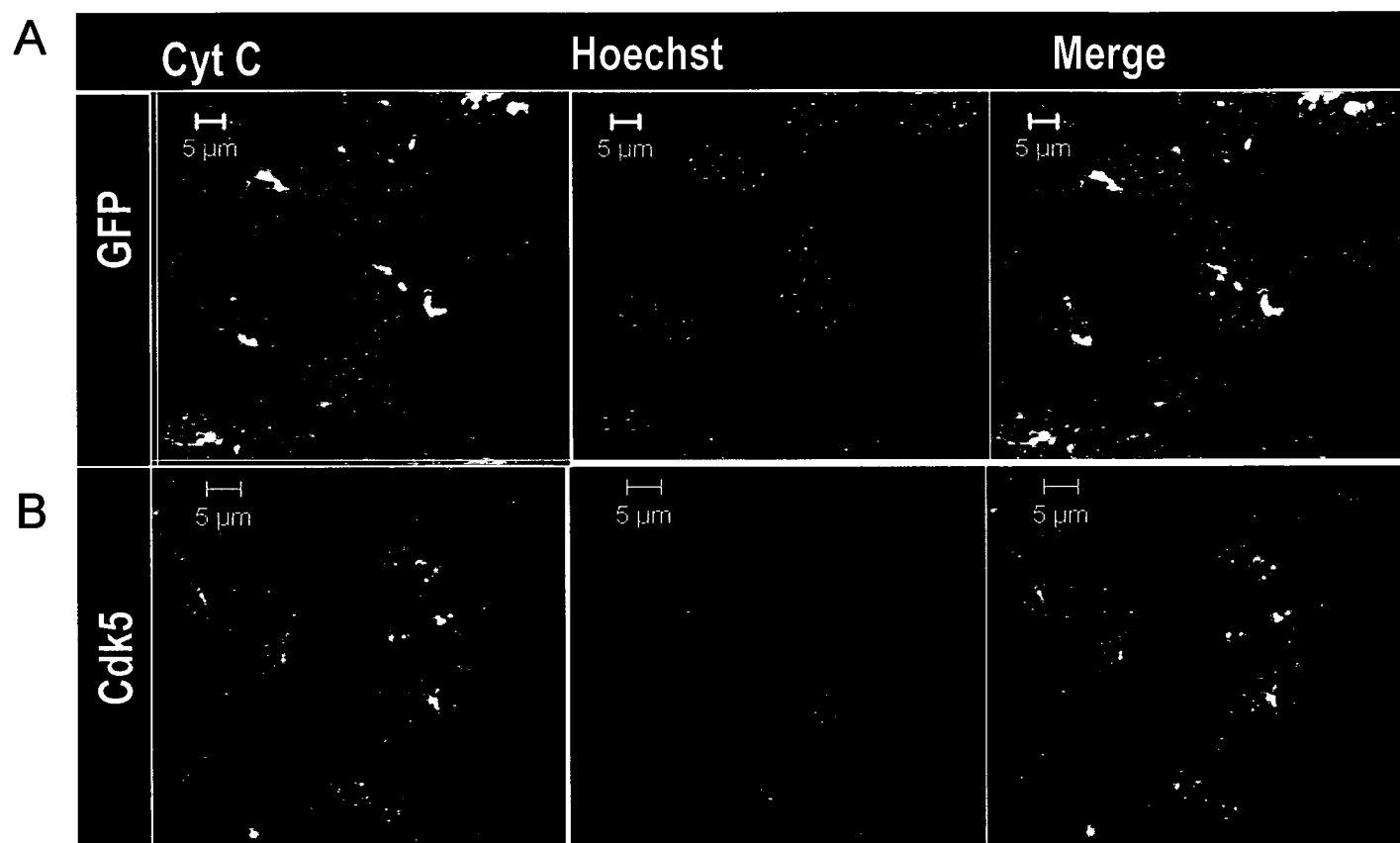


Figure 4.4

Figure 4.4. Cdk5 induces mitochondrial fragmentation in post mitotic neurons.

Cerebellar granule neurons were infected with adenoviruses carrying expression cassettes for either of GFP (panel A) or a cytoplasmic Cdk5 (Cdk5-NES) at the time of seeding (panel B). Neurons were fixed and mitochondrial morphology was assessed by an antibody against *cytochrome C*. Mitochondrial length was binned into different categories of <0.5, 1.0-2.0, 2.0-3.0, and > 3 μm . The graph (Panel C) compares the mitochondrial length shift upon induction of Cdk5. * indicates significance. For more detailed statistical significance see Table 1.

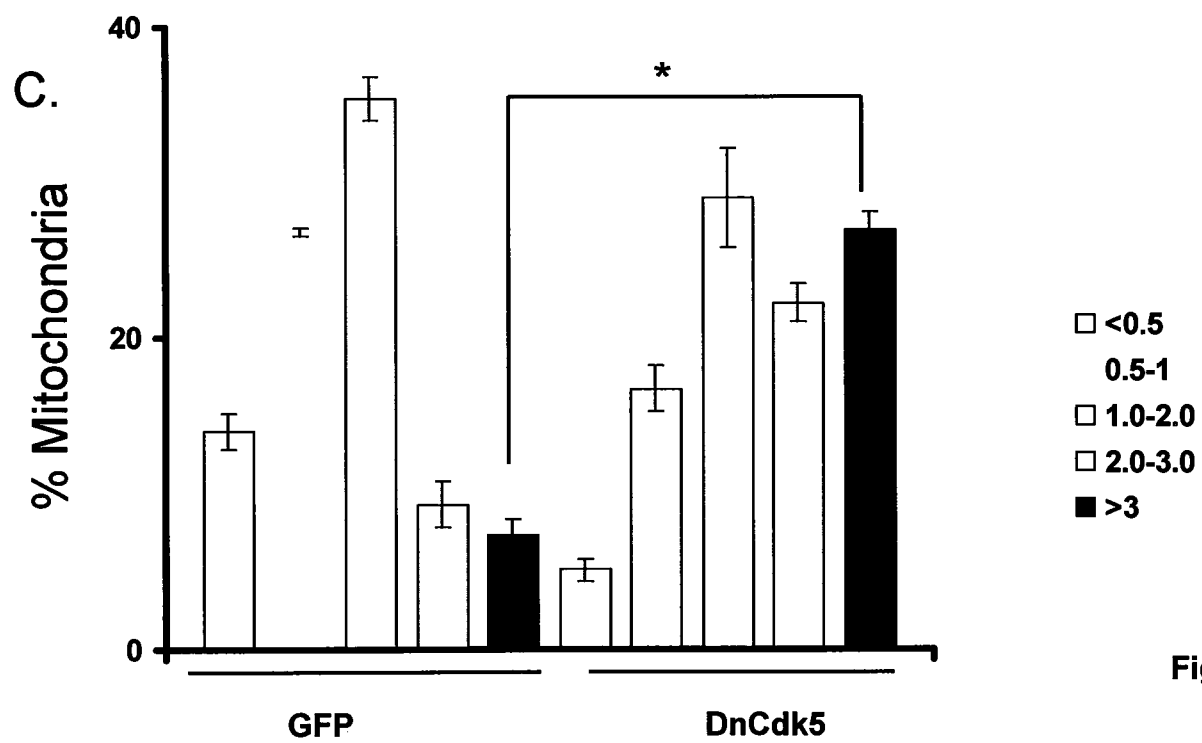
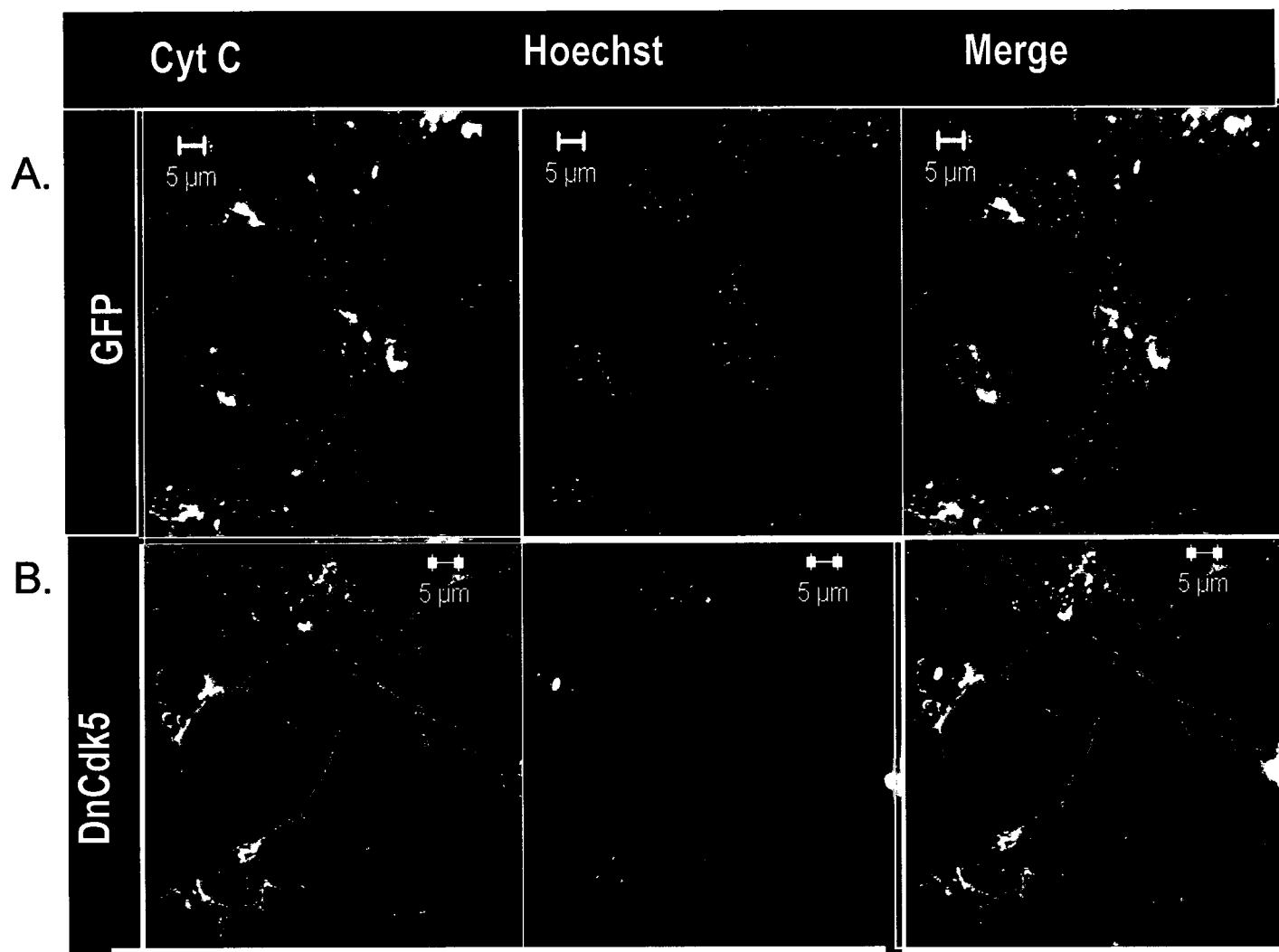


Figure 4.5

Figure 4.5. Downregulation of Cdk5 results in mitochondrial elongation in postmitotic neurons.

Cerebellar granule neurons were infected with adenovirus vectors carrying expression cassettes for either of GFP (panel A) or a cytoplasmic dominant negative mutant of Cdk5 (Dn-Cdk5-NES) at the time of seeding (panel B). Neurons were fixed and mitochondrial morphology was assessed by an antibody against *cytochrome C*. Mitochondrial length was binned into different categories of <0.5, 1.0-2.0, 2.0-3.0, and > 3 μm . The graph (Panel C) compares the mitochondrial length shift in Cdk5 ablated neurons compared with the control.

Length categories (μm)	GFP	Cdk5	Dn-Cdk5	GFP vs Cdk5	GFP vs Dn-Cdk5
< 0.5	13.95$\pm$ 1.15	33.34$\pm$5.9	5.07$\pm$0.65	P < 0.001	P < 0.001
0.5-1.0	26.8$\pm$0.27	27.8$\pm$3.3	16.73$\pm$1.5	P > 0.05	P < 0.001
1.0-2.0	35.4$\pm$1.45	22.16$\pm$1.19	28.99$\pm$3.16	P < 0.004	P > 0.05
2.0-3.0	14.8$\pm$1.4	9.28$\pm$.069	22.22$\pm$1.19	P < 0.008	P < 0.008
> 3.0	8.9$\pm$0.97	7.35$\pm$0.18	26.97$\pm$1.7	P < 0.09	P < 0.0004

Table 4.1

Table 4.1. Quantification of mitochondrial length in neurons expressing elevated or ablated levels of cytoplasmic Cdk5 versus GFP control.

Mitochondrial length and standard errors in each binned categories of < 0.5 , $0.5-1$, $1.0-2.0$, $2.0-3$, and $>3 \mu$ are shown for neurons expressing adenoviral vectors carrying expression cassettes for either of wtCdk5-NES, DnCdk5-NES or GFP control. The statistical significance for length comparison of each binned group is inserted ($n = 3$). p values of less than 0.05 are considered significant.

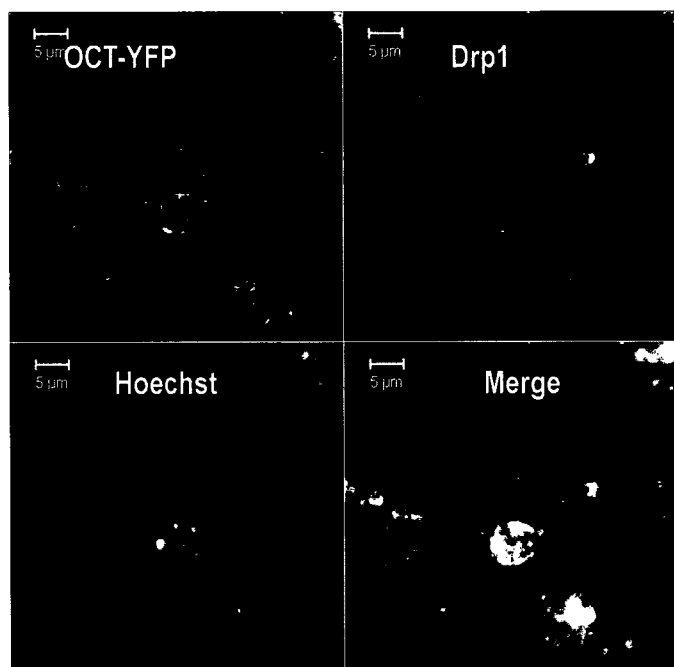
Cdk5 results in Drp1 recruitment from cytosol to the mitochondria

To identify the mechanism by which Cdk5 regulates mitochondrial length in primary neurons, neurons expressing wtCdk5-NES or GFP controls were subjected to DRP1 recruitment analysis by immunohistochemistry or sub-cellular fractionation. We hypothesized that phosphorylation by Cdk5 at Ser585 may be required for Drp1 recruitment to mitochondria at the site of scission. Neurons were co-infected with adenoviruses carrying a yellow fluorescence tagged (YFP) ornithine carbamyl transferase (OCT) (a mitochondrial enzyme targeted to the mitochondria (OCT-YFP) and either of wt-Cdk5-NES or GFP control. Samples were then fixed and stained for an antibody against Drp1. Targeting of OCT-YFP allowed us to visualize the mitochondrial network. To analyze recruitment of Drp1 in the mitochondria, multiple images throughout each neuronal section (i.e. Z stack of the field) was taken for analysis using a Ziess 510 meta confocal microscope. Drp1 staining in puncta with mitochondria was scored as recruitment (**Figure 4.6.B**) whereas a diffused or lack of Drp1 staining was scored as cytoplasmic (**Figure 4.6.A**). Our results indicated that in the Wt-Cdk5 group $53.66 \pm 16.38\%$ of neurons exhibited Drp1 recruitment compared to GFP control at $9.33 \pm 3.17\%$ (**Figure 4.6.C**). These results show that increased levels of cytoplasmic Cdk5 results in the recruitment of Drp1 from the cytoplasm to the mitochondria.

To confirm the role of Cdk5 in Drp1 recruitment as observed by confocal analysis, we performed subcellular fractionation on primary neurons infected with adenovirus vectors carrying a wt-Cdk5-NES or lacZ control constructs (**Figure 4.6.D**). Our results show that while Drp1 is found in both mitochondrial and cytoplasmic

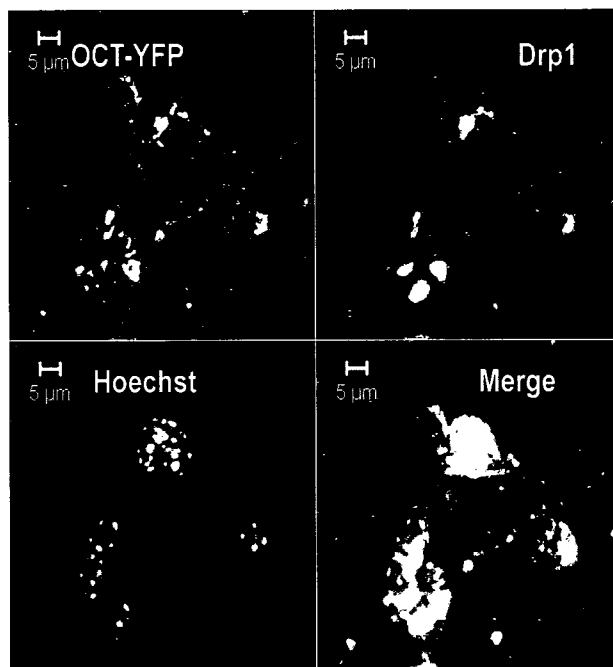
fractions in control lacZ group there is enhanced localization of Drp1 at the mitochondria upon increased activation of Cdk5. In conclusion our results demonstrate that Drp1 recruitment to induce mitochondrial fission can be mediated by Cdk5 in post mitotic neurons.

A.



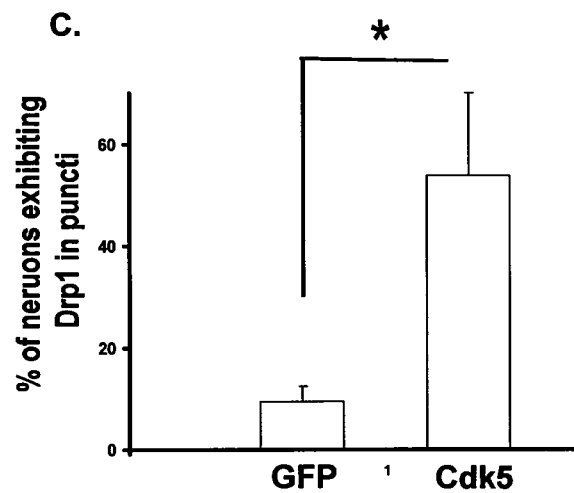
GFP, Drp1 diffused staining

B.



Cdk5, Drp1 puncta staining

C.



D.

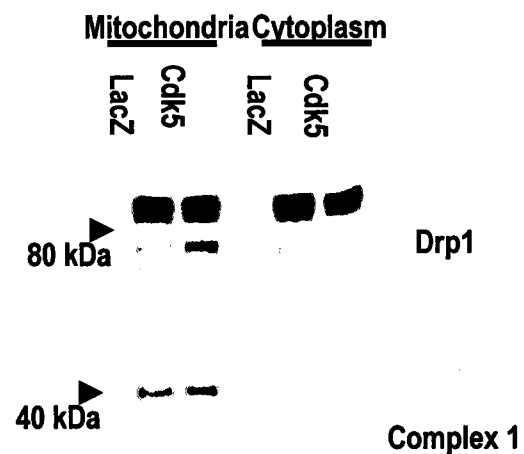


Figure 4.6

Figure 4.6. *Cdk5 induces Drp1 recruitment from the cytosol to the mitochondria in post mitotic neurons.*

Cerebellar granule neurons were co-infected with adenoviruses carrying expression cassettes for OCT-YFP and either of GFP control (panel A) or wt-Cdk5-NES (panel B) at the time of plating. Neurons were fixed and Drp1 recruitment was analyzed by an antibody against Drp1. Absence of staining or a diffused Drp1 staining was scored as lack of recruitment while detection of sharp puncti in mitochondria was scored as recruitment. Minimum of 100 neurons were scored in each replicate ($n = 3$, $p < 0.05$) and the difference in the recruitment between Cdk5 expressing neurons and GFP control was graphed (Panel C). Drp1 recruitment was assessed by subcellular fractionation (Panel D). Neurons infected with wt-Cdk5-NES or LacZ control were subjected to fractionation and analyzed by an antibody against Drp1. The blot was reprobbed with mitochondrial complex 1 as loading control.

4.5. DISCUSSION

Here we report a novel mechanism for the regulation of Drp1 fission activity in post mitotic neurons. First, we have identified Drp1 as a direct substrate of Cdk5 kinase. Cdk5 phosphorylates Drp1 at a conserved serine residue (Ser585 rat/Ser616 human). Second, this phosphorylation results in Drp1 recruitment from the cytosol to the mitochondria. Third, the phosphorylation and the resultant recruitment of Drp1 profoundly impacts mitochondrial morphology by inducing fission. This work identifies a novel mechanism by which Cdk5 regulates mitochondrial network at steady state. This finding has broad implications in regulation of synaptogenesis and synaptic strength.

Although our studies demonstrate a physical and functional interaction between Drp1 and Cdk5, the mechanism by which phosphorylation on Ser585 regulates mitochondrial morphology remains to be investigated. The conserved residue Ser585 has been also shown to be phosphorylated by cyclinB1/Cdk1 in mitotic cells (Taguchi et al., 2007). Similar to Cdk5 mediated phosphorylation, Cdk1-mediated Drp1 phosphorylation at Ser585 resulted in enhanced mitochondria fission and transfer into daughter cells. It is therefore possible that phosphorylation at this serine residue is fundamental in modifying the Drp1 GTPase activity and recruitment. These possibilities are currently being investigated in post mitotic neurons by constructing adenoviruses carrying wt or mutant Drp1.

In addition to Cdk5, Protein kinase A (PKA) has been shown to phosphorylate Drp1 at a different site, Ser 637 (Chang and Blackstone, 2007a; Cribbs and Strack, 2007;

Jahani-Asl and Slack, 2007). Although Ser 616 and Ser 637 are located in proximity of each other within the C terminal domain of the protein, unlike Cdk1, PKA-mediated phosphorylation of Ser 637 results in an elongated mitochondrial phenotype. It is thought that phosphorylation of Drp1 at Ser 637 inhibits the GTPase activity by decreasing the intermolecular interactions that direct GTP hydrolysis (Chang and Blackstone, 2007a). The Ser637 was also shown to be a target for calcineurin. Calcineurin mediated dephosphorylation of Drp1 was shown to induce its translocation from the cytoplasm to the mitochondria resulting in subsequent fission (Cereghetti et al., 2008; Cribbs and Strack, 2007). In contrast, phosphorylation of Drp1 at Serine 637 in neurons was shown to be regulated by Ca^{2+} /calmodulin-dependent protein kinase I α (CamKI α) and resulted in Drp1 translocation to the mitochondria. Although the physiological context relevant to these multiple phosphorylation sites requires further study, our results together with the studies performed in non neuronal cells support an important link between Drp1 phosphorylation status, mitochondrial fission and cell fate.

Next, we demonstrated that phosphorylation by Cdk5 can cause recruitment of Drp1 to the mitochondria resulting in mitochondrial fission in the absence of any cell death stimuli. Phosphorylation of Drp1 may account for one of the mechanisms by which Cdk5 confers its function in the regulation of synaptic plasticity. Since the size of synapses and their functional “strength” are important in learning and memory (Mattson, 2007; Mattson et al., 2008), and knowing that the content of dendritic mitochondria tightly regulates these parameters, our study opens new avenues for evaluation of a broad range of neurological diseases that involve loss of learning and memory.

Finally, Cdk5 and enhanced mitochondrial fission are both involved in the regulation of cell death following neuronal injury. For example following excitotoxic neuronal injury the cleavage product of the Cdk5 regulatory subunit, p25, targets Cdk5 to distinct cellular compartments to initiate death signaling. In addition, enhanced mitochondrial fission is documented as an early event in multiple modes of neuronal injury such as oxidative stress and excitotoxicity (Jahani-Asl et al., 2007). We propose a model whereby in response to neuronal injury Cdk5/p25 complex results in enhanced phosphorylation of Drp1 resulting in its recruitment to induce mitochondrial fission. This may in part explain the dramatic mitochondrial fragmentation associated with neuronal injury.

In conclusion, I have identified an important mechanism for the regulation of mitochondrial fission in post mitotic neurons. Since mitochondrial fission plays an active role in both synaptic plasticity and neuronal death, this work has shed light on a regulatory mechanism to drive these events. This study has also raised a number of important questions that require future investigation: 1) Does inhibiting the identified conserved serine residues (Ser585) targeted by Cdk5 rescues the dramatic mitochondrial fragmentation associated with injury and rescue neuronal death? How does Cdk5-mediated regulation of Drp1 at Ser585 differ from CamKII α mediated phosphorylation of Drp1 at Ser 637? Which of these sites dominate Drp1 GTPase activity under different physiological conditions (steady state versus neuronal injury)? These are the important questions that are currently under study in Dr. Slack's laboratory.

4.6. Materials and Methods

Primary neuronal cultures and adenoviral construction - Cerebellar granule neurons (CGNs) were cultured from CD1 mice at postnatal day 7 or 8 as described previously (Fortin et al., 2001). Recombinant adenoviral vectors carrying Ornithine Carbamyl Transferase (OCT), Cdk5 or DnCdK5 expression cassettes were prepared using AdEasy system, as described previously (He et al., 1998). Cells were infected at the time of plating with different multiplicity of infection (MOI) ranging from 25 to 150. 100 MOI was chosen based on the high efficiency and low toxicity. To measure the toxicity and efficiency of infection, Live/Dead assay (Molecular Probes, Eugene, OR) was performed two days post infection. Three random fields were chosen for each group and the images of cells in these fields were taken with a fluorescence microscope using the appropriate filters. Phase contrast micrographs of the same fields were taken with light microscopy and the number of cells infected was compared to the total number of cells in the field. To measure toxicity, the number of infected dead cells was compared to the total number of infected cells in the field.

Immunofluorescence - At each time point, neurons were fixed for 30 minutes with ice cold 4 % paraformaldehyde in 1X Phosphate Buffered Saline (1XPBS) and then rinsed twice with 1XPBS. Cells were permeabilized with 300 μ l of ice-cold 0.4% Triton-X in 1XPBS for 10 min and stained with the primary antibodies in 10% normal goat serum-0.4% Triton X/PBS for 1 h. Following three washes (5 min each) with ice cold 1XPBS, cells were incubated with the secondary antibodies to either Drp1 or *cytochrome C*

(1:250; BD Biosciences, Franklin Lake, NJ) in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed for 5 minutes and stained with Hoechst for 5 minutes. Following Hoechst staining, neurons were washed with 1X PBS for 3 x 5 min and mounted. Representative samples were photographed using a Zeiss 510 meta confocal microscope or a Zeiss Axiovert 100 (Oberkochen, Germany) fluorescence microscope equipped with a QiCam Digital camera (QImaging Corporation, Burnaby, Canada) and Northern Eclipse software (Empix Imaging Inc. Mississauga, ON, Canada).

Mitochondrial length measurement - CGNs were seeded on 4 well plates (Nalgene Nunc International, Rochester, NY) with attached glass coverslips coated with poly D Lysine (Fisher Scientific, Whitby, ON), and infected with adenoviral vectors carrying expression cassettes for either of Cdk5, DnCdK5, or GFP control. Neurons were fixed and stained with an antibody against *cytochrome c*. For each replicate, 2000-3000 mitochondria were counted from 5-8 different fields. Whole cell images were acquired by exciting at 488 nm with the GFP filter (Chroma Technology Corp., Rockingham, VT, USA). Mitochondrial length was measured by tracing the mitochondria using Northern Eclipse software. Mitochondrial length varied remarkably even in control neurons. For comparison purposes mitochondria were classified into different categories with a length ranging from less than 0.5 μm , 0.5 to 1 μm , 1 to 2 μm , 2 - 3 μm , and greater than 3 μm .

Analysis of Drp1 recruitment by Immunocytochemistry- Neurons were seeded on 4 well plates (Nalgene Nunc International, Rochester, NY) with attached glass coverslips coated with poly D Lysine (Fisher Scientific, Whitby, ON), and co-infected with viral vectors

carrying expression cassettes for YFP-tagged Ornithine Carbamyl Transferase (OCT-YFP), a mitochondrial matrix protein, and either of Cdk5, DnCdk5, or GFP control at the time of seeding. Neurons were stained and fixed with an antibody against Drp1 (BD Biosciences, Franklin Lake, NJ). The YFP was excited with 515 nm line of a multiple line Ar laser, the mitofluor Red was excited with the 543 nm line of He/Ne green laser and the Alexa 647 was excited with the 633 nm line of He/Ne red laser. All images shown demonstrate cells that are representative of moderate infection efficiencies, and that have been obtained from at least three independent experiments. In each case a Z stack of the field was taken for analysis using a Zeiss 510 meta confocal microscope. A diffused Drp1 staining or complete lack of staining was identified as cytoplasmic and staining of Drp1 colocalizing with OCT-YFP at puncti was scored as recruitment.

Biochemical analysis for Drp1 recruitment- Mitochondria were isolated from the primary neurons at 3 days in vitro (DIV) using subcellular fractionation as previously described (Cheung et al., 2006). Mitochondria and cytoplasmic fractions were run on SDS-PAGE gel side by side and the membranes were blotted for Drp1. The blots were standardized using mitochondrial complex 1 and LDH (Cheung et al., 2006).

In vitro Kinase assay- Recombinant His-Drp1 were expressed and purified by metal chelating resin and further purified by ion exchange chromatography using Q-fast flow (Pharmacia, Uppsala, Sweden). The in vitro kinase reaction was analyzed as described previously (Nishijima et al., 1997). Active Cdk5 complex was purchased from Upstate (Catalogue Number, 14-477).

Quantification and statistical analysis – For the assessment of mitochondrial length, a minimum of 1000 mitochondria for each treatment (per replicate) was scored (3 replicates). The data represent the mean and standard deviation from three independent experiments (n = 3). For mitochondrial Drp1 recruitment 100 neurons were scored from three independent experiments (n = 3). p values were obtained using two-way ANOVA and Students t test. A p value <0.05 was considered significant and was indicated on the graphs by an asterisk.

Sequence alignment

Drp1 protein sequences from different species were retrieved from the National Center for Biotechnology Information (NCBI) database and multiple sequence alignment was carried out using ClustalX, a windows interface of ClustalW (Thompson et al. 1994).

4.7. Acknowledgement

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CHAPTER 5
GENERAL DISCUSSION

5. DISCUSSION

Mitochondria are dynamic organelles that undergo continuous fusion and fission events to regulate their shape and function. The major goal of this PhD thesis has been to elucidate the role of mitochondrial fusion and fission machineries in the regulation of neuronal death. The results of these studies have culminated in a number of key findings: First, mitochondrial morphology defects are identified as key early events in the progression of neuronal death (Chapter 2 and 3). While enhanced fission contributes to the loss of mitochondrial integrity, defects in the fusion machinery are identified as a major cause of mitochondrial dysfunction. Second, the two key fusion proteins, Mfn2 and Opa1, protect against neuronal loss induced by multiple mechanisms. While Mfn2 acts as a signaling GTPase to promote neuronal survival (Chapter 2), Opa1 functions by maintaining the cristae structure (Chapter 3). Third, we show that mitochondrial remodeling as a result of loss of Opa1 oligomers are hallmarks of excitotoxic injury. Importantly, inhibition of calcium activated calpain proteases restores Opa1 oligomers, mitochondrial morphology and neuronal survival. Our findings are the first to identify Opa1 as a key regulator of neuronal fate following calcium deregulation (Chapter 3). *These studies underscore the potential therapeutic values of Mfn2 and Opa1 in a broad range of neurological diseases.*

The second goal of this PhD thesis has been to study how Drp1 is regulated to induce mitochondrial fission in neurons. We have identified Cdk5 as a key regulator of Drp1 recruitment (Chapter 4). Cdk5 phosphorylates Drp1 at a conserved Serine residue and results in Drp1 translocation from cytoplasm to the mitochondria to induce fission

(Chapter 4). This study highlights a neuronal intrinsic mechanism for the regulation of mitochondrial fission in post mitotic neurons. *Since mitochondrial fission is significant in the regulation of both neuronal plasticity and neuronal death, our studies describes a mechanism for how Cdk5 may perform its dual functions at the mitochondrial level.*

5.1. Mitochondrial Fusion Proteins Mfn2 and Opa1 Confer Neuroprotection against Different Modes of Neuronal Injury

Acute neuronal injury such as stroke and trauma results in neuronal death by multiple mechanisms. These mechanisms ultimately converge on the mitochondria to induce mitochondrial dysfunction and neuronal demise. Recent evidence suggested that mitochondrial fusion proteins Mfns and Opa1 may play a key role in the regulation of cell fate in cell lines and lower eukaryotes. For example, in non neuronal cells loss of mitochondrial fusion proteins, Mfn1, Mfn2 and Opa1 led to mitochondrial fragmentation and increased sensitivity to cell death stimuli (Olichon et al., 2003; Lee et al., 2004; Sugioka et al., 2004; Arnoult et al., 2005) while overexpression of Mfn2 inhibited Bax activation and more importantly protected against permeability transition in cultured cell lines (Neuspiel et al., 2005). In addition, overexpression of Opa1 in the mouse embryonic fibroblasts protected against different modes of cell death including those induced by etoposide and staurosporine (Frezza et al., 2006). Unlike cell lines, neurons have very high morphological complexities. It is therefore a challenging task to correlate the data obtained from the studies performed in non neuronal cells and/or to anticipate the consequences of improper mitochondrial targeting in the nervous system. Moreover, the

underlying mechanisms of neuronal death are more complex and extend far beyond the classical apoptotic signaling. Therefore assessment of the mitochondrial fusion following multiple mechanisms of neuronal injury such as excitotoxicity and oxidative stress required intensive investigations. In this thesis, Mfn2 and Opa1 were shown to rescue neuronal loss by distinct mechanisms in response to various modes of neuronal injury (**Figure 5.1**).

In an attempt to unravel the mechanism of Mfn2 function following neuronal injury, we employed a wild type (wt) and a hydrolysis deficient constitutively activated mutant of Mfn2 (Mfn2_{RasG12V})(Neuspiel et al., 2005). Comparative analyses between neurons expressing wt or mutant Mfn2 in various capacities (e.g. ability in rescuing mitochondrial defects, promoting neuronal survival, and regulation of MOMP) resulted in the identification of Mfn2 as a signaling GTPase (Jahani-Asl et al., 2007). While wt and mutant Mfn2 function equally to promote mitochondrial fusion, the active mutant provided two fold increases in protection against DNA damage and ROS induced neuronal death. In addition Mfn2 was shown to function upstream of cytochrome c release suggesting that it can directly or indirectly regulate the opening of MOMP in apoptotic signaling (**Figure 5.1**).

In follow up studies, we investigated the role of Mfn2 in excitotoxic injury (Jahani-Asl et al, Submitted). Excitotoxicity is distinct from classical apoptosis in a number of different ways. First, regulation of MOMP opening does not seem to be the most critical event following this mode of cell death as loss of the key regulators of

- Calcium
- ▶ Positively regulates
- | Negatively regulate
- ? Future Directions

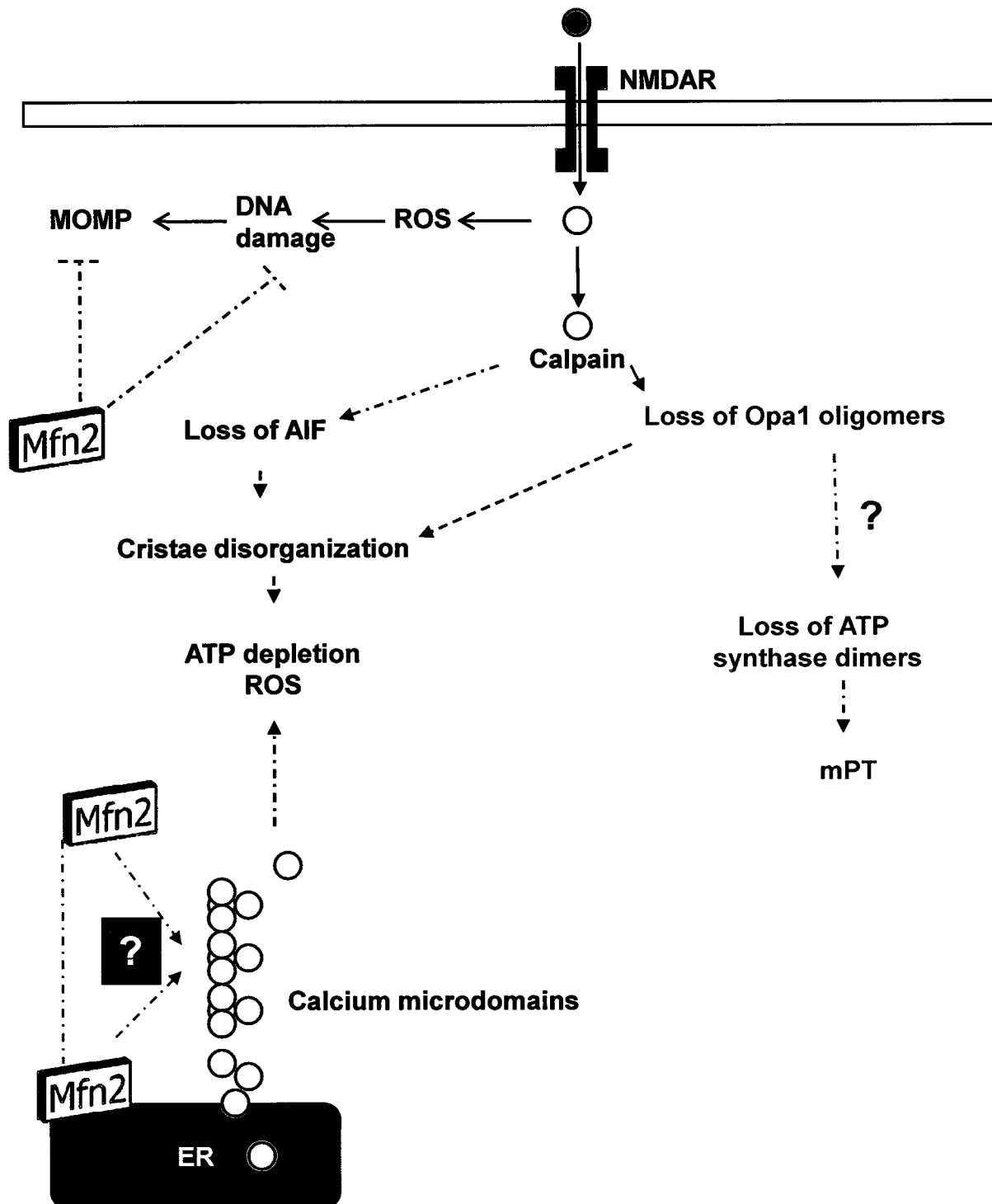


Figure 5.1

Figure 5.1. Proposed model for the mechanism of action of fusion proteins

Two fusion proteins, Mfn2 and Opa1, play important roles in promoting neuronal survival following multiple mechanisms of acute neuronal injury. Mfn2 acts as a signaling GTPase to promote neuronal survival against DNA damage and ROS. Mfn2 also inhibits the release of proapoptotic factors associated with apoptotic cell death. This model therefore proposes that Mfn2 functions upstream of MOMP opening and it may directly or indirectly regulates the opening of the pore in apoptotic neuronal death. In addition, Mfn2 bridges mitochondria and ER to facilitate calcium shuttling. We propose that following excitotoxic neuronal injury, Mfn2 may confer neuroprotection by removing calcium from the mitochondria and restoring the ER calcium levels. Opa1 promotes neuronal survival by maintaining IMM structure. Excitotoxic injury results in loss of Opa1 oligomers and collapse of mitochondrial network. Loss of Opa1 oligomers results in the widening of cristae junctions and loss of electrical gradient across the IMM. Mitochondria that have lost Opa1 oligomers therefore become fusion incompetent and suffer metabolic defects. We propose that Opa1 may confer this function in part by regulation of ATP Synthase dimers. Finally, inhibiting calpain activity restores Opa1 oligomers, mitochondrial structure and function. Whether calpain is involved in the processing of Opa1 or whether calpain modulates the Opa1 activity through other key regulators such as AIF requires future investigations.

MOMP pore formation (Bax and Bak) does not impact neuronal survival in response to AMPA, Kainate or NMDA induced injury (Cheung et al., 2005). Second, this mode of cell death is mainly attributed to the deregulation of the calcium transients due to excessive calcium uptake through glutamate receptors and to the modulation of calcium mediated signaling pathways as a result. Excessive intracellular calcium activates multiple mechanisms at multiple levels (cytoplasm, mitochondria, nuclei and endoplasmic reticulum) to ensure death of neurons. Mitochondria play a major role in the buffering of the intracellular calcium surplus and thus attenuate propagation of calcium waves within other compartments, however abnormal calcium accumulation by mitochondria is also a cause of mitochondrial dysfunction (Atlante et al., 2001). Third, activation of the calcium dependent pathways in different compartments of neurons (e.g. synaptic versus extrasynaptic) and the organelle specific responses can have different consequences. For example the density of the mitochondria in the vicinity of the glutamate receptors may impact the status of calcium microdomains within the cytoplasm and the resultant calcium dependent processes. We showed that overexpression of Mfn2 provides a mild protection against NMDAR overactivation (Chapter 3). Interpretation of how Mfn2 confers protection against excitotoxicity is complex and this complexity is due to the fact that the sequence of the events that lead to neuronal disintegration in this mode of cell death and their orderly occurrence in time and space is not well defined. We hypothesize that Mfn2 may function as a ROS scavenger as overexpression of Mfn2 in primary cortical neurons attenuated ROS levels following exposure to hydrogen peroxide (Cheung et al, submitted). Activation of Mfn2 also conferred two fold protection against H₂O₂ mediated neuronal loss in primary granular neurons (Jahani-Asl et al., 2007). In

further support of this hypothesis, overexpression of Mfn2 protected against mitochondrial permeability transition in cultured cell lines (Neuspiel et al 2006). A more tempting model to describe these results is that Mfn2 may be indeed responsible for restoring ER calcium stores. This is supported by a recent finding in which Mfn2 was shown to be enriched at the mitochondria-ER contact sites (de Brito and Scorrano, 2008). In this study Mfn2 was distinctively localized to both organelles. In addition, it was proposed that Mfn2 forms bridges between ER and mitochondria to facilitate mitochondrial calcium uptake, however mitochondrial calcium concentrations were measured to be higher in the Mfn2 knock out (Mfn2^{-/-}) mouse embryonic fibroblasts (MEF) when compared to their wt counterparts. If indeed Mfn2^{-/-} mediates mitochondrial calcium uptake, one would expect a lower concentration of calcium in the mitochondria taken from Mfn2^{-/-} MEF. It is therefore tempting to hypothesize that Mfn2 may be indeed responsible for restoring ER calcium stores rather than or in addition to its uptake. Interestingly, re-introduction of Mfn2 in the Mfn2^{-/-} MEFs decreased the mitochondrial calcium concentration, supporting the notion that Mfn2 may indeed attenuate mitochondrial calcium uptake.

In an attempt to unravel the mechanism of mitochondrial dysfunction following calcium influx associated with excitotoxicity, time lapse imaging, electron microscopy and loss and gain of function studies were performed. Defects in the IMM and cristae morphology were identified as key regulators of mitochondrial damage in NMDA induced neuronal death. Importantly, Opa1 oligomers which are previously shown to be critical in maintaining cristae morphology were lost in NMDA treated neurons.

Interestingly, Opa1 conferred two fold protections. Our results suggest that the IMM defects are upstream events that lead to the collapse of mitochondrial network in excitotoxic injury. Loss of the IMM integrity leads to the disturbance in the mitochondrial proton gradient. Since, mitochondrial fusion requires the proton gradient generated by a functioning electron transport chain (Meeusen and Nunnari, 2005), loss of IMM structure could result in fusion incompetent mitochondria and metabolically compromised neurons. This is supported by our data that fusion rate of the mitochondria was greatly blocked in NMDA treated neurons when compared to control neurons (Chapter 3). In further support, previous studies have demonstrated a role for Mfn2 in fuel oxidation (Pich et al., 2005).

Based on the studies presented in chapters 2 and 3 of this thesis, we envision a model in which Mfn2 protects against apoptotic signaling upstream of MOMP. Mfn2 may also confer protection against excitotoxicity by enhancing the metabolic capacity of neurons. Opa1 protects by maintaining cristae structure and resisting mitochondrial remodeling associated with calcium influx. These findings have broad implications for designing combinational neurotherapeutic modules for treatment of acute neuronal injury in which neuronal loss involves both apoptotic and excitotoxic pathways.

5.2. Mfn2 and Opa1: Relevance of our findings to other neurological disorders

The results obtained from these studies not only lay the foundation to investigate therapeutic values of these proteins for stroke patients, but it pertains to other

neurodegenerative diseases. This is primarily due to the fact that the death models studied in this PhD thesis (DNA damage, ROS and excitotoxicity) represent the underlying molecular mechanism of neuronal death in a broad range of neurological disorders such as Huntington's, Parkinson's and Alzhimers's disease (HD, PD and AD). In support of this, alterations in normal mitochondrial dynamics and dramatic fragmentation have been linked to HD, AD and PD (Wang et al., 2008; Cho et al., 2009). In addition, other laboratories have identified Mfn2 and Opa1 as molecules that rescue mitochondrial morphology defects in HD and AD death models. HD is a fatal neurodegenerative disorder, caused by abnormal expansion of a CAG trinucleotide sequence that encodes a polyglutamine tract (PGT) in the Huntington (htt) proteins (herein referred to as PGT-htt) (Imarisio et al., 2008). Overexpression of PGT-htt proteins sensitized Hela cells to oxidative stress mediated mitochondrial fragmentation and an arrest in the rate of mitochondrial fusion (Wang et al., 2009). Mfn2 was shown to rescue this fragmentation and motility defects following the PGT-htt induced cell death (Wang et al., 2009). Alzhimers's disease (AD) is a neurodegenerative disorder that results in neuronal loss in aged population. AD patients exhibit presence of plaques in the brain which are aggregates of amyloid β ($A\beta$), a cleavage product of amyloid precursor protein (APP)(Wang et al., 2007a). Overexpression of both $A\beta$ and APP were shown to lead to mitochondrial fragmentation in primary hippocampal neurons and this fragmentation was inhibited by overexpression of Opa1(Wang et al., 2008).

Our findings together with follow up studies by other groups have established the significance of mitochondrial dynamics as a paradigm for neurodegeneration research

and modulation of mitochondrial fission and fusion may have therapeutic values in intervention of a broad neurological diseases.

5.3. Mitochondrial Morphology Defects following Neuronal Injury

An imbalance in the rate of mitochondrial fusion and fission is shown to contribute to the collapse of mitochondrial network following cell death. In the studying of the mitochondrial dynamics following neuronal injury we have identified different pathways that result in the disintegration of mitochondrial pool: a) enhanced mitochondrial fission, b) attenuated rate of fusion, c) mitochondrial remodeling at ultrastructural level. Foremost among these is an arrest in the rate of mitochondrial fusion. Although Opa1 and Mfn2 are key GTPases that mediate mitochondrial fusion, it is not clear how their GTPase activities are regulated. Opa1 and Mfn2 rescue mitochondrial morphology defects induced by calcium deregulation, however there is no evidence to suggest a role for these proteins as calcium sensors. Recent studies suggest that the mitochondrial transport system (cytoskeletal elements, motor and accessory docking proteins) may play a key role in the regulation of mitochondrial dynamics in response to calcium transients (Hollenbeck and Saxton, 2005; Frederick and Shaw, 2007; Saotome et al., 2008; Macaskill et al., 2009; Wang and Schwarz, 2009).

For the tethering and fusion between the two mitochondria to occur, the organelles are first required to be repositioned in close vicinity of each other. In neurons, these relocations occur on the microtubule filaments and require two microtubule based

motor proteins Kinesin-1 (KIF5) and dynein (Hollenbeck and Saxton, 2005). In addition, mitochondrial Rho GTPase (Miro1 and 2), Milton and Syntabulin are identified as accessory protein that anchor mitochondria to the microtubule motor proteins (Cai et al., 2005; Guo et al., 2005; Glater et al., 2006). Miro was recently shown to regulate mitochondrial fusion in response to increased calcium influx (Wang and Schwarz, 2009). Like Mfn2, Miro is located on the OMM and possesses a GTPase domain, but additionally Miro contains two EF hand calcium binding domains. Excessive calcium halts mitochondrial motion by binding to Miro EF hand domain (Wang and Schwarz, 2009). Calcium-bound-Miro prevents motor/microtubule interaction and causes Miro-KIF5 complex to switch into an inactive state (Wang and Schwarz, 2009). It is therefore possible that the calcium influx associated with excitotoxicity may halt Mfn2 action through Miro. Considering the localization of Mfn2 and Miro on OMM, it is tempting to speculate that a functional interaction may exist between these two molecules. This hypothesis is supported by the findings that the Miro expressing neurons exhibit 20% increase in the percentage of elongated mitochondria relative to control (Saotome et al., 2008). Ectopic expression of Miro was also reported to enhance the fusion state of mitochondria at resting calcium levels (Saotome et al., 2008). Together these findings indicate that maintaining calcium concentrations within critical set points are crucial for the regulation of mitochondrial fusion.

In support of this, LETM1 (Leucine zipper-EF hand containing transmembrane protein 1), is required for the maintenance of mitochondrial morphology (Dimmer et al., 2008). LETM1 is an inner membrane mitochondrial protein with two calcium binding EF

hand motif (Endele et al., 1999). Loss of LETM1 leads to a fragmented mitochondrial phenotype suggesting a role for LETM1 in mitochondrial fusion (Dimmer et al., 2008). Opa1 is the only inner membrane space GTPase which is found in close association with the IMM. Similar to Mfn2, evidence is lacking to support a role for Opa1 in the regulation of mitochondrial fusion in response to calcium signaling. Whether there is a functional and physical interaction between LETM1 and Opa1 requires future studies.

We have identified an important link between Opa1 and calcium activated protease calpain. Opa1 oligomers which are required for the maintenance of mitochondrial structure and function are lost in response to calpain activation. Importantly, inhibition of calcium activated calpain proteases restores Opa1 oligomers, cristae morphology and mitochondrial length. Whether calpain directly target Opa1 or whether it requires other intermediate molecules to modulate Opa1 GTPase activity are interesting questions that remain to be tackled.

In addition to the defects in the mitochondrial fusion, an increase in the mitochondrial fission contributes to the loss of mitochondrial network following neuronal injury (Jahani-Asl et al 2007). A key required step in the mitochondrial fission is the recruitment of Drp1 from the cytoplasm to the mitochondria. The underlying mechanisms of Drp1 recruitment are just beginning to emerge. For example phosphorylation and sumoylation are two forms of post translational modifications that tightly regulate Drp1 activity (Chang and Blackstone, 2007b; Cribbs and Strack, 2007; Wasiak et al., 2007; Han et al., 2008). Recent reports suggest a calcium-mediated-regulatory mechanism for

Drp1 recruitment. For example calcium-activated Kinase CamKII α and Calcium-activated phosphatase calcineurin regulate Drp1 phosphorylation state (Cereghetti et al., 2008; Han et al., 2008). In addition, an increase in the mitochondrial calcium influx results in mitochondrial fragmentation in cell lines (Breckenridge et al., 2003; Hom et al., 2007; Cereghetti et al., 2008). This fragmentation is in part rescued by down-regulation of Drp1 (Breckenridge et al., 2003; Hom et al., 2007). On the contrary, the BH3 only protein, BIK, which acts on ER to facilitate mitochondrial calcium uptake results in mitochondrial dysfunction independent of the fission activity of Drp1 (Germain et al., 2005). Therefore specificity of Drp1's function in response to calcium transients in distinct physiological and pathological conditions requires future investigations.

In support of the involvement of cytoskeletal elements in the regulation of mitochondrial dynamics, motor protein complex dynein-dynactin has been reported to play a role in the distribution of mitochondria through Drp1 recruitment (Varadi et al., 2004). Disruption of dynein function through overexpression of the dynactin subunit (dynamitin) results in the redistribution of mitochondria to the nuclear periphery and translocation of Drp1 from mitochondrial membranes to the cytosol. Overexpression of a wild-type Drp1 restores normal mitochondrial distribution in dynamitin over-expressing cells. In addition disruption of F-actin reduces recruitment of DRP1 to the mitochondria and attenuates fission (De Vos et al., 2005). Studies in the budding yeast show that mitochondria predominantly interact with actin tracks for both anterograde and retrograde movements during cell division. Mutations in genes encoding actin or components of

actin filaments or treatment with actin depolymerizing agents result in an aberrant mitochondrial shape ((Boldogh et al., 1998).

In conclusion, although Mfn2, Opa1 and Drp1 are three key GTPases that mediate mitochondrial fusion and fission events, future studies must focus on a) how the GTPase activities of these proteins are regulated in both steady state and in response to neuronal injury? b) Do multiple stressors such as ROS and calcium directly modulate the GTPase activity of these proteins? And if not, what are the molecular events that link the devastating effect of ROS and calcium to breakdown of mitochondrial shape?

Based on our studies and those of other laboratories we propose a model whereby mitochondria function as a signaling platform for directing the death signals culminating in mitochondrial dysfunction. In response to certain stressors such as calcium, this is in part mediated through mitochondrial calcium sensors such as Miro and LETM1. We propose that the components of the cytoskeletal elements may play a significant role in the regulation of mitochondrial morphology defects following neuronal injury.

5.4. Cdk5 Mediated Regulation of Mitochondrial fission

Cyclin dependent kinase 5 (Cdk5) is a unique member of cyclin dependent kinases (CDKs) and plays an essential role in the development of the nervous system and the regulation of neuronal plasticity and death. (Dhavan and Tsai, 2001; Angelo et al., 2006; Cheung et al., 2006; Hawasli and Bibb, 2007; Zhang and Herrup, 2008). Recently,

mitochondrial fission was shown to contribute to the CDK5-mediated neurotoxicity (Meuer et al., 2007). In RNAi knock down experiments loss of cdk5 inhibited apoptosis-associated mitochondrial fission (Meuer et al., 2007). Whether there is a cross talk between Cdk5 and the key regulators of mitochondrial morphology remained unknown. In the last chapter of this PhD thesis we identified Drp1 as a substrate of Cdk5. Cdk5 phosphorylated Drp1 in *in vitro* kinase assay and in primary granule neurons. Using recombinant wild type and Drp1 mutants, Serine 585 (rat, Ser 616 human) was identified as the phosphorylation site for Cdk5. Our results revealed that Cdk5 induces mitochondrial fission in post mitotic neurons. Since Mitochondrial fission is initiated by the recruitment and assembly of Drp1 at the mitochondrial scission site, Drp1 recruitment was next evaluated in neurons expressing Cdk5. A combinational approach of immunostaining and biochemical analysis revealed that the phosphorylation of Drp1 by Cdk5 results in its recruitment from the cytoplasm to the mitochondria.

Cdk5 is an important regulator of neuronal death following acute injury. For example, activation of calpains associated with excessive calcium influx following excitotoxicity results in the cleavage of p35 regulatory subunit into p25. The cleavage product p25 targets Cdk5 to distinct cellular compartments to initiate death signaling. We propose a model whereby Cdk5/p35 complex regulates steady state mitochondrial fission while Cdk5/p25 complex regulates cell death associated fission (**Figure 5.2**). Mitochondrial fission in steady state by Cdk5/p35 complex is required for mitochondrial translocation to the dendritic spines and synapse development. Following neuronal injury, however, enhanced targeting of Drp1 to the mitochondria as a result of p35 cleavage into

p25 results in dramatic mitochondrial fission associated with cell death. We foresee that by inhibiting the identified conserved serine residues (targeted by Cdk5), the dramatic mitochondrial fragmentation associated with injury can be rescued and the devastating effect of Cdk5 can be in part eliminated.

These studies identify a neuronal intrinsic mechanism for the regulation of mitochondrial fission. Since both mitochondrial fission and Cdk5 have been documented to play an active role in synaptic plasticity and neuronal death, our studies have identified a potential mechanism through which these dual functions are regulated.

5.5. Concluding Remarks

In conclusion, the findings of this PhD thesis have expanded our knowledge of the novel field of mitochondrial dynamics and their significance in neuronal injury. This knowledge has resulted in further understanding the components of mitochondrial fission and fusion and their potential therapeutic implications to promote neuronal survival. At the same time that these findings have raised fundamental questions, it has also opened new avenues to revisit fundamental concepts in neurobiology.

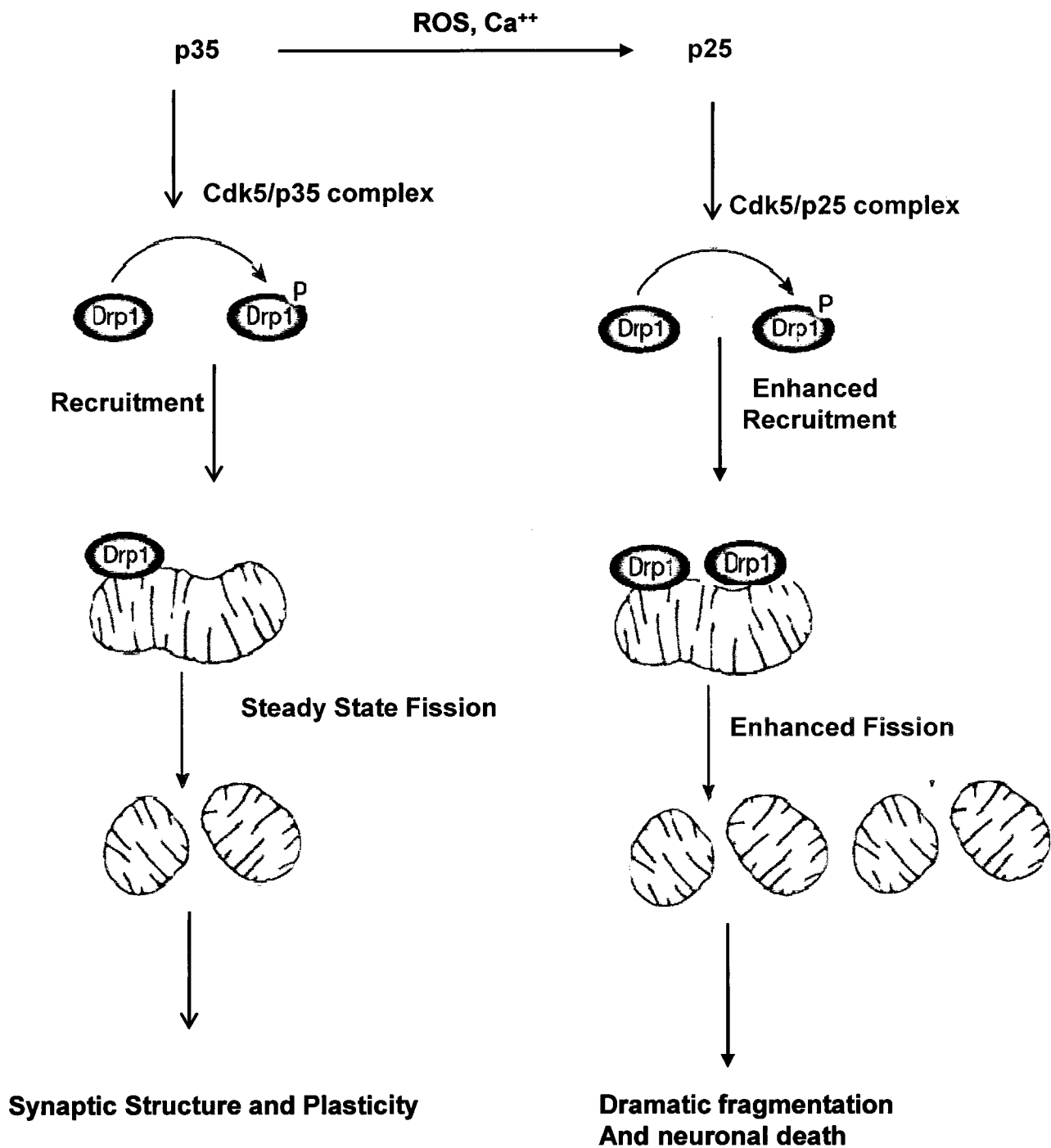


Figure 5.2

Figure 5.2. Proposed model for physiological relevance of Cdk5 mediated Drp1 phosphorylation

Drp1 phosphorylation by cdk5 results in the recruitment of Drp1 and its association with the mitochondrial membranes. This results in enhanced mitochondrial fission. Our studies proposes a model in which Cdk5/p35 complex results in steady state mitochondrial fission required for mitochondrial translocation to dendritic spines and their contribution to synaptogenesis. We propose that the Cdk5/p25 complex enhances the recruitment of Drp1 resulting in dramatic mitochondrial fission associated with cell death. Enhanced mitochondrial fission as a result of cdk5 mediated Drp1 phosphorylation could be a mechanism by which cdk5 regulates neuronal death.

APPENDICES

APPENDICE A

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APPENDICE B
CURRICULUM VITAE

NAME AND ADDRESS

First name: AREZU

Last name: JAHANI-ASL

ADDRESS: Post Doctorate Fellow

Department of Pathology, Azad Bonni's Laboratory

Harvard Medical School, Boston, MA

Email: arezu_jahani@yahoo.ca

SUMMARY

- Author on 10 manuscripts (6 published, 4 submitted or in preparation)
- Author on 24 published abstracts
- Recipient of 14 awards recognizing academics and research
- Significant involvement in academic community, teaching, supervision and leadership

EDUCATION

Sep 2005 – Present	Ph.D. Candidate, Neurosciences, University of Ottawa
Sep. 2002 – Mar. 2005	M.Sc. Cellular and Molecular Med., University of Ottawa
May 2001 – Apr. 2002	B.Sc. Hon. Biotechnology, University of Ottawa, Canada
Sep.1996 – Aug. 2000	B.Sc. (with distinction), Basic Medical Sciences, University of Toronto, Canada

HONOURS/ AWARDS & DISTINCTIONS

July 2009 Post doctorate Research Award, Canadian Institute of Health Research
(CIHR), (\$50,000 per annum, Total \$150,000).

Jun. 2007	Graduate Trainee Certificate of Merit (MSc)- In recognition of Outstanding Research Contribution, Department of Reproductive Biology, University of Ottawa
Sep. 2006	PhD seminar presentation award, in recognition of the outstanding Student seminar of 2005-2006 for skills in communication of ideas, clarity of presentation and general excellence in presentation, Neurosciences, University of Ottawa,
Apr. 2006	Doctorate Research Award (DRA), Canadian Institute of Health Research (CIHR), (\$ 22,000 per annum, Total \$66,000)
Apr. 2006	National Doctorate Excellence Scholarship, University of Ottawa, (in recognition of securing external funds)
May 2006	Young Investigators Award, Heart and Stroke Foundation of Canada (HSFC), Young Investigator Forum in Circulatory Health, Winnipeg, Canada, (\$1500)
May 2006	Canadian Stroke Network (CSN) Trainee Travel Award, CSN Annual General Meeting, St Johns, Canada
Aug. 2006	University of Ottawa Travel Award, Gordon Research Conference (GRC), Mitochondria and Chloroplast, Oxford, UK
Sep. 2005	Ontario Female Doctorate Scholarship in Biochemistry, Ministry of Education, (\$3000 per annum)
Sep 2005	Doctorate Entrance Scholarship, University of Ottawa, (\$5800 k Per annum)
May 2004	Best Trainee Presentation Award (National recognition): First

Place, Second Canadian Conference on Ovarian Cancer

Research, Ottawa, Canada (\$ 500)

July 2003 MSc. Travel Award, XVth international Ovarian Workshop, Vancouver, Canada

Sep. 1998 Dean's Honors List, University of Toronto, in recognition of academic excellence, Toronto, Canada

Sep. 1997 Golden Key National Honor Society at the University of Toronto, in recognition of scholastic achievements, Toronto, Canada

PEER REVIEWED PUBLICATIONS

Published

1) Arbour N, Vanderluit JL, Le Grand JN, **Jahani-Asl A**, Ruzhynsky VA, Cheung EC, Kelly MA, MacKenzie AE, Park DS, Opferman JT, Slack RS. Mcl-1 is a key regulator of apoptosis during CNS development and after DNA damage. J Neurosci. 2008 Jun 11; 28(24):6068-78.

2) **Jahani-Asl A**, Cheung EC, Neuspiel M, MacLaurin JG, Fortin A, Park DS, McBride HM, Slack RS (2007) Mitofusin 2 protects cerebellar granule neurons against injury-induced cell death. J Biol Chem. 2007 Aug 17; 282(33):23788-98.

3) **Jahani-Asl A**, Basak A, Tsang B.K. (2007) Caspase-3-mediated cleavage of Akt: involvement of non-consensus sites and influence of Phosphorylation, FEBS Lett: 26; 581(16):2883-8

4) **Jahani-Asl A.** and Slack RS (2007). The phosphorylation state of Drp1 determines cell fate. *EMBO Rep*, 8(10):912-3.

5) Fraser M., leung B., **Jahani-Asl A.**, Yan X, Thompson W.E., Tsang B.K. (2003) Chemoresistance in human ovarian cancer: the role of apoptotic regulators. *Reproductive Biology and Endocrinology* 2003, 1:66.

6) **Jahani-Asl A.**, Germain A, Slack RS. Joining forces to thwart cell death. *BBA* (In Press)

Manuscripts submitted or In Preparation

7) **Jahani-Asl A.**, Xu W, Pilon Larose K, McBride HM., Park DS, Slack RS, Regulation of mitochondrial dynamics following calcium deregulation through NMDAR (submitted *J cell Biol.*, Submission number JCB_2052491).

8) Irrcher I, Aleyasin H, Seifert E, Phillips M, Bevilacqua L, Hewitt S, **Jahani-Asl A.**, Rousseaux MWC, Callaghan S, Rizzu P, Rippstein P, Kim, RH, Slack RS, Harper ME, McBride HM, Mak TW, and Park DS. Loss of the Parkinson's Disease-linked gene DJ-1 results in abnormal mitochondrial dynamics.
(Submitted PNAS)

9) Jahani-Asl A, Ircher I, Rashidian J, Ishihara N, Park D, Slack RS. Identification of a neuronal intrinsic mechanism for the regulation of Drp1 activity and mitochondrial fission (In preparation).

10) Cheung et al. Submitted Mol. Cell.

PLATFORM PRESENTATION/INVITED SPEAKER

1) Jahani-Asl A, McBride HM, Park DS, Slack RS. Regulation of mitochondrial dynamics in neurodegeneration, University of Ottawa. Feb 2009.

2) Jahani-Asl A, McBride HM, Park DS, Slack RS. Regulation of mitochondrial dynamics in neurodegeneration, Dept. of Pathology, Harvard Medical School, Jan 2009.

3) Jahani-Asl A, McBride HM, Park DS, Slack RS. Regulation of mitochondrial dynamics in neurodegeneration, Department of cell biology and HHMI laboratory, Harvard Medical School, Jan 2009.

4) Jahani-Asl A, McBride HM, Park DS, Slack RS. Regulation of mitochondrial dynamics in neurodegeneration, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Jan. 2009

5) Jahani-Asl A, Cheung CC, Park DS, McBride HM, Slack RS. Mitofusin 2 protects against acute injury. Society for Neuroscience. November 2007, San Diego CA.

PUBLISHED ABSTRACTS

1) Jahani-Asl A, Pilon-Larose K, David S Park, Ruth S Slack. Regulation of Mitochondria dynamics in NMDA induced neuronal injury. Brain Health Research Day, June 2009, Ottawa, Ontario (**Local**)

2) Irrcher I, Aleyasin H, Seifert E, Phillips M, Bevilaqua L, Hewitt S, **Jahani-Asl A**, Rousseaux MWC, Callaghan S, Rizzu P, Rippstein P, Kim, RH, Slack RS, Harper ME, McBride HM, Mak TW, and Park DS. Loss of the Parkinson's Disease-linked gene DJ-1 results in abnormal mitochondrial dynamics. Mitochondria dynamics and physiology (KeyStone Symposia, Whistler BC, March 22-29, 2009). (**International**)

3) Jahani-Asl A, Pilon-Larose K, David S Park, Ruth S Slack. Regulation of Mitochondria dynamics in NMDA induced neuronal injury. Axonal Connections: Molecular cues for development and regeneration (Keystone Symposia, Colorado, Feb 17-22, 2009). (**International**)

4) Jacqueline Vanderluit; Nicole Arbour; Nicole Legrand; **Arezu Jahani-Asl**; Vladimir Ruzhynsky; Eric Cheung; Melissa Kelly; Joseph Opferman; Ruth Slack (2008). MCL-1 IS A KEY REGULATOR OF APOPTOSIS IN NEURAL PRECURSOR CELLS. 2nd Annual Canadian neuroscience meeting. Montreal Canada. (**National**)

5) Arezu Jahani-Asl, David S Park, Heidi McBride, Ruth Slack. Mitofusin 2 protects against acute injury. FASEB summer research conference, Assembly of the Mitochondrial respiratory chain, Tucson, Arizona, August 5-10, 2007. **(International)**

6) Legrand N, Arbour N., Vanderluit J, **Jahani-Asl A**, Cheung EC., Opferman JT, Park DS, Slack RS (2007) MCL1 is required for neuronal survival. Society for Neuroscience, San Diego, CA Nov. 2007. **(International)**

7) Arezu Jahani-Asl, David S Park, Heidi McBride, Ruth Slack. Mitofusin 2 protects against acute injury. Stroke Network Annual General Meeting. Montebello QC. June 11-13, 2007. **(National)**

8) Legrand N, Arbour N., Vanderluit J, **Jahani-Asl A**, Cheung EC., Opferman JT, Park DS, Slack RS (2007) MCL1 is required for neuronal survival. Cold Spring Harbour Laboratories Cell Death meeting, September 26th-30th, 2007, Cold Spring Harbour, Long Island, NY. **(International)**

9) Arezu Jahani, Eric C.C. Cheung. David S Park, Heidi McBride, Ruth Slack. Mitofusin 2 protects against acute neuronal injury. Canadian Association for Neuroscience meeting, Toronto May 23-25, 2007. **(National)**

10) Jahani-Asl A, Park DS, McBride HM, Slack RS. Mitofusin 2 protects against acute injury. Society for Neuroscience. November 3-7, 2007, San Diego CA. **(International)**

11) Arezu Jahani Asl, Eric C.C. Cheung, Jason G. MacLaurin, David S. Park, Heidi McBride and Ruth S. Slack, Dynamic fusion of mitochondria protects neurons against oxidative stress and excitotoxicity. 6th Annual OHRI research day, Nov, 30th 2006, Ottawa, Ontario. **(Local)**

12) Arezu Jahani Asl, Eric C.C. Cheung, Margaret Neuspiel, Jason G. MacLaurin, William Xu, Andre Fortin, David S. Park, Heidi McBride, Ruth S. Slack. Dynamic fusion of mitochondria protects neurons against oxidative stress and excitotoxicity. Society for Neuroscience, (October 14-18 2006), Atlanta, Georgia **(International)**.

13) Arezu Jahani-Asl, Eric C.C. Cheung, Margaret Neuspiel, Jason G. MacLaurin, , Andre Fortin, David S. Park, Heidi McBride, Ruth S. Slack. Dynamic fusion of mitochondria protects neurons against oxidative stress and excitotoxicity. GRC: Mitochondria and chloroplast, Magdalen Collage, Oxford, UK, August 13- 18, 2006. **(International)**.

14) Arezu Jahani-Asl , Eric C.C. Cheung, Jason MacLaurin, Andre Fortin, William Xu, David S.Park, Heidi McBride, Ruth S. Slack. Activation of mitochondrial fusion protects neurons against injury induced cell death.. 5th Annual OHRI Research day, (May 3-4, 2006)Ottawa, Ontario. **(Local)**

20) Jahani-asl A, Basak A, Tsang BK. Novel Caspase-3 Cleavage Sites in Akt1: Influence of Phosphorylation. Second Canadian Conference on Ovarian Cancer Research, Ottawa, Ontario, May 16-18, 2004 **(National)**

21) A. Jahani-Asl, A. Basak, B.K. Tsang. Caspase-3-Mediated Akt1 Cleavage: Influence of phosphorylation. Canadian Workshop on Human Reproduction and Reproductive Biology “ From Physiology to Genes to Therapy” May 3-4 2004, Ottawa, Canada **(National)**

22) Jahani-Asl A, Basak A, Tsang B.K. Novel caspase-3 cleavage sites in Akt: Influence of phosphorylation. 3rd Annual OHRI Research Day, Ottawa Canada. Nov, 28 , 2003. **(Local)**

23) A.A. Jahani, A. Basak, B.K. Tsang. Influence of Phosphorylation on Caspase-3-mediated Akt1 Cleavage. 21st Annual Ottawa Reproductive Biology Workshop, May , 12, 2003. **(National)**

24) Jahani A.A. , Basak A., Tsang B. Caspase-3-Mediated Akt1 Cleavage: Influence of phosphorylation. 2nd Annual OHRI Research Day, Ottawa Canada Jan, 24, 2003 . **(Local)**

RESEARCH EXPERIENCE

Sep. 2005 –Present; PhD candidate

Neuroscience Research Institute, University of Ottawa, Ottawa, Canada

Project: Role of mitochondrial Fission/Fusion in neuronal injury.

Technical skills: Microscopy: Deconvolution, light/dissection; Confocal (Olympus and Zeiss softwares), Tissue Culture skills: Cerebellar granule neurons, cortical neurons, apoptosis assays, Immunoprecipitation, Immunocytochemistry, Western blot, cellular fractionation, Purification of recombinant proteins, *In vitro* kinase assays, Lentivirus production. Experience with different injury models including DNA damage, Oxidative stress and excitotoxicity. Advanced courses: Stroke injury models, SPIN 2006

May. 2002- Dec 2004; MSc. candidate,

Ottawa Health Research Institute (LOEB), Ottawa, Canada

Project: Caspase-3-mediated cleavage of Akt1: Influence of phosphorylation.

Technical Skills: Spectrometry: Matrix assisted laser desorption Ionization time of flight mass spectrometry (MALDI-TOF), Surface enhanced laser desorption ionization mass spectrometry (SELDI-M.S.); protein-staining (Silver, Sypro Ruby, Coomassie blue), protein desalting using Zip-Tips, Western Blot, Immunoprecipitation, Caspase-3 assay.

May 2001- May 2002; Honors student

Department of Biology, University of Ottawa, Ottawa, Canada

Project: Investigating molecular mechanisms of senescence in soybean.

Technical skills: DNA and RNA extraction, quantification, and separation by gel electrophoresis, Restriction digest and mapping, cloning

SUPERVISORY AND TEACHING EXPERIENCE

Contribution to student supervision

2009: Carmen Hamze, University of Ottawa, MSc student-Slack lab

- Technical training and supervision for her project
- Discussion sessions on data interpretation and literature review

2008: Haitham Wahbe, University of Ottawa, MSc student- Slack lab

- Technical training and supervision for his project resulting in contribution to ongoing project

2006: William Xu, University of Ottawa, Honours student- Slack lab

- Providing technical training and supervision for his project
- Contributing to providing critical feedback on poster & seminar presentations, as well as honors' thesis

Teaching appointment

May 2008 – Aug 2008

Lecturer, University of Ottawa, Canada, teaching ANP1105: Human Anatomy and Physiology I

Sep 2007- Oct. 2007

Lecturer, University of Ottawa, Canada, teaching ANP1105: Human Anatomy and Physiology I

May. 2007- Jul. 2007

Lecturer, University of Ottawa, Canada, Teaching ANP1105: Human Anatomy and Physiology; Course Content: An introduction to tissue and cell morphology, biochemistry of the cell and physiological concepts including membrane transport mechanisms. Anatomy & physiology of cardiovascular, blood, lymphatic and respiratory systems. Introduction to the control mechanisms: concepts of homeostasis, nervous and endocrine systems.

Responsibilities included: designing the course notes, exams and assignments

Sep. 2006- Dec. 2006

Course coordinator, Lecturer, University of Ottawa, Canada, Teaching ANP1101: Introduction to General Anatomy and Physiology; Course Content: An introduction to tissue and cell morphology, biochemistry of the cell, Metabolic pathways; Anatomy and physiology of neurons, muscles and blood including immune system, inflammatory responses and homeostasis.

The responsibilities included: designing the course, the website, exams and assignments for a class of 250 first year nursing students.

Oct. 2005- Dec. 2005

Lecturer, University of Ottawa, Canada, Teaching ANP1101: Introduction to General Anatomy and Physiology. The responsibilities included: designing the course notes, the website, exams and assignments

Sept 2005- 2007

Instructor (volunteer), Lets talk science, Ottawa, Canada . Teaching basic scientific concepts and demonstrating experiments to grade school students.

Sep. 2001- 2002

Science tutor in the classroom, Carleton District School Board, Ottawa,

Introduced grade-school students to the world of science and research;

Leading Scientific projects for the grades 2 to 6.

Sep.1999 -2002

Laboratory Demonstrator, Department of chemistry, University of Toronto,

Designing and demonstrating the laboratory work for the Chemistry Olympiad competitions.

Sep.1996-1998

Teaching Assistant, University of Toronto, Department of continuing education

INVOLVEMENT IN ACADEMIC

COMMUNITY/COMMITTEES/ASSOCIATIONS

2005 – Present

Ottawa Health Research Institute (OHRI) trainee committee, the committee consisting of Scientists, and trainees meets every four month to discuss the concerns of the OHRI trainees

2007 –2008

VP communication, Graduate student council - University of Ottawa; Editor of CMM/NSc Biweekly newsletter, overseeing publication of CMMNSC Biweekly

2007-2008

Member of Canadian Association for Neuroscience/Association Canadienne des Neurosciences,

2005-2008

Member of Association of Part Time Professors at the University of Ottawa
(APTPU)

2006-2007

Member of Society for Neurosciences (SFN)

2005- 2007

Member of Lets Talk Science

1997-2008

Golden key National honor society, University of Toronto Chapters,

APPENDIX C
PERMISSION TO REPRINT PUBLISHED MATERIAL

Permission to reprint EMBO report article as part of dissertation

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APPENDIX D
ADDITIONAL PUBLICATIONS

Mcl-1 Is a Key Regulator of Apoptosis during CNS Development and after DNA Damage

Nicole Arbour,^{1,3*} Jacqueline L. Vanderluit,^{2,3*} J. Nicole Le Grand,^{2,3} Arezu Jahani-Asl,^{2,3} Vladimir A. Ruzhynsky,^{2,3} Eric C. C. Cheung,^{2,3} Melissa A. Kelly,^{2,3} Alexander E. MacKenzie,⁴ David S. Park,^{2,3} Joseph T. Opferman,⁵ and Ruth S. Slack^{2,3}

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Despite the importance of Mcl-1, an anti-apoptotic Bcl-2 family member, in the regulation of apoptosis, little is known regarding its role in nervous system development and injury-induced neuronal cell death. Because germline deletion of Mcl-1 results in peri-implantation lethality, we address the function of Mcl-1 in the nervous system using two different conditional Mcl-1 mouse mutants in the developing nervous system. Here, we show for the first time that Mcl-1 is required for neuronal development. Neural precursors within the ventricular zone and newly committed neurons in the cortical plate express high levels of Mcl-1 throughout cortical neurogenesis. Loss of Mcl-1 in neuronal progenitors results in widespread apoptosis. Double labeling with active caspase 3 and Tuj1 reveals that newly committed Mcl-1 deficient neurons undergo apoptosis as they commence migration away from the ventricular zone. Examination of neural progenitor differentiation *in vitro* demonstrated that cell death in the absence of Mcl-1 is cell autonomous. Although conditional deletion of Mcl-1 in cultured neurons does not trigger apoptosis, loss of Mcl-1 sensitizes neurons to an acute DNA damaging insult. Indeed, the rapid reduction of Mcl-1 mRNA and protein levels are early events after DNA damage in neurons, and maintaining high Mcl-1 levels can protect neurons against death. Together, our results are the first to demonstrate the requirement of Mcl-1, an anti-apoptotic Bcl-2 family protein, for cortical neurogenesis and the survival of neurons after DNA damage.

Key words: neurogenesis; cell death; neuronal progenitors; neuron; apoptosis; development

Introduction

Myeloid cell leukemia 1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 family of proteins. Germline knock-outs (KOs) of Mcl-1 are peri-implantation lethal at embryonic day 3.5 (E3.5) (Rinkenberger et al., 2000), which is the most severe phenotype among the anti-apoptotic Bcl-2 proteins. Furthermore, Mcl-1 is essential for the development and maintenance of B and T lymphocytes and for the survival of hematopoietic stem cells (Opferman et al., 2003, 2005). Mcl-1 is believed to inhibit cell death through interactions with proapoptotic Bcl-2 family members, including multidomain (Youle and Strasser, 2008) and BH3-only (for review, see Letai, 2008). The affinity of interaction between Bcl-2 family members varies significantly, with Mcl-1

showing increased binding affinity for Noxa, PUMA (p53-upregulated modulator of apoptosis), Bim_{EL}, and Bcl-2 modifying factor (Chen et al., 2005; Kuwana et al., 2005).

The peri-implantation lethality of Mcl-1 germline mutant mice suggests a unique requirement for Mcl-1 in early embryogenesis (Rinkenberger et al., 2000). Bcl-xl germline deficient mice are also embryonic lethal, at E13, showing increased apoptotic activity in the brain and hematopoietic system (Motoyama et al., 1995). More recent studies have suggested that the requirement for Bcl-xl may vary depending on the cell type examined. For example, conditional deletion of Bcl-xl in catecholaminergic neurons resulted in viable mice with the catecholaminergic neuronal population reduced by one-third (Savitt et al., 2005). Mice with a Bcl-2 targeted deletion show normal neuronal development but exhibit significant loss of sympathetic, motor, and sensory neurons postnatally (Michaelidis et al., 1996). Although these studies demonstrate the importance of Bcl-2 family proteins in the maintenance of certain cell populations within the CNS, none have been shown to have a direct role in neural development.

Despite the importance of Mcl-1 in apoptosis during early development, little is known regarding its role in nervous system development and injury-induced neuronal cell death. Recent studies, however, have shown that Mcl-1 upregulation may play a role in Notch-1-mediated survival of neural precursor cells

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*N.A. and J.L.V. contributed equally to this work.

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(Oishi et al., 2004), as well as in maintaining the survival of granule cells during migration and differentiation (Zhang and D'Ercole, 2004). Heterozygous Mcl-1 germline deletion also results in increased susceptibility of neurons to pilocarpine-induced seizure injury (Mori et al., 2004). These studies suggest that Mcl-1 may play an important role in nervous system development, and after acute neuronal injury, however, the impact of Mcl-1 deficiency during nervous system development or after acute injury has never been examined.

In this study, we show for the first time that Mcl-1 is required for neural development. Loss of Mcl-1 in neuronal progenitor cells results in widespread apoptosis of progenitors expressing Nestin and β III tubulin (Tuj1), demonstrating that Mcl-1 is required for the survival of newborn neurons. We also show that Mcl-1 plays a key role in the regulation of neuronal survival after injury. Loss of Mcl-1 sensitizes neurons to apoptosis induced by DNA damage, whereas maintenance of high Mcl-1 levels protects neurons against death. Together, our results demonstrate that Mcl-1 is required for neural precursor survival and the regulation of injury-induced neuronal cell death.

Materials and Methods

Mice and primary neuronal cultures. For embryonic time points, the time of plug identification was counted as E0.5. All experiments were approved by the University of Ottawa Animal Care Ethics Committee adhering to the Guidelines of the Canadian Council on Animal Care. Cortical and cerebellar granule neurons (CGNs) were cultured as described previously (Cregan et al., 1999; Fortin et al., 2001).

Generation of transgenic mice. Floxed Mcl-1 mice were described previously (Opferman et al., 2003). The generation of telencephalon-specific Mcl-1 conditional mutants was accomplished by breeding floxed Mcl-1 mice with Foxg1:Cre mice (Hebert and McConnell, 2000) to generate Foxg1^{cre/+};Mcl-1^{lox/lox} mice. The generation of nestin-specific conditional mutants, floxed Mcl-1 mice were crossed with Nestin:Cre mice (Berube et al., 2005) to generate Nestin^{cre/+};Mcl-1^{lox/lox} mice. The genotyping of these mice was performed as described previously (Hebert and McConnell, 2000; Casanova et al., 2001; Opferman et al., 2003). The floxed Mcl-1 mice were on a C57BL/6 background, the Nestin:Cre lines were on a pure FVB/N background, and the Foxg1:Cre mice were a mixed C57BL/6 and FVB/N background. Double heterozygous littermates (Cre^{+/+};Mcl-1^{lox/+}) were used as controls for *in vivo* experiments, because they were indistinguishable from wild-type embryos (results not shown).

Tissue processing, immunohistochemistry, and *in situ* hybridization. Tissue fixation and cryoprotection of embryonic tissue was performed as described previously (Ferguson et al., 2002). Briefly, embryos were dissected and postfixed overnight in 4% PFA and cryoprotected in a sucrose gradient of 12, 16, and 22% sucrose (Sigma-Aldrich) in PBS. Tissue was frozen, and 14 μ m coronal cryosections were collected on Superfrost Plus slides (12–550–15; Fisher Scientific). Sections were stained in 0.1% cresyl violet for light microscopy or immunohistochemistry as described previously (Ferguson et al., 2002) for active caspase 3 (AC3) (1:100; Cell Signaling Technology 9664), phospho-Histone H3 (PH3) (1:500; Upstate Biotechnology, 06–570), Nestin (mouse monoclonal, 1:200; Research Diagnostics, 21714), Tuj1 (1:100), and Doublecortin (DCX) (1:200; Santa Cruz Biotechnology). For double immunofluorescence of AC3 and DCX, sections were postfixed in 4.0% paraformaldehyde before staining. PH3-positive cells were counted along a 1000 μ m length of the lateral ventricle from the dorsal midline extending laterally at three distinct regions through the rostrocaudal extent of the lateral ventricle. The mean number of PH3-positive cells in Foxg1:Cre Mcl-1 mutant and control brains was compared by Student's *t* test. *In situ* hybridization was performed with a riboprobe to Mcl-1 (Rinkenberger et al., 2000) according to previously published protocols (Vanderluit et al., 2004; Ruzhynsky et al., 2007).

Confocal microscopy. All images were taken using a Zeiss 510 meta confocal microscope. To identify colocalization of active caspase 3 with Tuj1, Nestin, or doublecortin-positive cells, Z stack sections (0.5–1 μ m

Table 1. Primer sequences for RT-PCR gene expression analysis

Gene	Sequence	
	5'-sense-3'	5'-anti-sense-3'
Mcl-1	TCTGAGATGTTGGCTGCGGAGA	TCTAGACTATCTTATTAGATATGCCAGAC
Bcl-2	CTCGTCGCTACCGTCGTGACTTCG	CAGATGCCGTTTACGGTACTCAGTC
Bcl-W	CGAGTTTGAGACCGTTTCCGCC	GCAGTTTCCCAACAAAGGCTCC
Bcl-xL	AGGCAGGCGATGAGTTTGAA	CGGCTCTCGGCTGCTGCATT
Bax	AAGCTGAGCGAGTGCTCCGGCG	GCCACAAGATGGTCACTGCTGCC
Bak	CACCCACCTCATCGTCCATTTC	GCCACCCCTCCCTGTCTATA
S12	GGAAGGCATAGCTGCTGG	CCTCGATGACATCCTTGG

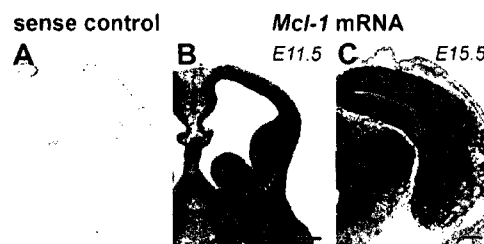


Figure 1. Mcl-1 is expressed in neural precursors and postmitotic neurons in the developing mouse telencephalon. *In situ* hybridization analysis of Mcl-1 mRNA expression in telencephalic coronal sections of E11.5 (B) and E15.5 (C) mouse embryos. Note the absence of the positive signal in the section of the brain hybridized with sense control riboprobe (A). Scale bar, 250 μ m.

per section) were taken on different fields using an 63 $\times$ oil immersion objective. The counts were performed individually analyzing six different sections of telencephalon, including ventral (V), dorsal (D), lateral ventral (LV), lateral dorsal (LD), medial ventral (MV), and medial dorsal (MD) areas (see Fig. 5B). Colocalization for active caspase 3 and doublecortin was counted in the ganglionic eminence. For each animal, two different brain sections were analyzed for three different animals per experiment.

Cell culture and recombinant adenovirus infection. The cDNA for Mcl-1 (mouse) (Rinkenberger et al., 2000) was a gift from Dr. S. Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Recombinant adenoviral vectors carrying expression cassettes for Mcl-1 or Cre were constructed, purified, and titered as described previously (Cregan et al., 2000). Adenoviral vectors were added to cell suspensions immediately before plating. Multiplicity of infection (MOI) refers to the number of plaque forming units (pfu) per cell, as calculated from viral titers (pfu/ μ l).

Semiquantitative reverse transcription-PCR analysis. Total RNA was isolated from cells using Trizol isolation reagent according to the manufacturer instructions (Invitrogen). Pilot experiments were performed to determine the linear range of amplification with respect to quantity of starting template and PCR cycles. The primers used for the detection of genes are shown in Table 1. Total RNA (25–100 ng) was used for cDNA synthesis and targeted gene amplification using the SuperScript One-Step Reverse Transcription (RT)-PCR kit (Invitrogen). cDNA synthesis was performed at 48°C for 45 min followed by a 2 min initial denaturation step at 94°C. This was followed by 24 cycles (Bax), 25 cycles (Mcl-1, S12), 29 cycles (Bcl-2), 32 cycles (Bcl-w), 30 cycles (Bcl-xL), and 30 cycles (Bak) at 94°C for 30 s, 55–64°C for 30 s, and 72°C for 1 min. The resulting products were sequenced to confirm identity.

Western blot analysis. Western blot analysis was performed as described previously (Cregan et al., 1999) with antibodies against Mcl-1 (1:10,000; Rockland Immunochemicals) (Opferman et al., 2003, 2005) and actin (1:2000; SC-1616, Santa Cruz Biotechnology) as a loading control.

Camptothecin treatment and cell viability assays. For inducing acute DNA damage, neurons were treated with 10 μ M camptothecin (CPT) after 2 d *in vitro* (DIV). Cell survival was measured by the following: LIVE/DEAD viability/cytotoxicity kit (Invitrogen) as described previously (Cregan et al., 2004), and Hoechst nuclear staining with condensed

Table 2. Recovery of embryos from Mcl-1 conditional mutants

Mcl-1 conditional mutant	Embryonic time point						
	E9.5	E10.5	E12.5	E15.5	E16.5	E17.5	E19.5
Foxg1:Cre Mcl-1 KO							
Total number of embryos			20	32	11	11	4
Number of knock-outs (%)			3 (15%)	8 (25%)	1 (9%)	0 (0%)	0 (0%)
Expected number Mendelian ratio (%)			4 (20%)	7 (21%)	2 (25%)	2 (25%)	1 (25%)
Nestin:Cre Mcl-1 KO							
Total number of embryos	9	30	74	20			
Number of knock-outs (%)	2 (22%)	8 (27%)	9 (12%)	0 (0%)			
Expected number Mendelian ratio (%)	2 (25%)	8 (27%)	17 (24%)	5 (25%)			

nuclei as an indicator of apoptosis. For Hoechst counts, cells were fixed with 4% PFA in PBS for 30 min and then washed with $1 \times$ PBS and stained with Hoechst (33258; Sigma Chemical). For LIVE/DEAD assays and Hoechst counts, data represent the mean and SE from a minimum of three independent experiments.

Cortical progenitor cultures. Cortical progenitor cells were cultured as described previously (Callaghan et al., 1999). Briefly, cortices were dissected from Foxg1:Cre Mcl1 mutant and littermate control embryos at E12.5. Cortices were mechanically dissociated by trituration, and cell aggregates were plated on polyornithine-coated four-well dishes and cultured in media containing Neurobasal medium (Invitrogen), 0.5 mM glutamine, 0.5% penicillin-streptomycin, 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), and 10 ng/ml FGF-2 (Sigma). On day one, the media was replaced with fresh media without FGF2. After 3 d, cultures were fixed in 4% PFA, and cell nuclei were stained with Hoechst (Sigma) to identify healthy (diffuse nuclear staining) versus apoptotic (condensed nuclei) cells. The number of apoptotic and healthy cells were quantified per aggregate and represented as the total number of cells per aggregate. Newly committed neurons were identified by immunostaining with antibodies to Tuj1. Cell counts were performed to quantify the total number of Tuj1-positive cells, and the number of apoptotic neurons (Tuj1 positive with condensed nuclei) per aggregate. The number of apoptotic cells/aggregate and the number of apoptotic neurons/total number of neurons in each aggregate were represented as a percentage.

Results

Mcl-1 is required for cortical neurogenesis

During nervous system development, Bcl-2 family proteins play critical roles in shaping the developing brain by regulating the survival of neural precursor cells and neuronal number after neurogenesis (Akhtar et al., 2004). Although Mcl-1 has been shown to play an important role in the development of the immune system, the question as to whether Mcl-1 has any role in nervous system development remains unknown. To address this question, we first asked whether Mcl-1 was expressed in the developing brain and in which population of cells. *In situ* hybridization with a riboprobe to Mcl-1 was performed on sections from developing mouse brains during the period of cortical neurogenesis from E11 to E15.5 (Fig. 1). Robust expression of Mcl-1 was observed within the neural precursor population in the ventricular zone (VZ) at E11.5. By midneurogenesis at E15.5, Mcl-1 is still highly expressed within the ventricular zone and in postmitotic neurons in the developing cortical plate (CP) (Fig. 1). The high levels of Mcl-1 in both proliferating neural precursors and postmitotic neurons during this critical period of brain development lead us to question whether Mcl-1 regulates the survival of these populations.

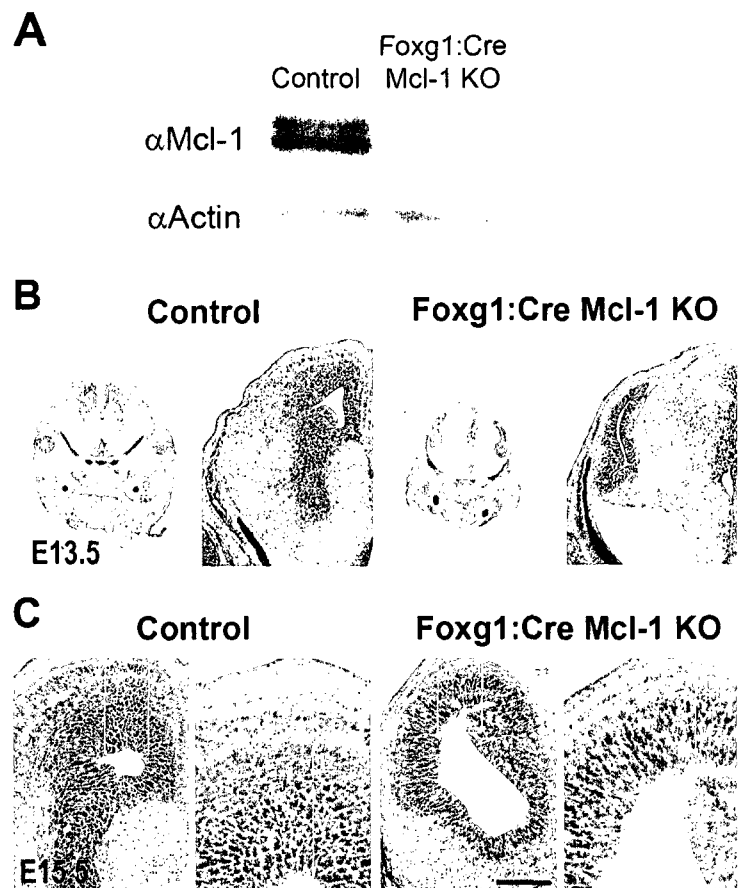


Figure 2. Loss of Mcl-1 in developing neurons results in severe defects in cortical neurogenesis. **A**, Protein lysates were collected from cortical neurons cultured from Foxg1:Cre mutant or control mice 48 h after plating and were blotted for Mcl-1 expression then for actin as a loading control, showing efficient excision of Mcl-1 in mutant cortical neurons. **B**, Cresyl violet-stained coronal sections of E13.5 Foxg1:Cre mutant mice and littermate controls showing significant loss of cortical size and morphology. **C**, Cresyl violet-stained coronal sections of E15.5 Foxg1:Cre mutant mice and littermate controls showing Mcl-1 deficiency has a pronounced effect on CP formation. The right panels are a magnification of the boxed areas. Scale bar, 250 μ m.

To assess the role of Mcl-1 in nervous system development, we used conditional mutants as Mcl-1 germline mutation results in preimplantation lethality (Rinkenberger et al., 2000). Animals in which Cre-recombinase was inserted at the Foxg1 locus (Foxg1:Cre) (Hebert and McConnell, 2000) were interbred with mice carrying a floxed Mcl-1 allele (Opferman et al., 2003). In these animals, Cre is expressed in neural progenitors throughout the developing telencephalon beginning at E8 and reaching maximal expression levels at E17 (Tao and Lai, 1992; Shimamura and

Rubenstein, 1997). Previous studies have revealed efficient Cre-mediated recombination of floxed alleles specifically using this Foxg1:Cre mouse model (Hebert and McConnell, 2000; Ferguson et al., 2002). By interbreeding floxed Mcl-1 mice with Foxg1:Cre, we could selectively assess the role of Mcl-1 in neural precursor cells during forebrain development.

Conditional deletion of Mcl-1 specifically within the forebrain resulted in embryonic lethality at E16–E17; however, embryos collected at earlier time points were found at expected Mendelian ratios (Table 2). The expression of Foxg1 in the midbrain–hindbrain junction, a region important for regulating cardiovascular and respiratory function, would result in Cre-mediated Mcl-1 deficiency in this region and may account for the embryonic lethality. Indeed, we and others have shown that Foxg1:Cre-mediated deletion of Rb (Ferguson et al., 2002) or Smo (Fuccillo et al., 2004) results in perinatal lethality.

To assess the role of Mcl-1 in brain development, embryos were collected at E15.5 corresponding to midneurogenesis. This stage is characterized by a large population of Nestin-expressing progenitors within the VZ and subventricular zone (SVZ), early committed neuroblasts within the SVZ/intermediate zone (IZ), and a growing population of postmitotic neurons in the developing CP. Efficient recombination of the floxed Mcl-1 allele in the telencephalon was confirmed by Western blot analysis (Fig. 2A). Animals lacking Mcl-1 in the developing telencephalon exhibit a dramatic neural phenotype with the overall size of the mutant telencephalon being significantly smaller relative to littermate controls (Fig. 2B). Morphological assessment of coronal sections through the telencephalon at E15 revealed a striking reduction in the size of the cortical plate. In contrast to littermate controls in which layering of the VZ/SVZ, IZ, and CP are delineated clearly, Mcl-1 mutant mice lacked a clear distinction between the IZ and CP (Fig. 2C). These results demonstrate that Mcl-1 is essential for telencephalic development.

The dramatic reduction in cortical plate formation led us to question whether this defect occurred before or after precursor commitment to a neuronal fate. A defect before neuronal commitment could be associated with Mcl-1 having a role in neural progenitor proliferation or survival, whereas a defect after commitment would be attributed to Mcl-1 playing a role in the survival of newly born neurons. We first determined whether Mcl-1 has a crucial role in the regulation of uncommitted neural precursor cells. Previous studies have suggested that Mcl-1 may modulate the rate of cell division (Fujise et al., 2000; Jamil et al., 2005). To determine whether Mcl-1 affects neural progenitor proliferation, we quantified the number of cycling cells in Mcl-1 deficient brains using the M-phase cell cycle marker PH3 (Fig. 3). Quantitative analysis of the number of cycling cells revealed no significant difference between Mcl-1 deficient (40.2 ± 5.1) and littermate controls (27.5 ± 1.7), demonstrating that Mcl-1 does not perturb neural precursor cell proliferation. We next asked whether Mcl-1 was required for neural precursor cell survival.

We assessed the role of Mcl-1 in neural precursor cell survival *in vivo* in the Foxg1:Cre Mcl-1 mutant and in a second conditional knock-out in which Cre is expressed in neural precursor cells. Transgenic mice in which Cre-recombinase is expressed under the control of the nestin promoter (Nestin:Cre) (Berube et al., 2005) were interbred with mice carrying a floxed Mcl-1 allele (Opferman et al., 2003). Nestin is expressed in neural progenitors throughout the developing nervous system at E7.5, after preplate formation (Dahlstrand et al., 1995). The use of two conditional mutants allowed us to drive Cre expression very early in development and examine the role of Mcl-1 in neural precursor cells. The

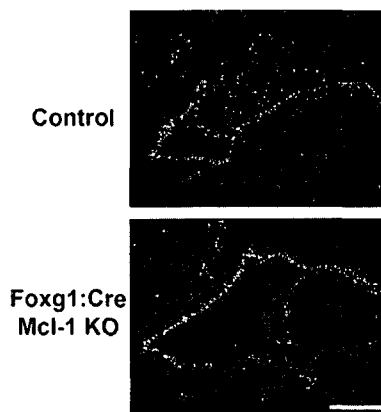


Figure 3. Mcl-1 deficiency does not affect proliferation of cortical progenitor cells. Coronal sections of E15.5 Foxg1:Cre Mcl-1 mutant mice and littermate controls immunostained for PH3. Quantitative analysis of PH3-positive cells in the ventricular zone revealed no significant difference in the number of proliferating cells between mutant and control mice. Scale bar, 100 μ m.

absence of Mcl-1 in the developing CNS with the Nestin:Cre resulted in embryonic lethality occurring before E15 (Table 2). Morphological analysis of coronal sections through the brains of E12.5 embryos revealed a similar cortical phenotype as the Foxg1:Cre mutant embryos with a dramatic reduction in cortical plate formation (Fig. 4A). Deletion of Mcl-1 in neural progenitor cells by either Foxg1:Cre or Nestin:Cre conditional mutants seriously impaired the development of the cortex. The striking reduction in the telencephalon in Mcl-1 mutants suggests that Mcl-1 is required for the survival of precursors in the developing brain.

To determine whether the observed cell loss in the ventricular zone is caused by apoptosis, sections from Foxg1:Cre and Nestin:Cre mutant embryos along with control littermates at E12.5 were immunostained with antibodies to active caspase 3, a hallmark of classical apoptotic cell death. Numerous active caspase 3-positive cells were present within the VZ/SVZ of Mcl-1 mutant mice, whereas only an occasional apoptotic cell was observed in littermate controls (Fig. 4B). The total number of active caspase 3-expressing cells was quantified in two distinct regions of the developing cortex (Fig. 4C). A dramatic increase in the number of apoptotic cells was found in both Foxg1:Cre and Nestin:Cre Mcl-1 deficient brains compared with littermate controls (Fig. 4D). Our results show that Mcl-1 mutants exhibit extensive apoptotic cell death in the developing cortex relative to littermate controls, which demonstrate an essential role for Mcl-1 in the maintenance of neural precursor survival during cortical development.

Because apoptotic cells are seen throughout the cortex, we next asked which cell types require Mcl-1 for survival: the newly committed neurons or the uncommitted precursors. To distinguish these possibilities, sections were double labeled with antibodies to active caspase 3 and Nestin to identify neural precursor cells, or active caspase 3 and Tuj1 to identify newly committed neurons. Sections were examined for coexpression of cell type-specific markers and active caspase 3, and the percentage of cells exhibiting caspase activation within each population was quantified. We first asked whether uncommitted neural precursor cells required Mcl-1 for survival. Double labeling revealed that a large proportion of Nestin-expressing cells underwent cell death in the absence of Mcl-1 (Fig. 5A). To determine whether Mcl-1 deficiency resulted in region-specific cell death, we quantified the percentage of Nestin-expressing apoptotic cells in different re-

gions surrounding the lateral ventricles, including the dorsal, lateral dorsal, medial dorsal, lateral ventral, and medial ventral regions (Fig. 5B). Examination of cell death around the ventricles revealed that a lack of Mcl-1 induces a highly significant increase in cell death of uncommitted neural precursor cells. The highest percentage of precursors double stained for active caspase 3 and Nestin was in the ventral and ventral lateral regions in the Mcl-1 deficient brains with 24.7 ± 7.73 and $32 \pm 8.8\%$, respectively, relative to 0.4 ± 0.41 and $0.5 \pm 0.34\%$ of controls (Fig. 5C). These results demonstrate that neural precursors require Mcl-1 for survival during development and that the ventral populations at E12 are most affected.

Because many caspase 3-expressing cells were found in the intermediate zone where progenitors migrate away from the ventricle, we next asked whether precursor cells undergo apoptosis after commitment to a neuronal fate as they commence migration. To determine whether these newly born neurons required Mcl-1 for survival, coronal sections from E12.5 embryos were double labeled for Tuj1 and active caspase 3, and newly born neurons undergoing cell death were counted and compared with littermate controls (Fig. 6A). Consistent with a reduction in the cortical plate, the number of newly born neurons undergoing cell death was significantly increased in Mcl-1 mutants, whereas $12.0 \pm 0.86\%$ of Tuj1-expressing cells colabeled with the apoptotic marker relative to only $1.2 \pm 0.86\%$ in littermate controls. In the lateral ventral region, the percentage of dying cells was 7.03 ± 2.1 versus $1.0 \pm 0.33\%$ in the control group (Fig. 6B). To confirm these findings, we assessed whether committed neurons undergo cell death as they initiate migration to their final destination. Double labeling with doublecortin, a marker for newly migrating populations, and active caspase 3 revealed that these migratory precursor cells were undergoing cell death as they left the ventricular zone and initiated migration (Fig. 7A). The number of doublecortin-expressing cells undergoing cell death was 64.5 ± 6.4 in Mcl-1 mutant mice relative to 0.3 ± 0.3 in littermate controls. These results explain the reduction in cortical thickness observed in Mcl-1 mutant mice, where Mcl-1 deficient neural precursor cells undergo cell death as they commit to a neuronal fate and migrate away from the ventricular zone.

To determine whether neural precursor cell death observed *in vivo* is caused by a cell autonomous or noncell autonomous mechanism, we cultured primary cortical progenitor cells and monitored their survival *in vitro* as they differentiated. Cortical progenitor cell aggregates from E12.5 Foxg1:Cre Mcl-1 mutant

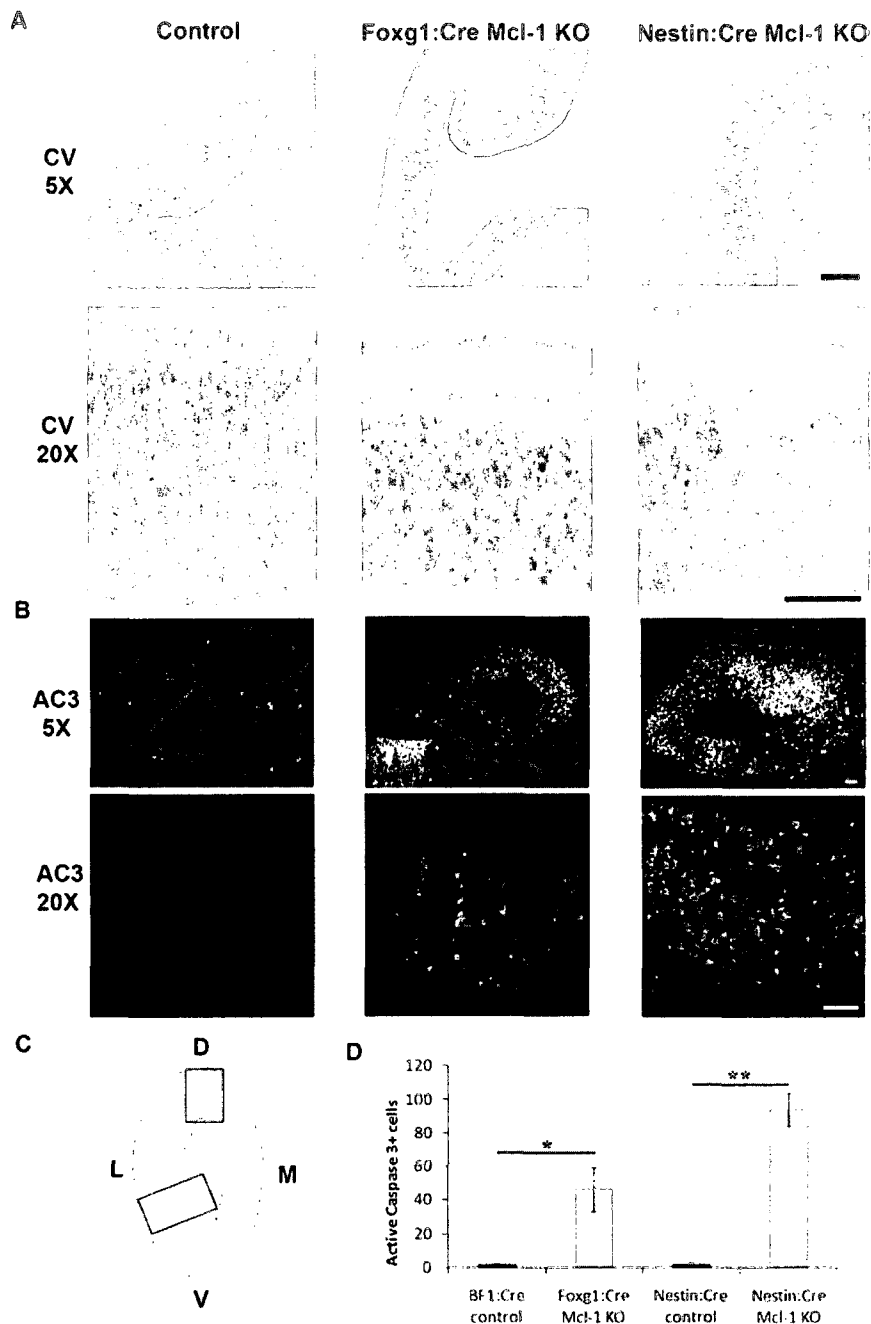


Figure 4. Loss of Mcl-1 in developing neurons results in increased apoptotic activity in neuronal progenitors. **A**, Cresyl violet-stained coronal sections through the telencephalic hemisphere of E12.5 Foxg1:Cre and Nestin:Cre Mcl-1 mutant embryos and littermate controls showing a significant reduction in the thickness of the developing cortex in the Mcl-1 mutants. The bottom panel represents high-magnification photomicrographs of the developing cortex. **B**, Coronal sections from E12.5 Foxg1:Cre and Nestin:Cre Mcl-1 mutants and littermate controls were immunostained for AC3, a marker of apoptotic cell death. Numerous active caspase-3-positive cells are dispersed throughout the VZ in Mcl-1 deficient mice. The bottom panels are higher-magnification photomicrographs. **C**, Schematic representation of areas quantified. **D**, Quantitative analysis of AC3-positive cells reveals a significant increase in apoptotic cells in mutant animals. L, Lateral; M, medial. Scale bars: **A**, 100 μ m; **B**, 50 μ m. * $p < 0.05$, ** $p < 0.01$.

embryos and littermate controls were plated at similar plating densities and allowed to differentiate *in vitro*. After 3 DIV, significant cell loss was observed in the Mcl-1 deficient cultures, at which point cultures were fixed and stained. Quantification of the total number of cells per aggregate revealed a twofold reduction in Mcl-1 deficient cultures (Fig. 8A, B). To assess whether the

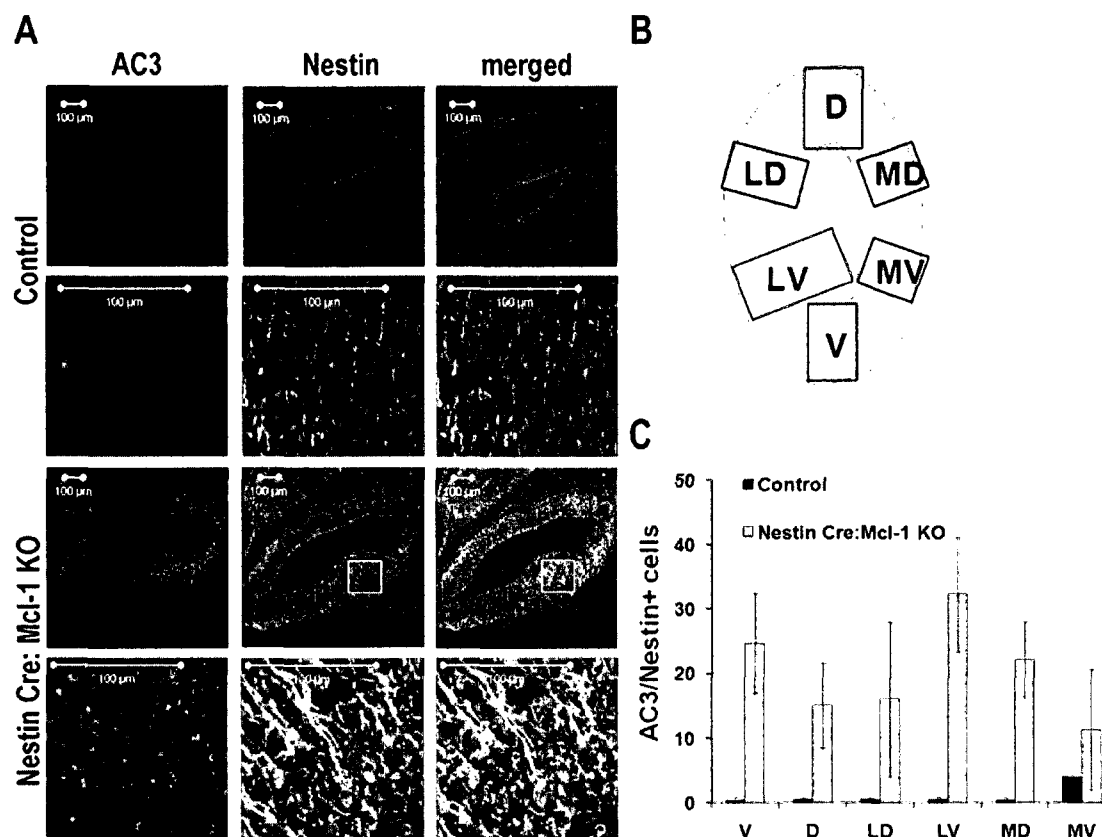


Figure 5. Mcl-1 deficiency results in increased cell death of Nestin-expressing progenitor cells. **A**, Confocal photomicrographs of coronal sections from E12.5 Nestin:Cre Mcl-1 mutant and control brains, immunostained for active caspase 3 and Nestin, a marker for neuronal progenitor cells. **B**, Schematic of the six counting areas around the ventricle: ventral, dorsal, lateral dorsal, lateral ventral, medial dorsal, and medial ventral. **C**, Quantitative analysis of AC3 and Nestin+ cells in the six different regions. Within the mutant brain, the LV region had significantly greater numbers of Nestin+ apoptotic cells.

cells in each aggregate were viable, cultures were stained with the Hoechst nuclear stain to identify healthy versus apoptotic cells. Quantification revealed that $52 \pm 3\%$ of cells in Mcl-1 deficient cell aggregates were apoptotic versus $15 \pm 1\%$ in aggregates from littermate controls. This massive cell death is comparable with the apoptosis observed *in vivo* and demonstrates that cell death from Mcl-1 deficiency is cell autonomous.

To determine whether Mcl-1 deficient neural precursors *in vitro* were capable of committing to a neuronal phenotype before apoptosis, cultures were immunostained with antibodies to Tuj1. The percentage of Tuj1-positive cells that are also apoptotic was 2.5-fold higher in Mcl-1 deficient cultures (Fig. 8B). As apoptosis progresses, cells rapidly lose their phenotypic markers; therefore, our results likely under-represent the true number of apoptotic neurons. Our *in vitro* findings along with our *in vivo* observations indicate that Mcl-1 is necessary for the survival of newly committed neurons as well as maintaining the survival of neural progenitor cells. These studies identify a novel cell autonomous requirement for the anti-apoptotic Bcl-2 family protein, Mcl-1, in neurogenesis.

Mcl-1 regulates the onset of cell death after neuronal injury

Because Mcl-1 plays an essential role in the regulation of survival of neural precursor cells and newly committed neurons during telencephalic development, we were unable to evaluate the requirement for Mcl-1 in mature neurons in these mice. To assess whether Mcl-1 is required to maintain survival of postmitotic neurons, cortical neurons were cultured from E14.5 Foxg1:Cre Mcl-1 KO mice and cerebellar granule neurons homozygous for

the floxed Mcl-1 allele and then infected with an adenoviral vector expressing Cre-recombinase to induce recombination *in vitro* (Fig. 9G). Unlike neural progenitor cells, our results reveal that neither isolated cortical neurons, nor cells lacking Mcl-1 through Cre-mediated recombination of Mcl-1 *in vitro* undergo apoptosis but remained viable for at least 3–4 d (Fig. 9A,B). Because Mcl-1 deletion did not induce apoptosis in postmitotic neurons within the timeframe of the experiment, we asked whether Mcl-1 deficiency modulated neuronal sensitivity to acute injury. Although spontaneous apoptosis was not observed in the absence of Mcl-1 in mature postmitotic neurons, Mcl-1 deficient neurons were more sensitive to exposure to a DNA damaging agent, camptothecin ($10 \mu\text{M}$), than control cells ($8.9 \pm 1.7\%$ Mcl-1 deficient vs $25.25 \pm 3.0\%$ control) (Fig. 9C,D). Although Mcl-1 deficiency alone did not induce an apoptotic response in more mature neurons, our results suggest that Mcl-1 may play an important role in the regulation of survival in the context of acute neuronal injury.

Because our results reveal that the absence of Mcl-1 accelerates the rate of cell death in response to neuronal injury, we asked whether Mcl-1 or any of the multidomain Bcl-2 family members were transcriptionally regulated after DNA damage induced neuronal cell death. Primary cortical neurons were treated with $10 \mu\text{M}$ camptothecin after 2 DIV. In contrast to the other Bcl-2 family members where there is no change in mRNA, Mcl-1 was rapidly downregulated after DNA damage (Fig. 9E). This change in mRNA levels is followed by a steady decline in Mcl-1 protein levels (Fig. 9F). The rapid downregulation of Mcl-1 before the onset of neuronal cell death suggests that downregulation of

Mcl-1 may have a pivotal role regulating the onset of neuronal cell death after injury.

Mcl-1 plays an important role in injury-induced neuronal cell death

If Mcl-1 downregulation is a key event in controlling the onset of apoptosis after acute neuronal injury, then maintaining Mcl-1 levels should protect neurons from cell death. To determine this, we constructed an Mcl-1 adenoviral vector (AdMcl-1). Efficient protein expression of the Mcl-1 vector in neurons was confirmed by Western blot analysis (Fig. 10A). To assess the protective role of Mcl-1 in neurons, CGNs were infected with AdMcl-1 or a LacZ control adenovirus (AdLacZ) (MOI 75) at the time of plating. After 2 DIV, neurons were treated with 10 μ M camptothecin, and cell survival was assessed at 12 h intervals. Cells maintaining high expression of Mcl-1 showed a nearly twofold greater protection against DNA damage-induced cell death relative to cultures expressing LacZ (AdLacZ 24.7 $\pm$ 2.3% cell death and AdMcl-1 at 14.8 $\pm$ 2.0% cell death); in addition, Mcl-1-mediated resistance to injury is maintained at 48 h (AdLacZ, 53.3 $\pm$ 2.3%; AdMcl-1, 25.5 $\pm$ 1.9%) after injury (Fig. 10B). These results show that Mcl-1 downregulation may be involved in controlling the onset of apoptosis after injury, because maintaining Mcl-1 levels in injured neurons can extend neuronal viability. Furthermore, these findings demonstrate a role for Mcl-1 in maintaining neuronal survival against acute injury, and that loss of Mcl-1 increases neuronal sensitivity to DNA damage-induced cell death.

In these studies, we have shown that Mcl-1 is required to maintain neural precursor survival during forebrain development and after acute neuronal injury. In neuronal progenitors and immature neurons, loss of Mcl-1 results in apoptosis as progenitor cells commence migration away from the ventricular zone. Additionally, we show that Mcl-1 is downregulated in response to DNA damage-induced injury, and maintaining levels of Mcl-1 can reduce the rate of apoptosis, whereas loss of Mcl-1 increases sensitivity to stress. In conclusion, the results of our studies provide the first demonstration of a requirement for the anti-apoptotic Bcl-2 family protein, Mcl-1, for neural development and implicate Mcl-1 as a prime therapeutic target for the protection of neural precursors and neurons from apoptosis.

Discussion

The involvement of Mcl-1 in apoptotic cell death has been well documented (for review, see Craig, 2002; Michels et al., 2005);

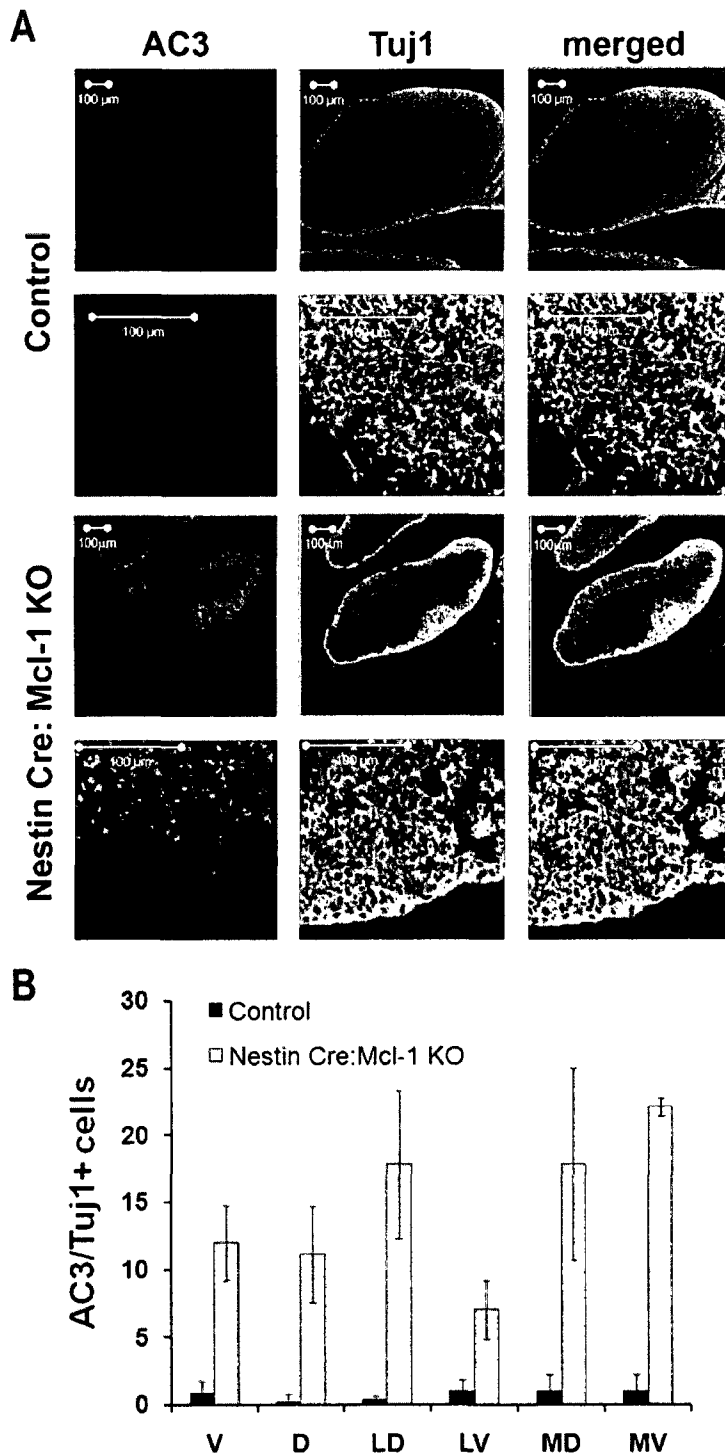


Figure 6. Mcl-1 deficiency results in cell death in newly committed neurons. *A*, Confocal photomicrographs of coronal sections from E12.5 Nestin:Cre Mcl-1 mutant and control brains immunostained for active caspase 3 and Tuj1, an early neuronal marker. *B*, Quantitative analysis of AC3/Tuj1-positive cells in each of the regions around the cortex. Nestin:Cre mutant brains have significantly more apoptotic immature neurons relative to control littermates.

however, little is known regarding the role of Mcl-1 in the regulation of neuronal apoptosis during development or after acute injury. In this study, the striking cortical phenotype of Mcl-1 conditional mutant mice clearly demonstrates the importance of Mcl-1 in neural development. Specifically, Mcl-1 deficiency results in the apoptotic death of Nestin+ neural progenitors and

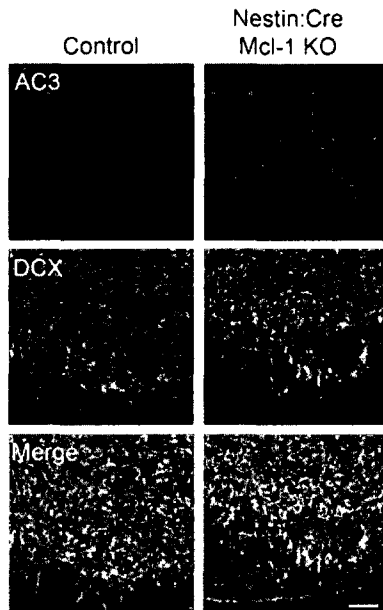


Figure 7. Loss of Mcl-1 results in death of migratory neuroblasts. Confocal photomicrographs of coronal sections from E12.5 Nestin:Cre Mcl-1 mutant and control brains immunostained for active caspase 3 and doublecortin, a migratory neuroblast marker. Quantitative analysis revealed significantly more AC3/DCX-positive cells in Mcl-1 mutant brains relative to control littermates. Scale bar, 40 μ m.

Tuj1+ newly committed neurons, indicating that Mcl-1 is required for the survival of both cell populations. Furthermore, we also demonstrate a role for Mcl-1 in postmitotic neurons during acute DNA-induced injury. Our results show that Mcl-1 down-regulation is an early event in acute neuronal injury and that maintaining Mcl-1 levels can protect neurons against DNA damage-induced cell death. These studies demonstrate a novel mechanism by which neural precursors are regulated in the developing brain and highlight the importance of Mcl-1 in the regulation of neuronal survival.

Neural progenitor cell numbers are tightly regulated by a balance between proliferation and cell death. During embryogenesis, developmental progenitor cell death has been reported to be as high as 50–70% (Blaschke et al., 1996). In the healthy adult brain, with each progenitor cell division, one daughter cell dies by apoptosis (Morshead et al., 1994). Gene knock-out studies have demonstrated the essential role apoptosis plays in regulating the precursor population, where mice deficient for key proapoptotic genes caspase 3, caspase 9, or Apaf-1 exhibit dramatically reduced apoptosis leading to the production of excessive neural precursor cells and brain malformation (Kuida et al., 1996, 1998; Cecconi et al., 1998). Although the apoptotic trigger has yet to be defined, proapoptotic members of the Bcl-2 family have shown some involvement. Single gene deletion of either proapoptotic proteins Bax or Bak does not produce a nervous system phenotype; deletion of both Bax and Bak, however, significantly reduces developmental apoptosis resulting in hypercellularity of specific CNS populations (Lindsten et al., 2000, 2003). The excess cells are located in the germinal zones of the brain (i.e., the SVZ) and have been identified with markers specific for neural precursors (Nestin), astrocytes (GFAP), and neurons (β III tubulin) (Lindsten et al., 2003). These studies show that apoptosis plays an important role in the regulation of neural precursor cells and their differentiated progeny.

Presently, little is known regarding which anti-apoptotic Bcl-2

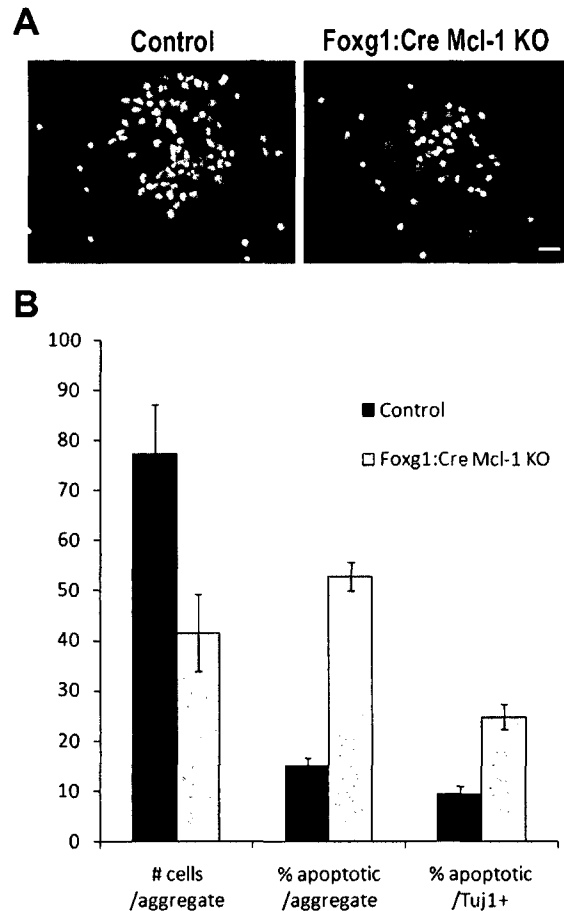


Figure 8. Mcl-1 deficient cortical progenitor cell death is cell autonomous. Cortical progenitor cell aggregates were plated at similar plating densities and allowed to differentiate in culture while survival was monitored. Cell survival was assessed with Hoechst nuclear staining to distinguish both viable (diffuse Hoechst nuclear staining) and apoptotic (condensed nuclei) cells. **A**, Representative photomicrographs of Hoechst-stained control and Mcl-1 deficient E12.5 cortical precursor cultures after 3 DIV. **B**, Counts were performed to quantify the total number of cells (viable and apoptotic) per aggregate (represented as mean number of cells/aggregate) and the number of apoptotic cells per aggregate (represented as percentage of apoptotic). Tuj1 immunostaining was performed to determine whether cortical precursors were capable of committing to a neuronal phenotype before dying. The number of apoptotic cells that were also Tuj1 positive (Tuj1+) were counted and compared with the number of Tuj1+ cells per aggregate (represented as percentage of apoptotic/Tuj1+). Results are summarized from six different platings of each genotype derived from two independent animals. Scale bar, 25 μ m.

family protein is important in maintaining neural precursor cell survival. Previous studies using germline deletions of various Bcl-2 family members have not revealed a crucial role for any single Bcl family member in the development and survival of the CNS. Bcl-2-targeted deficiency studies suggest that Bcl-2 is not essential for development; however, there is loss of peripheral nervous system (PNS) neurons in early postnatal life, including motor, sensory, and sympathetic neuron populations (Michaelidis et al., 1996). This suggests a requirement for Bcl-2 in the maintenance and survival of these postmitotic populations. Bcl-xl germline knock-out mice exhibit extensive neuronal cell death at E13.5 (Motoyama et al., 1995). Unlike the dramatic phenotype of Bcl-xl whole embryo knock-outs, a neuron-specific Bcl-xl conditional mutant mouse revealed a less severe phenotype in which the population of catecholaminergic neurons was reduced by one-third, suggesting that the requirement for Bcl-xl may be cell type specific (Savitt et al., 2005). The results of our

studies demonstrate Mcl-1 to be independently essential for neuronal development. In the present study, we identified Mcl-1 as a key regulator of neural precursor cell survival, because conditional mutations of Mcl-1 result in massive apoptosis of neural precursor cells. Mcl-1 is the first anti-apoptotic Bcl-family protein with a critical role in the maintenance of the neural precursor cell population.

In this study, we identify a change in Mcl1 requirement as neural progenitor cells differentiate into postmitotic neurons, and we show that this transitional period requires the anti-apoptotic Bcl-2 family member Mcl-1. In both conditional Mcl-1 mutant mice, we observed widespread apoptosis of Nestin+ neural progenitors and Tuj1+ newly committed neurons. *In vitro* differentiation of neural progenitors also resulted in extensive apoptosis, indicating a cell autonomous mechanism. In contrast, mature neurons in which Mcl-1 loss was induced by adenovirus delivery of Cre were able to survive in culture for up to 4 d with no evidence of apoptosis. These results indicate that the susceptibility to apoptosis changes during progenitor cell differentiation. Previous studies have shown that the expression profiles of anti-apoptotic and proapoptotic Bcl family proteins change as neural precursors become postmitotic neurons. During nervous system development, proapoptotic Bax and Bid are expressed in neural precursor cells within the ventricular zone, with Bax expression peaking at E12–E15 (Krajewska et al., 2002) corresponding to the early neurogenic interval when we see widespread apoptosis of neural precursors in the Mcl-1 conditional mutants. Although Mcl1 and Bcl-xL have been shown to block Bax- and Bak-mediated apoptosis, Bcl-xL is expressed at very low levels in the neural precursor population (Motoyama et al., 1995; Roth et al., 2000). The extensive neural precursor apoptosis we observe at E12 may be attributed to the relatively high levels of the proapoptotic proteins, such as Bax, and therefore the loss of Mcl-1 has a major impact on their survival. One might predict, therefore, that during neurogenesis, the majority of newly committed (Tuj1+) neurons in Mcl-1 conditional mutants die by apoptosis likely before sufficient Bcl-xL expression, because in the Bcl-xL null mutant, apoptosis is observed in more mature neuronal populations at E12.5 (Motoyama et al., 1995; Roth et al., 1996). The results of this study therefore suggest that Mcl-1 regulates the survival of cells during this transition from progenitor cell to postmitotic neuron.

Although Mcl-1 deficient neurons did not die when Mcl-1 was deleted, these neurons were more sensitive to injury-induced apoptosis. Cells lacking Mcl-1 died at an accelerated rate relative to

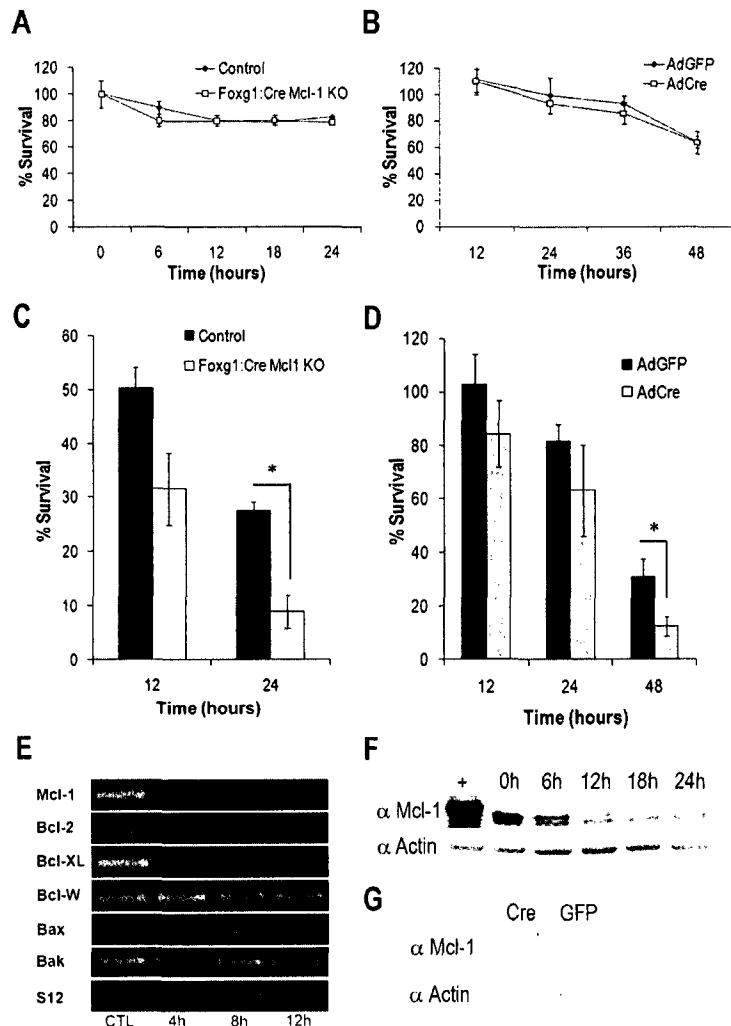


Figure 9. Loss of Mcl-1 in neurons results in increased sensitivity to DNA damage-induced cell death. **A**, Quantitative analysis of cortical neuron survival by LIVE/DEAD assay revealed Mcl-1 mutant cortical neurons survive in culture similarly to cortical neurons cultured from littermate control animals. **B**, CGNs were cultured from Mcl-1 flox/flox mice and infected with an adenovirus expressing Cre recombinase (AdCre) or green fluorescent protein (GFP) (AdGFP) as a control, and cell survival was assessed at 12 h intervals by LIVE/DEAD assay. Deletion of Mcl-1 in CGNs did not induce cell death, because cell survival was similar in cultures infected with AdGFP. **C**, Cortical neurons were isolated from Foxg1:Cre Mcl-1 knock-out or control mice, treated with 10 μM CPT, and collected at the times indicated. Neuronal survival was determined by LIVE/DEAD assay ($n = 3$). Significantly fewer Foxg1:Cre Mcl-1 knock-out neurons were alive at 24 h after camptothecin treatment than neurons from littermate controls ($*p < 0.05$). **D**, CGNs were isolated from Mcl-1 flox/flox mice and infected with AdCre or AdGFP (MOI 75). Neuronal survival was determined at the indicated times by LIVE/DEAD assay ($n = 3$). **E**, Cortical neurons were cultured from CD1 mice and treated with 10 μM camptothecin, and total RNA was collected at the indicated times and analyzed by semiquantitative RT-PCR for Mcl-1, Bcl-2, Bcl-XL, Bcl-W, Bax, Bak, and S12 transcript expression. CTL, Untreated controls. **F**, Cortical neurons were treated with 10 μM camptothecin, and protein lysates were collected at indicated times after treatment. Western blot analysis was performed with antibodies for Mcl-1 and actin (loading control). **G**, Western blot analysis of Mcl-1 protein expression in CGNs infected with AdCre or AdGFP (MOI 75) 48 h after plating, showing efficient excision of Mcl-1 in cultured neurons infected with AdCre.

littermates expressing Mcl-1. After DNA damage-induced injury, cells mount a strong apoptotic response, including a dramatic upregulation of BH3-only proteins, including Puma and Noxa (Cregan et al., 2004). In the context of this changing environment, the absence of Mcl-1 results in a rapid onset of cell death. Indeed, after DNA damage-induced injury, Noxa and Puma are induced, whereas Mcl-1 is rapidly downregulated. Previous studies have shown that Mcl-1 is at the apex of Bcl-2-mediated apoptotic signaling and that the downregulation of Mcl-1 is required for Bax activation (Nijhawan et al., 2003). Consistent with this model, our results show that maintaining Mcl-1 expression can

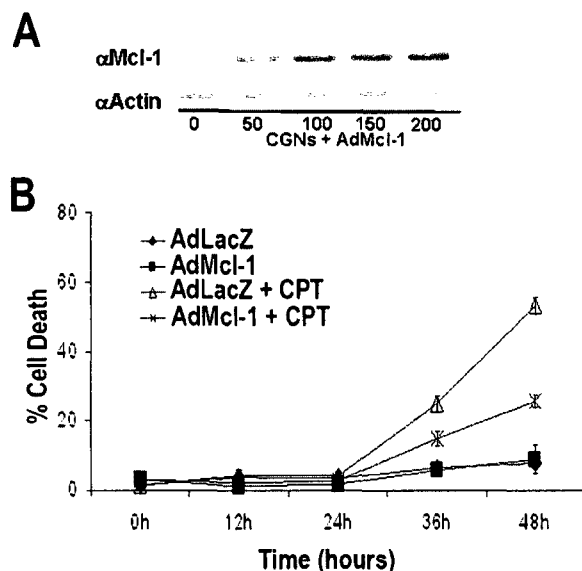


Figure 10. Sustained expression of Mcl-1 protects neurons against DNA damage-induced cell death. **A**, Western blot showing efficient transduction of AdMcl-1. CGNs were infected with the indicated MOI of AdMcl-1. Protein lysates were collected 48 h postinfection and immunoblotted for Mcl-1 expression and then for actin as a loading control. **B**, CGNs were infected with AdMcl-1 or AdLacZ control at 75 MOI at the time of plating and 48 h postinfection were treated with 10 μ M CPT. LIVE/DEAD viability/cytotoxicity assay was performed at the indicated times ($n = 3$).

preserve neuronal survival for extended time periods. These studies demonstrate the importance of Mcl-1 in regulating the onset of neuronal apoptosis after acute injury such as DNA damage.

Recent work by Mori et al. (2004) demonstrating increased neuronal sensitivity to seizures in Mcl-1 heterozygous mice has prompted questions regarding the role of Mcl-1 in the regulation of injury-induced neuronal apoptosis. This observed sensitivity suggests that Mcl-1 plays an important role in neuronal survival after insult; however, the mechanism by which this occurs remains unknown. In contrast to the other anti-apoptotic Bcl-2 family members, Mcl-1 is very tightly regulated both transcriptionally and posttranslationally (for review, see Craig, 2002). The rapid reduction in Mcl-1 transcript levels after DNA damage can be attributed to several mechanisms. Previous studies have revealed that camptothecin treatment leads to inactivation of Rb through activation of cyclin-dependent kinases (Park et al., 2000). Rb inactivation results in enhanced activity of E2F transcription factors, which have been shown previously to repress Mcl-1 transcription (Croxtan et al., 2002). In addition to regulation by E2F, Mcl-1 is also a regulatory target for HIF1 α , (Piret et al., 2005), cAMP response element-binding protein, and sis-inducible element (Wang et al., 1999), which may play important roles in Mcl-1 regulation in acute neuronal injury such as hypoxia. The precise mechanism by which Mcl-1 transcript levels are rapidly downregulated after DNA damage is not known; however, our results suggest that Mcl-1 levels may be sensitive indicators of neuronal injury and impending cell death. This is further supported by the demonstration that sustaining Mcl-1 expression can provide significant protection against neuronal cell death and highlight the importance of Mcl-1 in neuronal cell death regulation.

The results of our studies show a novel requirement for Mcl-1 in the development and maintenance of neurons within the CNS.

Our *in vivo* results demonstrate that Mcl-1 is required for cortical neurogenesis and the survival of newly committed neurons. Additionally, our data show that Mcl-1 regulates apoptotic cell death after a DNA damaging insult. Most importantly, our results implicate Mcl-1 as a key regulatory target with which to expand the neural precursor pool and to maintain neuronal survival in the damaged brain.

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literature report

The phosphorylation state of Drp1 determines cell fate

Recent research has opened new avenues for the evaluation of mitochondrial function; for example, mitochondria are no longer perceived as thread-like static entities within the cytosol, but instead are viewed as highly dynamic organelles that can change in shape and size, and are transported to strategic locations within the cell. Mitochondrial morphology, size and position within cells are maintained through a balance of fission and fusion events. Perturbation of the steady state between these opposing processes has been directly implicated in several human disorders (Chan, 2006). Although the list of genes for mitochondrial morphogenesis is rapidly increasing, dynamin-related protein 1 (Drp1)—a cytosolic dynamin GTPase—was among the first fission proteins to be discovered; however, the mechanism by which Drp1 function is regulated is poorly understood.

In this issue of *EMBO reports*, Cribbs & Strack identify a new mechanism by which second messengers—cAMP and calcium—modulate mitochondrial shape and function through the regulation of Drp1 phosphorylation. Cyclic-AMP-dependent protein kinase (PKA)-mediated phosphorylation of Drp1 at Ser656 induces mitochondrial elongation and resistance to apoptotic stimuli, whereas dephosphorylation of Ser 656 by calcineurin promotes mitochondrial fragmentation and increases cell vulnerability to apoptosis. These studies provide a new mechanistic insight into the link between the mitochondrial fission machinery and cell death signalling.

Drp1 is recruited to the mitochondrial surface at potential fission sites (Ingberman *et al*, 2005; Okamoto & Shaw, 2005). The energy generated by GTP hydrolysis is believed to provide the mechanical force required to execute fission (Ingberman *et al*, 2005). Although gain- and loss-of-function studies of Drp1 correlate mitochondrial fission with apoptosis (Frank *et al*, 2001; Germain *et al*, 2005), there is no evidence to show that Drp1 alone, or mitochondrial fission by itself, can induce apoptosis. In addition, although Drp1 GTPase can be regulated by ubiquitination and sumoylation (Nakamura *et al*, 2006; Wasiak *et al*, 2007), there is little insight as to how Drp1 activation might be regulated during apoptosis signalling. In this issue, Cribbs & Strack have addressed some of these important questions and provide a mechanistic link for second messenger regulation of Drp1 GTPase activity and apoptosis signalling.

First, Cribbs & Strack show that PKA-mediated-phosphorylation of Drp1 at Ser656 attenuates the GTPase activity of Drp1 and promotes cell survival, suggesting that cAMP might mediate survival partly through the inhibition of Drp1 (Fig 1). An independent study (Chang & Blackstone, 2007) corroborates these findings by showing that PKA-dependent phosphorylation of Drp1 within the GED domain at Ser637 blocks Drp1 GTPase activity. Phosphomimetic substitution at Ser637Asp was also shown to block mitochondrial fission (Chang & Blackstone, 2007). The question of how phosphorylation at Ser656 and Ser637 might differ in regulating the GTPase activity remains to be resolved. One possibility is that a spatiotemporal relationship exists whereby phosphorylation at one site regulates modification of the second site. In addition, a third Drp1 phosphorylation site has been reported and is believed to be involved in breaking down the mitochondrial network during mitosis (Taguchi *et al*, 2007). It is likely that Drp1 phosphorylation at different sites might have different physiological consequences. This hypothesis is supported further by the recent finding that phosphorylation modulates substrate processing

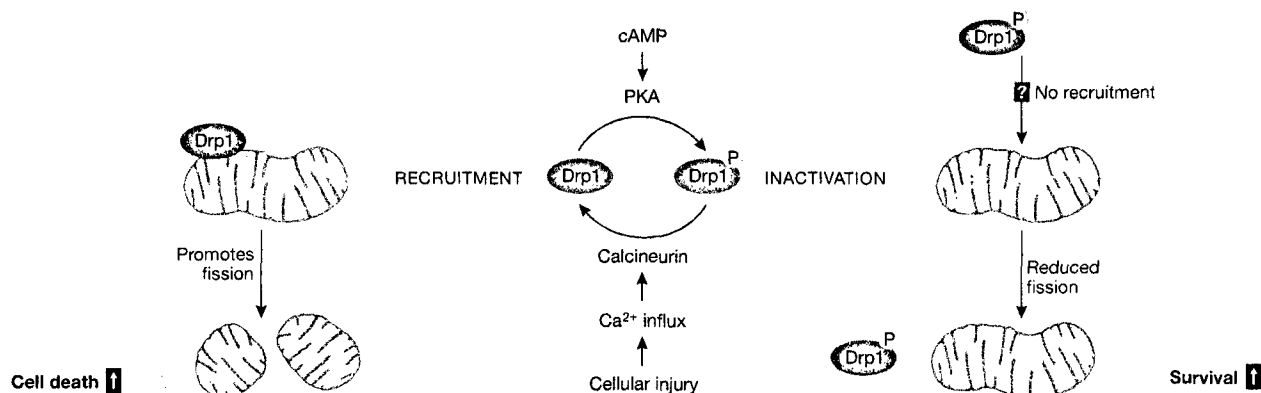


Fig 1 | Missing links between mitochondrial fission and apoptosis. Cyclic-AMP-dependent protein kinase (PKA) phosphorylates dynamin-related protein 1 (Drp1) and induces mitochondrial elongation and resistance to apoptosis. Calcineurin dephosphorylates Drp1, promotes mitochondrial fragmentation and cell vulnerability to apoptosis. Whether phospho-Drp1 confers protection through inhibiting mitochondrial fission or through other regulatory signalling molecules remains to be discovered.

in a site-specific manner (Jahani-Asl *et al.*, 2007a). The complexity imposed by phosphorylation on the three-dimensional structure and oligomerization of Drp1 protein requires further investigation.

A link between mitochondrial fission and death signalling pathways converging on mitochondria has been a hotly debated topic for several reasons. Mitochondrial fission is required under normal physiological conditions to ensure biogenesis and to respond to changes in energy demands (Yaffe, 1999). Although mitochondrial fission has been shown to occur as an early event during cell death (Barsoum *et al.*, 2006; Jagasia *et al.*, 2005; Jahani-Asl *et al.*, 2007b), and Bax/Bak-mediated Drp1-induced mitochondrial fission promotes cell death (Arnoult *et al.*, 2005), it has also been shown that inhibiting the fission machinery does not prevent Bax/Bak-dependent apoptosis (Parone *et al.*, 2006). In addition, Drp1-mediated fission of mitochondria has been reported to protect against cell death (Szabadkai *et al.*, 2004). More importantly, although *in vitro* studies often focus on death pathways evoked by single inducers, the scenario *in vivo* is quite complex (Cheung *et al.*, 2007). Previous findings show that recruitment of Drp1 at the scission sites occurs simultaneously with Ca^{2+} uptake by mitochondria (Breckenridge *et al.*, 2003). In this issue, Cribbs & Strack show that calcium induces mitochondrial fission through Drp1 dephosphorylation (Fig 1). This finding has broad implications pertaining to several modes of cell death including death induced by staurosporine (a kinase inhibitor), etoposide (topoisomerase inhibitor), calcium ionophore A23187 and oxidative stress. The question of how Drp1 phosphorylation might protect against cell death remains open: one possibility is that phosphorylation might modulate Drp1 interaction with other regulatory proteins that assist in targeting Drp1 to mitochondria. Finally, Cribbs & Strack show that Drp1 phosphorylation protects against apoptotic insult despite the fact that a population of mitochondria exhibit ultrastructure abnormalities. This apparent inconsistency suggests that Drp1 might have other roles in addition to regulating mitochondrial fission.

In summary, these new studies suggest that components of the mitochondrial fission–fusion machinery are linked to cellular signalling pathways and identify a new mechanism by which second messengers might regulate mitochondrial structure and function. Future research towards identifying upstream and downstream regulators of the fission–fusion machineries might identify new approaches to modulate the onset of cell death that becomes deregulated in many human diseases.

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