

Mitofusin 1 and Mitofusin 2 function in the Context of Brain Development

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Abstract

Mitofusin 1 and 2 are outer-mitochondrial membrane proteins that have been shown to be involved in fusion. Mitofusin 2 has also been associated with apoptosis and development. When Mfn1 and Mfn2 were each conditionally knocked out from the cerebellum, Purkinje cells in Mfn2 deficient cerebellum during development had undergone neurodegeneration. Mutations in Mfn2 have also been associated with the Charcot Marie Tooth Type 2A (CMT2A). We want to assess the effect Mfn2 and Mfn1 might have on the development of other regions of the brain such as the telencephalon. We generated Mfn1 and Mfn2 conditional knockouts in the telencephalon by crossing them with Foxg1 Cre - a cre expressed in the telencephalon. We found that Mfn1 deficient mice have lost their corpus callosum at the midline, but survive over 6 months with a decrease in progenitor cells postnatally. Mfn2 deficient mice die between P9 and P12 with a decrease in progenitor cells postnatally and a decrease in number of neurons in the cortex. Therefore, our results suggest that Mfn1 and Mfn2 play a significant role in the development of the telencephalon.

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List of abbreviations

Adenosine triphosphate ATP

Apoptosis inducing factor AIF

Cerebellar Granular Neuron CGN

Charcot Marie Tooth Type 2A CMT2A

Dynamin related protein 1 Drp1

Empty spiracles homologs Emx1

Endoplasmic reticulum ER

Fibroblast growth factor fgf8

Flavin adenine dinucleotide FAD

Fission 1 protein Fis1

Gamma-aminobutyric acid(A) receptor-interacting factor GRIF

heparan sulfotransferase Hs2st and Hs6st1

Opa1 optic atrophy 1

Mouse embryonic fibroblasts MEF

Mitochondria DNA mtDNA

Mfn Mitofusin

MOM Mitochondrial outer membrane protein

Nicotinamide adenine dinucleotide NAD

Nuclear factor I family Nfia

Poly (ADP-ribose) polymerase-1 PARP1

Ras associated protein RAB7

Reactive Oxygen Species ROS

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Chapter I: Introduction

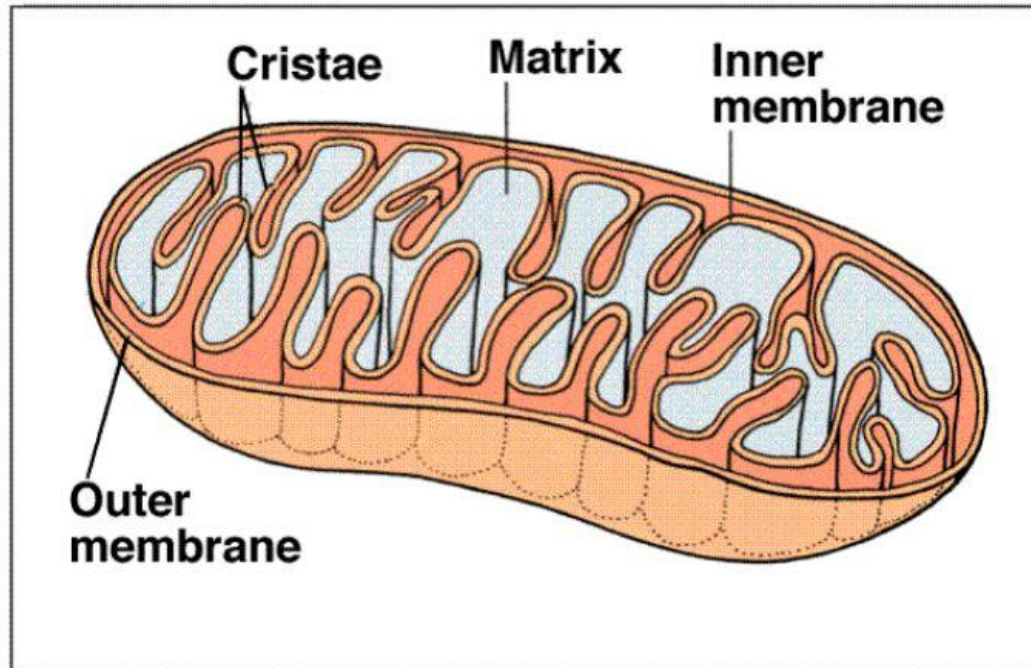
1. Mitochondria

Mitochondria are dynamic organelles that divide and fuse (Bereiter-Hahn and Voth, 1994). They are semiautonomous organelles that possess their own genome and DNA. Mitochondrial structure is tightly regulated and its compartments include the cristae, outer membrane, inner boundary membrane, intermembrane space, and matrix (Frey and Mannella, 2000) (Fig1.1). In my thesis I will be studying the role of the two outer mitochondrial membrane proteins Mfn1 and Mfn2 in the telencephalon during development.

1.1 Mitochondrial Function: classical- energy metabolism

Mitochondria are involved in energy production and metabolism; they provide the cell with the necessary amount of ATP through oxidative phosphorylation (Ernster and Schatz, 1981; Westermann, 2010). When glucose enters the cell, it is oxidized to pyruvate in the cytosol by a process known as glycolysis. Pyruvate is then imported into the mitochondria where it is decarboxylated by pyruvate dehydrogenase into acetyl CoA and CO₂ and a molecule of NADH is released. Acetyl CoA enters the citric acid cycle in the mitochondrial matrix, a series of reactions occur that oxidize the acetyl group (Alberts, 2002; Harold, 2001). High energy electrons are released from those reactions and taken up by NAD⁺ and FAD²⁺ along with H⁺ ions that convert to NADH and FADH₂ (Saraste, 1999; Schultz and Chan, 2001). An NADH molecule acts as an electron donor to the electron transport chain to facilitate the transport of H⁺ from the matrix to the intermembrane space. Oxidation of NADH to NAD⁺ generates an electrochemical proton gradient (Saraste, 1999; Schultz and Chan, 2001).

Mitochondrion



From Widmaier, E.P., H. Raff, and K.T. Strang. 2006. *Vander's human physiology : the mechanisms of body function*. McGraw-Hill, Boston. xxxi, 827 p. pp.

Figure 1.1 The internal structure of mitochondria. A mitochondrion is composed of the outer membrane, the intermembrane space, the inner membrane that folds to form the cristae, and the matrix.

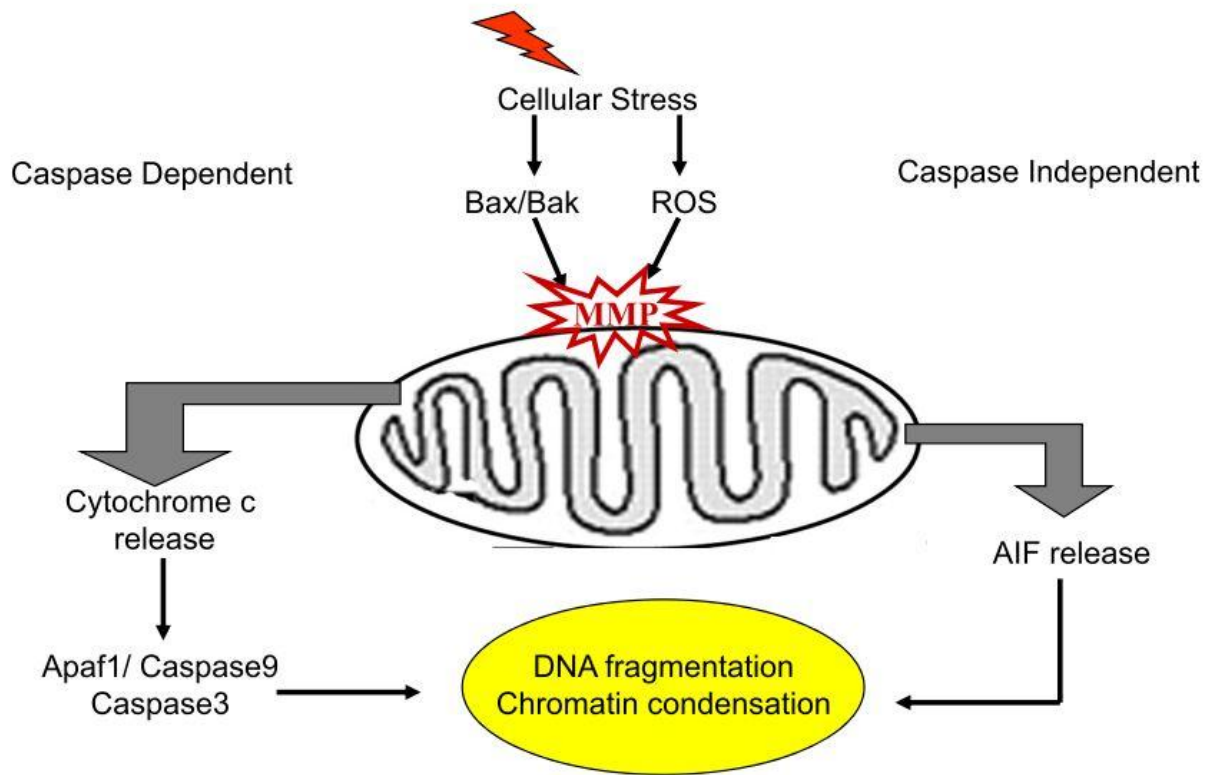


Figure 1.2 Caspase dependent and Caspase independent Apoptosis

Caspase dependent cell death. Upon cellular stress Bax/ Bak translocate to the outer mitochondrial membrane and permeabilize it, promoting the release of cytochrome c, activation of Apaf1 and caspases and consequently inducing cell death.

Caspase-independent cell death. AIF is released from the mitochondria to the nucleus to induce cell death.

The electrons are taken up by the electron transport chain in a stepwise manner in order to preserve the energy and not lose it as heat (Lowell and Spiegelman, 2000). When an electrochemical proton gradient is formed, H^+ flows down the gradient through the ATP synthase pump, and the mechanical energy of the flow of H^+ through the pump is transferred to chemical energy catalyzing the synthesis of ATP from ADP and inorganic phosphate (Pi)(Boyer, 1997). The electrons that were transferred through the electron transport chain reach oxygen that is reduced to generate water (Alberts, 2002). In total, this process generates 38 ATP per glucose molecule, thus providing the cell with the necessary energy for viability and biochemical processes.

1.2 Mitochondrial Function: more recent role in apoptosis signaling

In addition to their role in metabolism and energy production, mitochondria are involved in regulating apoptosis and cell death. Apoptosis is a programmed cell death that is either triggered by intrinsic (inside) or extrinsic (outside) stimulus. The extrinsic cell death pathway is activated when specific ligands bind the death receptors outside the cell and initiate the apoptotic cascade to eliminate unwanted cells during development or immuno-surveillance (Boatright and Salvesen, 2003). Intrinsic pathways are triggered by certain stress stimuli such as mitochondrial damage or UV radiation (Boatright and Salvesen, 2003). The intrinsic and sometimes the extrinsic pathways converge at the mitochondria to activate the caspases and complete apoptosis. Apoptotic cell death includes the caspase-dependent and caspase-independent modes of cell death (Aarts and Tymianski, 2004; Cregan et al., 2004; Yuan et al., 2003).

1.3 Caspase-dependent apoptosis

Caspase-dependent apoptosis involves the activation of pro apoptotic Bcl 2 family of proteins such as Bax and Bak and the inactivation of anti apoptotic Bcl2 family members by the BH3 only proteins (Brunelle and Letai, 2009). In healthy cells, Bax is located in the cytosol or loosely attached to the mitochondrial outer membrane (MOM) (Sharpe et al., 2004). When apoptosis is induced, Bax and Bak translocate to the outer membrane of the mitochondria and oligomerize (Saikumar et al., 1998; Wolter et al., 1997). Upon stimulation of cell death, BH3 only proteins function to activate Bak and Bax. For example, when caspase 8 is activated, it cleaves the amino terminal of Bid- a BH3 only Bcl2 protein, and produces truncated tBid that interacts directly with Bax and Bak to permeabilize the MOM and trigger the release of cytochrome c from the intermembrane space (Li et al., 1998; Luo et al., 1998). Cytochrome c activates the apoptosis-protease activating factor 1 (Apaf-1) that initiates the formation of an apoptosome (Boatright and Salvesen, 2003) to recruit procaspase 9 and activate the caspase cascade (Danial and Korsmeyer, 2004; Green and Kroemer, 2004). The caspases turn on other apoptotic proteins such as nucleases to mediate chromatin condensation, DNA fragmentation and finally cell death (Riedl and Salvesen, 2007) (Fig. 1.2).

1.4 Caspase- Independent Apoptosis

The caspase-independent cell death pathway is suggested to be a secondary response to ensure the completion of apoptosis (Cregan et al., 2002). As previously mentioned, when apoptosis is activated, the MOM swells and ruptures (Cregan et al., 2004). This prompts the release of the apoptotic inducing factor (AIF) from the intermembrane space (Cregan et al., 2004). AIF is a mitochondrial protein located at the inner mitochondrial membrane and during apoptosis is cleaved by the cystein proteases cathepsins and calpains to

become a soluble protein (Otera et al., 2005; Uren et al., 2005; Yuste et al., 2005). AIF release is mediated by poly (ADP-ribose) polymerase-1 (PARP1) where PARP1 deficient cells failed to induce the translocation of AIF from the mitochondria to the nucleus (Yu et al., 2002). PARP1, a nuclear enzyme is usually activated when there is excessive DNA damage and plays a role in DNA repair and regulation of cell death (de Murcia and Menissier de Murcia, 1994; Lautier et al., 1993; Shall and de Murcia, 2000). AIF along with several mitochondrial proteins such as Endo G, translocate to the nucleus when apoptosis is triggered to induce chromatin condensation and cell death (Li et al., 2001; Susin et al., 1999) (Fig 1.2).

1.5 Mitochondrial Structure: how function influences structure

It is well established so far that mitochondria play key roles in metabolism and apoptosis. Recent findings suggest that the mitochondrial architecture has a major effect on these functions. Mitochondria constantly change their morphology in response to stimulus and extracellular and intracellular factors. Studies suggest that mitochondrial structure has a significant impact on function. For example, during apoptosis, the pro-apoptotic BH3 only protein tBid induces the remodeling of the mitochondrial cristae and widens the cristae junctions to allow the mobilization of cytochrome c (Scorrano et al., 2002). BIK another BH3 only protein that activates apoptosis from the ER and causes increase in calcium influx from the ER to the mitochondria induces cristae remodeling and mitochondrial fragmentation (Germain et al., 2005). Changes in mitochondrial structure can protect against cell death. Optic atrophy 1 (Opa1), the mitochondrial inner membrane fusion protein which I will be discussing in detail below on has been shown to play a protective role in controlling the cristae structure by maintaining tight cristae junctions

through oligomerization of the soluble intermembrane space Opa1 oligomer with an integral inner membrane Opa1 (Frezza et al., 2006). Recent advances in electron tomography have now identified that the cristae structure with its junctions also effects the energy production efficiency of the mitochondria (Frey et al., 2002; Mannella, 2006; Mannella et al., 1994). For example, under high respiratory rate conditions the cristae become highly condensed and we observe large intracristal spaces allowing the presence of more substrates, oxygen and ADP (Frey et al., 2002; Mannella, 2006). Furthermore, studies in our lab have shown that the over expression of mitochondrial fusion protein Mitofusin 2 (Mfn2) causes elongation of the mitochondria and promotes survival in cerebellar granular neurons (CGN) (Jahani-Asl et al., 2007).

2. Mitochondrial Dynamics

The shape of mitochondria in living cells is diverse and fluctuates from small rounded up spheres to elongated interconnected tubules, and so mitochondria undergo constant fission and fusion.

2.1 Mitochondrial Fission

Fission is the separation of a long tubular mitochondrion (2-25 μm in length) into two or more segments (0.5 μm). It is an important process that occurs in a cell at steady state, and has been shown to be necessary during cell division and development (Jagasia et al., 2005; Youle and Karbowski, 2005). For example, fission facilitates the rapid translocation of the mitochondria to their target sites in axons and dendrites (Chang and Reynolds, 2006). Fission is essential for mitochondrial renewal, redistribution, and proliferation into synapses (Chan, 2006; Hoppins et al., 2007). During development, immature neurons have small motile mitochondria which aids in the rapid translocation to

high energy demanding regions (Chang and Reynolds, 2006). The molecules that have been found to comprise the fission machinery in mammals include Fis1 and the dynamin related protein 1(Drp1) (Karbowski et al., 2004). Fis1 is a mitochondrial protein inserted on the outer mitochondrial membrane, and Drp1 is a cytosolic dynamin related GTPase that is recruited to sites of fission i.e. along punctate foci (Ingerman et al., 2005; Okamoto and Shaw, 2005). Over expression of Fis1 causes mitochondrial fragmentation and decreased expression results in mitochondrial elongation (James et al., 2003; Stojanovski et al., 2004). Similarly, inhibition of Drp1 promotes elongation of mitochondria (Santel and Fuller, 2001) Studies have suggested that Fis 1 interacts with Drp1, and other proteins to activate fission (James et al., 2003; Yoon et al., 2003). The exact fission machinery in mammals still requires extensive research, but studies in yeast have shown that Fis1 forms complexes on the mitochondria by recruiting Mdv1 and Caf4 the adapters of Dnm-the homolog of Drp1 in yeast (Griffin et al., 2005; Tieu and Nunnari, 2000; Zhang and Chan, 2007) . Therefore, Dnm colocalizes and form spirals on the mitochondria and fission occur by constricting the mitochondria into two (Roux et al., 2006) (Fig 1.3).

Fission has also been associated with the regulation of apoptosis. Fragmented mitochondria are observed around times of Bax activation at the mitochondria but before the mitochondrial outer membrane permeabilization and the release of cytochrome c (Barsoum et al., 2006; Martinou and Youle, 2006). Furthermore, it has been shown that Drp1 co- localizes with Bax when cell death is promoted and studies with a dominant-negative form of Drp1, which inhibits fission, also prevented cytochrome c release and

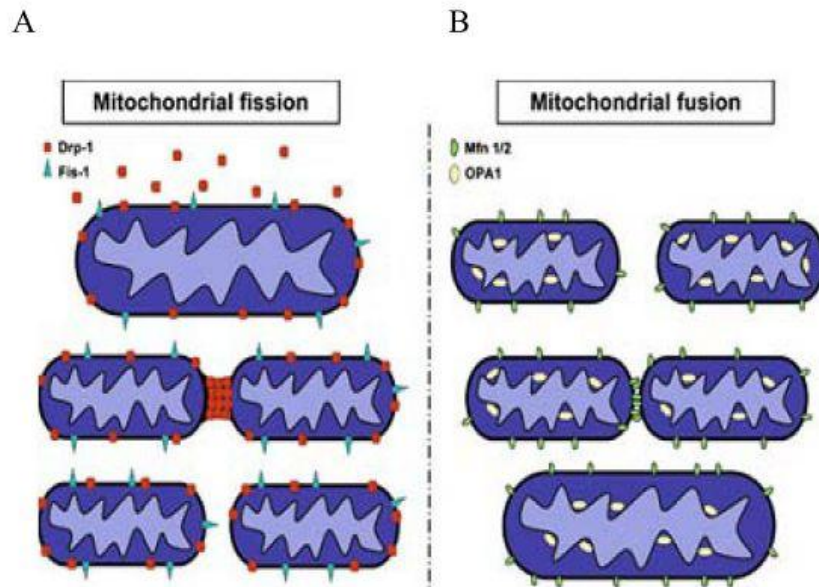
cell death (Frank et al., 2001). On the other hand, other studies have shown that fission occurs independently from cell death.

Down regulation of Fis1 and Drp1 prevented mitochondrial fission and fragmentation, but did not prevent cell death (Delivani et al., 2006). Moreover studies in *C. elegans* have shown that induction of fission does not affect cell survival (Parone et al., 2006). Cell death induced by actinomycin D in HeLa cells produced Bax/Bak dependent apoptosis and cytochrome c release before mitochondrial fragmentation (Arnoult et al., 2005; Esseiva et al., 2004). This suggests that these fission proteins may have functions in cell death and fission independently. Studies in neurons have shown that mitochondrial fission occurred prior to activation of the cell death machinery (caspase dependent and caspase independent), and that mutant Drp1^{k38A} that harbors an inactive GTPase domain which inhibits fission, promoted cell survival and decreased fusion rate after ROS treatment (Barsoum et al., 2006). In summary, two views have been proposed in regards to the role of fission in apoptosis (Cheung et al., 2007): 1) Drp1 is first recruited from the cytosol to the mitochondria, fission is activated and then Bax is translocated to the fission site (Barsoum et al., 2006; Frank et al., 2001). 2) Bax is recruited first to the mitochondria causing alteration in mitochondrial structure, and then Drp1 is activated and fission (Delivani et al., 2006; Esseiva et al., 2004; Parone et al., 2006). Future studies should give more insight and clarify these mechanisms.

2.2 Mitochondrial Fusion

Fusion occurs when two mitochondria converge to form a single mitochondrion. Fusion occurs in several steps: 1) the outer mitochondrial membranes of two mitochondria tether and fuse. 2) The mitochondrial inner membranes dock and fuse. 3) The contents of each

mitochondria are mixed (Ishihara et al., 2004) (Fig3). Proteins involved in fusion include Mfn1, Mfn2, and Opa1. Mfn1 and Mfn2 are large GTPases localized to the outer mitochondrial membrane (Knott et al., 2008; Rojo et al., 2002) whereas Opa 1 (optic atrophy 1) a GTPase, is localized to the intermembrane space (Alexander et al., 2000; Delettre et al., 2001; Delettre et al., 2000; Olichon et al., 2002). I will discuss each of the fusion proteins in detail in the sections that follow. Fusion plays a role in development and survival; it serves as a protective mechanism to injury and mitochondrial dysfunction by allowing rapid mixing of membranes, mitochondrial DNA, and soluble contents that may have been damaged during cellular stress (Chan, 2006; Chen et al., 2005). Fusion acts as a quality control mechanism. This was shown in an experiment using two HeLa cell lines carrying a respiratory defect, with different mutations of a tRNA gene. When these two cell lines were mixed, respiration was restored because of fusion of each cell line's mitochondria (Ono et al., 2001). As a complementation mechanism, the fusion of the two mitochondria occurs, enabling one mitochondrion to complement the other with a functional allele to restore normal respiration (Westermann, 2010). It was also shown that when there was sustained fusion, premature senescence was induced as well as an increase in elongated mitochondria (Lee et al., 2007a; Lee et al., 2007b). The life span of yeast *saccharomyces cerevisiae* and fungus *Podospora anserine* was prolonged after experimentally elongating their mitochondria (Scheckhuber et al., 2007). These, studies have suggested that mitochondrial length may have a functional role in senescence. In the following section I will talk about the mitochondrial fusion proteins Opa1, Mfn1, and Mfn2.



From Autret, A., and S.J. Martin. Bcl-2 family proteins and mitochondrial fission/fusion dynamics. *Cell Mol Life Sci.* 67:1599-606.

Figure 1.3. Mitochondrial fission and fusion dynamics. A) Drp-1 is recruited to mitochondria to promote membrane constriction and mitochondrial fission. B) Mfn1 and Mfn2 are involved in outer mitochondrial membrane fusion, whereas Opa1 is responsible for inner mitochondrial membrane fusion via its interaction with Mfn1.

3.Mitochondrial Fusion Proteins

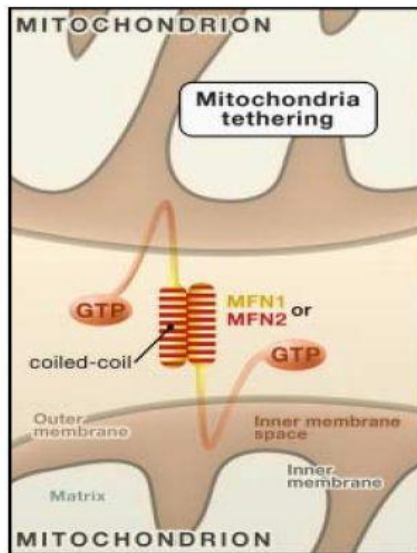
3.1 Opa1

Optic atrophy 1 (Opa1) is a protein located either in the intermembrane space or the inner mitochondrial membrane and interacts directly with Mfn1 to promote fusion (Griparic et al., 2004; Olichon et al., 2002). It belongs to the dynamin family with Mgm1p as its yeast orthologue in *Saccharomyces cerevisiae* and Msp1 as its yeast orthologue in *Schizosaccharomyces pombe*. Opa1 has a mitochondrial import sequence (MIS) at the amino terminus, a transmembrane domain (TM1), followed by GTPase domain, a middle domain, and a GTPase effector domain (GED) which is a coiled-coil domain at the carboxy terminal that is required for oligomerization and dynamin activation (Delettre et al., 2000; Jones and Fangman, 1992; Pelloquin et al., 1998; Praefcke and McMahon, 2004). Two isoforms of Opa1 exist: a short form Opa1 (s-Opa1) and a long form of Opa1 (l-Opa1). There are eight different splice variants of Opa1 and these give rise to l-Opa1 that is imported and anchored to the inner mitochondrial membrane (Olichon et al., 2002; Satoh et al., 2003). l-Opa1 undergoes proteolysis by the rhomboid protease PARL to produce s-Opa1 that is found freely in the intermembrane space (Cipolat et al., 2006; Olichon et al., 2002; Satoh et al., 2003). Mutations in Opa1 are associated with Type 1 Optic Atrophy (ADOA-1), a neurodegenerative disease that affects the retinal ganglion cells (Delettre et al., 2000). Several studies have shown the importance of Opa 1 in fusion. Inhibiting its expression via RNAi causes mitochondrial fragmentation (Griparic et al., 2004; Olichon et al., 2003). Mitochondrial fusion in Opa1^{-/-} is impaired resulting in mitochondrial fragmentation while over-expression of Opa1 induces mitochondrial elongation (Cipolat et al., 2004; Olichon et al., 2002; Song et al., 2009). Opa1 has been involved in the process of apoptosis independent from fusion where treatment of Mfn1^{-/-}

cells with a fusion inactivated form of Opa1 protected against apoptosis (Landes et al., 2010) Furthermore, decreased expression of Opa1 increases the cell's susceptibility to apoptosis (Lee et al., 2004; Olichon et al., 2003). Opa1 has also been implicated in the regulation of cristae junctions. Loss of Opa1 or over expression of BH3 only proteins, such as BIK or BID, lead to widening of the cristae and the release of cytochrome c (Frezza et al., 2006). Over expression of Opa1 is believed to protect the cell by tightening the cristae junctions through enhanced Opa1 oligomerization thereby decreasing cytochrome c release (Frezza et al., 2006; Yamaguchi et al., 2008). Since Opa1 is found on the inner membrane, Opa1 mutations have been shown to affect respiration. It is not surprising that cells treated with Opa1 RNAi exhibit reduced respiration and oxygen consumption (Chen et al., 2005). Thus, Opa1 plays a key role in several mitochondrial functions including survival, respiration, and architecture.

3.2 Mfn1 and Mfn2

Mfn1 and Mfn2 are outer mitochondrial transmembrane GTPases that play a major role in mitochondrial fusion. They have high homology (63%) and similar topology (Rojo et al., 2002; Santel and Fuller, 2001). They are composed of a GTPase domain at the amino terminus, followed by a hydrophobic coiled coil heptad repeated domain (HR1) (Koshiba et al., 2004; Rojo et al., 2002), a bipartite transmembrane domain and a hydrophobic coiled-coil HR2 domain at the carboxyl terminal (Fritz et al., 2001; Rojo et al., 2002). The amino and carboxyl terminals are exposed to the cytosol and the middle region passes through the intermembrane space. Mfn1 and Mfn2 form homo-oligomeric and hetero-oligomeric complexes in trans (Chen et al., 2003; Ishihara et al., 2004; Koshiba et al., 2004; Meeusen et al., 2004) (Fig 4). They mediate mitochondrial fusion



From Merkwirth, C., and T. Langer. 2008. Mitofusin 2 builds a bridge between ER and mitochondria. *Cell*. 135:1165-7.

Figure 1.4. Mitochondrial interactions. Mitochondrial fusion can be promoted by homo and hetero-oligomeric tethering of Mfn1 and Mfn2 in trans via their coiled-coil domain.

by tethering outer membranes together through interactions of their coiled-coil HR2 domain in a buckle and molecular zippering mechanism at the carboxyl terminal (Koshiba et al., 2004). Mfn2 has also been shown to be located on the endoplasmic reticulum (ER) and can tether the ER and mitochondria by homo or hetero dimers with Mfn2 or Mfn1 (de Brito and Scorrano, 2008). It has been shown that Mfn1 directly interacts with Opa 1 thus allowing the tethering of the outer and inner mitochondrial membranes during fusion (Chen et al., 2003; Cipolat et al., 2004; Ishihara et al., 2004).

Defects in Mfn2 have been associated with the Charcot- Marie- Tooth type 2A (CMT2A) disease and CMT4 in humans (Zuchner et al., 2004). CMT is a neuromuscular degenerative disease, characterized by distal weakness of the lower limbs, sensory loss, decreased reflexes, and foot deformities and results in degeneration of sensory and motor axons and thus the degeneration of neurons (Zuchner and Vance, 2006). CMT was first classified as CMT1 and CMT2 (Zuchner and Vance, 2006). CMT1 is characterized by defects in nerve conduction velocity and thus has been associated with the demyelinating form of CMT (Zuchner and Vance, 2006). CMT2 is characterized by abnormal conduction amplitude since the axon is damaged in this condition, and thus the total number of axons that generates a nerve impulse decreases (Zuchner and Vance, 2006). CMT2 can be further classified into CMT2A and CMT2B (Zuchner and Vance, 2006). CMT2B is a result of mutation in the Ras associated protein (RAB7) with a severe sensory neuropathy (Zuchner and Vance, 2006), while mutations in Mfn2 are associated with CMT2A. These mutations are characterized by missense mutations within the GTPase and Ras- binding domain of Mfn2 (Kijima et al., 2005; Lawson et al., 2005). It has also been shown that in CMT2A, Mfn1 is able to complement the mutated Mfn2 and

maintain fusion activity because of the ability of Mfn1 and Mfn2 to form hetero oligomers (Detmer and Chan, 2007). This suggests that Mfn1 may be redundant and additional roles may exist for Mfn2.

As discussed above, Mfn1 and Mfn2 both play a role in mitochondrial fusion, but differences in the role each play in fusion has been seen. Although Mfn2 has a higher GTPase activity than Mfn1, experiments measuring fusion rates in Mfn1^{-/-} and Mfn2^{-/-} mouse embryonic fibroblasts (MEF) showed that cells expressing only Mfn1 have higher fusion activity than those expressing Mfn2 only (Chen et al., 2003). Studies have shown that Mfn1 promotes more competent mitochondrial tethering than Mfn2, and thus induces fusion more efficiently than Mfn2 (Ishihara et al., 2004; Neuspiel et al., 2005). Mitochondrial morphology studies of MEFs from Mfn1^{-/-} and Mfn2^{-/-} cells have shown that loss of Mfn1 results in a severe fragmentation and sphere-shaped mitochondria which were greater than the fragmentation seen in Mfn2 deficient cells (Chen et al., 2003). However, Mfn2 deficient cells showed variable lengths of mitochondrial spheres suggesting that Mfn2 may also be required for maintaining tubular shape in addition to fusion (Chen et al., 2003). In both the Mfn1^{-/-} and Mfn2^{-/-} MEFs the rate of fusion was less than that of wild type MEFs (Chen et al., 2003). In summary, studies have shown that both Mfn1 and Mfn2 are required for proper tethering and fusion of mitochondria, but certain differences in the role each play in fusion has been shown.

3.3 Mitofusins in mitochondrial DNA stability

It has been shown that skeletal muscle lacking Mitofusins may develop mitochondrial myopathies (Chen et al., 2010). These studies have shown the importance of mitochondrial fusion in protecting against mitochondrial DNA (mtDNA) mutations

(Chen et al., 2010). Experiments using double mutant Mfn1^{-/-} Mfn2^{-/-} MEFs showed fewer number of copies of mtDNA per nuclear genome compared to their wild type (wt) control (Chen et al., 2010). The re-introduction of either Mfn1 or Mfn2 into Mfn1^{-/-} Mfn2^{-/-} MEFs restored the mtDNA to wild type levels. This suggests that in the absence of fusion there is less mixing of mitochondrial contents which could explain the loss of mtDNA (Chen et al., 2010). It has also been suggested that Mitofusins may play a role in respiration. For instance, respiratory complex I contains seven mtDNA encoded subunits. When PolgA D257A/D257A, the knock in alleles that encode a deficiency in the proofreading domain of a DNA polymerase found in complex I, were introduced into Mfn1^{-/-} cells, oxygen consumption was severely decreased emphasizing its role in maintaining mitochondrial DNA (Chen et al., 2010). Therefore, these studies have shown that Mfn1 and Mfn2 play a role in maintaining mtDNA stability, and proper respiration.

4. Other roles of Mitofusin 2

4.1 Role of Mfn2 in axonal Transport

Mitochondria are in constant motion to reach regions in the cell that require high energy. For example in neurons, mitochondria have to translocate along the axons to regions of high energy demand and thus any disruption of this process might lead to neurodegenerative diseases (Berthold et al., 1993; Duncan and Goldstein, 2006; Misgeld et al., 2007). Studies have shown that adapters connect the mitochondria to motor proteins to facilitate the transport to their destined area (Hollenbeck and Saxton, 2005; Kang et al., 2008; Li et al., 2009) In *Drosophila*, one of the mitochondrial outer membrane proteins dMiro attaches to kinesin through the Milton adaptor protein (Glater et al., 2006; Guo et al., 2005). Furthermore the Miro and Milton mammalian orthologue

have been identified. For Miro, the two mammalian isoforms are Miro1 and Miro2, and for Milton, the two isoforms are 2OIP106 and GRIF1 (Fransson et al., 2006). These isoforms have been suggested to act in the same manner as the *Drosophila* Miro and Milton orthologue (Fransson et al., 2006).

Experiments performed by Misko et al showed that Mfn2 is important for mitochondrial axonal transport and Mfn2 interacts with the Miro/Milton complex (Misko et al., 2010). By culturing neurons from Mfn2^{-/-} cells or expressing CMT2A associated Mfn2 mutants in neurons, they found that the mitochondria's anterograde and retrograde motion was slower and mitochondria paused more frequently in these conditions (Misko et al., 2010). This finding led them to suggest that this is because of the inability of the mitochondria through Mfn2 to attach to the microtubule transport system (Misko et al., 2010). Furthermore, other experiments provided evidence that the function of Mfn2 in transport is independent of its role in fusion. Treating neurons with an Opa1 siRNA and disrupting fusion showed no change in mitochondrial motility (Misko et al., 2010), demonstrating that the underlying mechanism for Mfn2-mediated axonal transport is independent of fusion.

4.2 Role of Mfn2 in apoptosis

Mfn2 is an outer mitochondrial membrane protein and mitochondria are involved in apoptosis, and several studies have shown that Mfn2 plays a role in apoptosis. Mfn2 co-localizes with Drp1 and Bax on mitochondrial foci sites (Karbowski et al., 2002), and Bax has been shown to inhibit the activity of Mfn2 (Karbowski et al., 2002; Karbowski et al., 2006). Studies in the literature have shown that Mfn2 may have an anti apoptotic activity. Mfn2 has been linked to anti apoptotic Bcl2 family of proteins where it was

shown to directly interact with CED-9 the worm orthologue of Bcl2 to promote fusion (Delivani et al., 2006). A dominant active form of Mfn2- Mfn2_{RasG12V} that has a GTP hydrolysis rate deficiency and still promotes fusion has shown to protect against Bax mediated cytochrome c release and decrease injury due to free radical release (Neuspiel et al., 2005). In neurons, over expression of Mfn2_{RasG12V} also promoted fusion and protected against neuronal cell death upon neuronal injury suggesting that Mfn2 has a protective role in addition to fusion (Jahani-Asl et al., 2007). In contrast, there is also evidence that Mfn2 may have an apoptotic effect in certain instances. Mfn2 levels were shown to be increased in myocytes after myocardial infarction and oxidative stress caused by hydrogen peroxide that activates the apoptotic machinery after releasing free radicals (Shen et al., 2007). Thus whether Mfn2 has an apoptotic or anti apoptotic role could be due to tissue specificity where it can be apoptotic in one type of tissue and anti-apoptotic in another.

4.3 Role of Mfn2 in development

It has been suggested that Mfn2 may have a role during development. Studies in yeast and flies have shown that Fzo (orthologue of mammalian mitofusins) is required during development. Fzo was specifically and transiently expressed in spermatids; inhibition of Fzo interrupted fusion during development of postmeiotic spermatids and thus led to male sterility (Hales and Fuller, 1997). Furthermore, it has been shown that fusion is important during development. For instance, during the embryonic stages, the mitochondria in rat cardiac muscle and diaphragm skeletal muscle appear like tubules, but later on in adult stages the mitochondria progress into reticular networks (Bakeeva et al., 1981; Bakeeva et al., 1978; Bakeeva et al., 1983).

Furthermore, Mfn1 and Mfn2 are required during different phases of embryonic development of mice, but Mfn1 is required during embryogenesis whereas Mfn2 is required for placentation (Chen et al., 2003; Chen et al., 2007). Mfn1 and Mfn2 heterozygous animals are viable, whereas whole body Mfn1 and Mfn2 knockouts are embryonically lethal because of a defect in the placenta (Chen et al., 2003). By E8.5 Mfn1^{-/-} embryos are significantly smaller and die by E11.5. For this reason floxed Mfn1 mice were crossed with Meox2 Cre mice which express Cre Recombinase in the embryo by E7 in all cell lineages except the cell lineages giving rise to the placenta (Tallquist and Soriano, 2000). Mfn1 deficient pups mice that only expressed Mfn1 in the placenta were generated and these mice survived normally with no apparent difference compared to their wild type littermates (Chen et al., 2007). Similarly Mfn2 whole body knockouts die by E11.5 and embryos at E9.5 were also smaller than their respective littermates (Chen et al., 2003). Therefore, floxed Mfn2 were also crossed with Meox2 Cre mice. One third of the Mfn2 deficient pups die at P1 and the surviving pups show severe defects in movement, balance, as well as uncoordinated limb movement. Anatomical and histological analysis revealed a defect in the cerebellum which is responsible for movement and balance thus providing an explanation for the observed phenotype. For this reason conditional knockout for Mfn1 and Mfn2 were generated in the cerebellum using En1 Cre (Chen et al., 2007; Kimmel et al., 2000). These Mfn2 deficient pups exhibited similar motor movement defects as well as smaller cerebella with normal lobular organization and layer formation, while Mfn1 deficient pups were normal (Chen et al., 2007). This clearly demonstrated that Mfn2 plays a role in the cerebellar development.

After observing morphological and phenotypic defects in the cerebellum, Chen et al performed *in vitro* and *in vivo* experiments to further study the affect of Mfn2. Purkinje cells are important for the proper function of the cerebellum. They relay the efferent cellular output of the cerebellum to the rest of the central nervous system. Purkinje cells form elaborate dendritic arbors that spread into the molecular layer and synapse with axons of climbing fibers and granule cells (Sotelo, 2004). The dendrites of Mfn2 deficient Purkinje cells showed reduced growth and deterioration over time with reduced branching dendrites (Chen et al., 2007). Functionally, mitochondrial changes were observed in the Mfn2 deficient mice where complex V and cytochrome c were expressed at higher levels. *In vitro* studies confirmed *in vivo* findings; cell cultures of Mfn2^{-/-} cerebellar neurons revealed dendritic shrinking reduced branching as well as Purkinje cells cell death. The mitochondria aggregated in the cell body of Purkinje cells and did not enter the dendritic tracts (Chen et al., 2007). Over expression of Mfn2 lentivirus rescued the phenotype causing dendrites to become more branched and the mitochondria were expressed in the dendritic tracts. In summary, in the cerebellum, Mfn2 is important during development and Purkinje cells require Mfn2 for dendrite outgrowth, spine formation and cell survival (Chen et al., 2007).

4.4 Corpus callosum development

The commissural plate consists of all the telencephalic commissures that cross the midline between the two hemispheres (Rakic and Yakovlev, 1968). This includes the corpus callosum, hippocampal commissure, and anterior commissure. During development, and around E15, the anterior commissure and hippocampal commissure cross the midline whereas the corpus callosum start to cross at E17 (Moldrich et al.,

2010). Moreover, the cerebral cortical axons cross the midline around E16.5, and then more axons will continue to cross until the first few days after birth. Fusion of the cerebral hemispheres creates the midline, bridge-like structure (Lindwall et al., 2007; Paul et al., 2007). The corpus callosum development is a coordinated process that requires regulation between tissue fusion, neural and glial cell production as well as proper axon guidance (Bagri et al., 2002; Lindwall et al., 2007; Serafini et al., 1996). Furthermore, several transcription factors have been related with the development of the corpus callosum. These include the nuclear factor I family (Nfia), and empty spiracles homologs (Emx1) (Moldrich et al., 2010). Nfia is important for the formation of glial populations at the midline (Shu et al., 2003), the development of corpus callosum and hippocampal commissure. Emx2 is a transcription factor required for correct development of the cortex as well as the formation of commissural projections (Bishop et al., 2000; Mallamaci et al., 2000; Yoshida et al., 1997). The fibroblast growth factor (Fgf8) is expressed in the commissural plate and thus has also been shown to be involved in commissure development (Cholfin and Rubenstein, 2008; Tole et al., 2006). Furthermore, studies have shown that the heparan sulfate is essential for the proper development of the corpus callosum (Inatani et al., 2003). When heparan sulfate biosynthesis was inhibited during brain development, the corpus callosum was not properly developed (Inatani et al., 2003). Moreover, recent studies by Conway et al have shown that heparan sulfotransferase Hs2st and Hs6st1 are required for the proper crossing and projection of the axons across the corpus callosum (Conway et al., 2011). Furthermore, the axon guidance molecule Slit2 is required for the proper development of the corpus callosum where, in the absence of Slit2, fewer axons are able to cross and

probst-like bundles of axons are formed ectopically (Bagri et al., 2002). Thus, corpus callosum development is a highly regulated process that starts embryonically and continues after birth and involves several transcription factors, enzymes, tissue fusion and axon guidance molecules.

Rational

Mitofusins 1 and 2 are expressed throughout the brain during development and postnatally. The importance of proper Mfn2 expression in brain function has been observed where mutations in Mfn2 have been implicated in CMT2A neurodegenerative disease. Furthermore, conditional deletion of Mfn2 in the cerebellum causes a reduction in its size, movement defects, and Purkinje cell degeneration (Chen et. al 2007). We therefore asked if Mitofusin 1 and 2 play an important role in telencephalic development, which is the region affected by several neurodegenerative diseases. In my thesis I will characterize and study the role of Mfn1 and Mfn2 during telencephalic development.

Hypothesis I hypothesize that Mfn1 and Mfn2 will play an essential role in telencephalic development.

Objectives

To examine this hypothesis there are two objectives:

Objective #1 To generate Mfn1 and Mfn2 conditional knockouts in the telencephalon

Objective #2 To characterize the phenotype of mice carrying a conditional null mutation for Mfn1 and Mfn2 in the developing telencephalon.

Chapter II Materials and Methods

Mice

Floxed Mfn1 (Mfn1^{flox/flox}) and floxed (Mfn2^{flox/flox}) (Chen et al., 2003; Chen et al., 2007) were bred on a mixed 129 and C57 BLC/6 genetic background. Telencephalic specific Mfn1 deficient mice were generated by crossing Mfn1^{flox/flox} mice with mice expressing Cre recombinase from the Foxg1 promoter bred on a C57BLC/6 and FVBN mixed background (Foxg1:Cre) (Hebert and McConnell, 2000) to produce Foxg1^{cre+/-} Mfn1^{f/f}. Foxg1 is a transcription factor belonging to the fork head family (Tao and Lai, 1992) and is activated mid gestation at E8.5. By E10, Foxg1 is expressed in all of the telencephalon and in lower levels in the developing ear, olfactory epithelium, foregut, and mid-hindbrain (isthmus) region (Hebert and McConnell, 2000). The Foxg1 Cre mouse was used because Cre is expressed in the telencephalon, the region in which the requirement for Mfn1 and Mfn2 was to be examined in these studies. The controls for the in vivo experiments were double heterozygous littermates (Foxg1^{cre+/-} Mfn1^{f/+}) and the Foxg1^{cre+/-} littermates. Telencephalon specific Mfn2 deficient mice were generated by a similar protocol. For embryonic time points, mice were taken at E16.5. The day the plug was discovered is considered embryonic day E0.5. Experiments were approved by the University of Ottawa Animal Care Ethics which adheres to the Canadian Council on Animal Care Guidelines.

Real time PCR

Genotyping for Cre was carried out using the following primers and PCR protocol:

Foxg1 Cre genotyping was carried out using the following Primers: CRE-3B 5'-TGACCAGAGTCATCCTTAGCG - 3' and CRE -5B 5'-AATGCTTCTGTCCGTTTGCC

- 3' (Hebert and McConnell, 2000); 94°C for 2.00 min followed by 30 cycles of 94°C for 1.00 min to denature the DNA template, 56 °C for 1.00 min for annealing the primers, 72 °C for 1 min, 30 sec for primer extension, and finally 72°C for 5.00 min. Each reaction mix contained 1X reaction buffer (Invitrogen) that contains 2.5µM of each primer, 0.2mM dNTP's, 1.75mM MgCl₂, and Taq polymerase (Invitrogen, 10342-020), and 1µg of the DNA template (Hebert and McConnell, 2000). Genotyping for Mfn1 was carried out using the following primers: DC285 5'- AGC AGT TGG TTG TGT GAC CA-3' and HC105 5'- TTG GTA ATC TTT AGC GGT GCT C- 3'(Chen et al., 2003; Chen et al., 2007); 94°C for 30 min followed by 35 cycles of 94°C for 30 min to denature the DNA template, 58 °C for 1.00 min for annealing the primers, 72 °C for 1 min for primer extension, and finally 72°C for 1 min. Each reaction mix contained 1X reaction buffer (Invitrogen) that contains 2.5µM of each primer, 0.2mM dNTP's, 1.75mM MgCl₂, Taq polymerase (Invitrogen, 10342-020), and 1µg of the DNA template (Chen et al., 2003; Chen et al., 2007; Detmer and Chan, 2007). Genotyping for Mfn2 was carried out using the following primers: HC106 5'- GAA GTA GGC AGT CTC CAT CG-3' and HC107 5'- AAC ATC GCT CAG CCT GAA CC-3'(Chen et al., 2003; Chen et al., 2007); 94°C for 30 min followed by 35 cycles of 94°C for 30 min to denature the DNA template, 58 °C for 1.00min for annealing the primers, 72 °C for 1 min for primer extension and finally 72°C for 1 min. Each reaction mix contained 1X reaction buffer (Invitrogen) that contained 2.5µM of each primer, 0.2mM dNTP's, 1.75mM MgCl₂, Taq polymerase (Invitrogen, 10342-020), and 1µg of the DNA template (Chen et al., 2003; Chen et al., 2007; Detmer and Chan, 2007).

Mfn2 samples were run on a 3.25% agarose gel whereas Mfn1 and Cre were run on a 2% agarose gel. The resulting DNA bands were visualized with an Alphamager 2200 (Alpha Innotech Corporation) at around 700bp for Cre, 450bp for floxed Mfn1, 350 for Mfn1 wild type, 180bp for floxed Mfn2, and 145bp for Mfn2 wild type

Tissue Fixation and Cyroprotection

Animals were sacrificed by injection of sodium pentobarbital (900mg/kg body weight). Postnatal animals (P9) were perfused and brains were dissected and fixed in 4% Paraformaldehyde (PFA) in phosphate buffer saline (PBS) at pH7.4 overnight and then cyroprotected by saturation in 20% sucrose. Tissue was frozen on dry ice in OCT (Tissue Tek 4583), sectioned into 10µm sections at -20° C using cryostat, and placed on Superfrost Plus (R) slides and stored at -80° C.

Dissected embryos were fixed in 4% Paraformaldehyde (PFA) in phosphate buffer Saline (PBS) at pH7.4 overnight and cyroprotected by saturation in 20% sucrose. Tissue was frozen on dry ice in OCT, sectioned into 10 µm sections at -20° C using cryostat (Microm HM500) and placed on Superfrost Plus (R) slides and stored at -80° C.

Cresyl Violet staining

Tissue was stained with 0.25% Cresyl Violet solution in 200mM acetate buffer for 15 minutes at room temperature and subsequently washed in water, followed by dehydration in ethanol gradient (70%, 95%, and 100%) 30 seconds each. Sections were ultimately immersed in xylene, and coverslips were mounted with permount.

Immunohistochemistry

Slides were prepared by rehydrating in 1 time PBS for 10 minutes followed by blocking with blocking solution (1XPBS, 10% normal horse serum, 0.1% PBS- tween, 0.1% triton

X) for 20 minutes. Slides were incubated with primary antibody overnight at 4° C. The primary antibodies used include: Active Caspase 3(AC3) (Rabbit, Cell Signaling, 1:200), ki67 (Mouse, Pharminogen, 1:200), ki67 (Rabbit, Cell Marque, 1:200), Cux1 (Mouse, Santa Cruz Biotechnology, 1:100), Foxp2 (Rabbit, Abcam, 1:1000), NeuN (Mouse, Millipore, 1:200), and myelin basic protein (Rabbit, Millipore, 1:500). The slides are then washed with PBS for 10 minutes at room temperature and then incubated with the appropriate secondary antibody for 1 hour at room temperature. Secondary antibodies used include goat anti-rabbit 594 Alexa (Invitrogen Molecular Probe, 1:500), goat anti-rabbit 488 Alexa (Invitrogen Molecular Probe, 1:500), goat anti-mouse 594 Alexa (Invitrogen Molecular Probe, 1:500), goat anti-mouse 488 Alexa (Invitrogen Molecular Probe, 1:500). Slides were finally washed with 1XPBS for 10 minutes, and treated with Hoechst for 3 minutes then mounted with 1:3 glycerol: PBS.

Western blot

Protein was extracted from the cortex of E16.5 embryos using a lysis buffer (Germain et al., 2011) and protein concentration was determined using the Bio-Rad Bradford Assay. 40ug of whole cell lysate proteins were resolved on a 10% polyacrylamide gel. Gels were run in a 1x SDS-PAGE running buffer (25uM Tris Base, 200uM glycine and 0.1% SDS) at 200V for 1 hour. The proteins were transferred on a nitrocellulose membrane for 1 hour at 4° C at 100 volts in a 1Xwestern blot transfer buffer (48uM Tris Base, 386 uM glycine, 0.1% SDS page and 20% methanol). The membrane was blocked in 1XTPBS (NaHPO₄, NaCl, Tween-20) with 5% skim milk powder. Primary antibodies were diluted in 5% milk and 1XTPBS. The membranes were blotted against the following primary

antibodies: Mfn1 (Chicken, Novous, 1:1000) and Mfn2 (Rabbit polyclonal, Sigma, 1:1000), and actin (Mouse monoclonal, sigma, 1:5000) as loading control.

The membrane was then washed with 1XTPBS for 15 minutes then blotted with secondary antibody rabbit IgG (Santa Cruz Biotechnology, 1:500) and mouse IgG (Santa Cruz Biotechnology 1:500) that was prepared in 1XTPBS containing 5% skimmed milk. The antibodies bound to the membranes were detected by Enhanced Chemi-Luminescence (ECL) , by mixing equal amounts of solution A (0.1M Tris (pH8.5), 390uM coumaric acid, and 2.46mM Luminol) and solution B (0.1M Tris (pH8.5) and 0.02% H₂O₂) and applying the mixture to the membrane for 1 minute. Finally, the membrane was exposed to Hyperfilm xray film (Amersham Biosciences) and developed.

Microscopy

Sections were examined using Carl Zeiss Axioskop 2 MOT microscope (Karl Zeiss MicroImaging Inc). The images were captured using QICAM from QIMAGING, and were analyzed using Northern Eclipse software from Empix Imaging Inc

Quantification and Statistical analysis

The Ki67 cells were quantified by counting the positively labeled cells along the dorsal lateral surface of the ventricle. The NeuN positively labeled cells were quantified through the total cortical thickness in the dorsal lateral boxed area of the cortex with respect to the length of the ventricle. Four different sections were taken per animal and 3 animals were used for every specific genotype.

All data are expressed as the mean of all animals examined. The bars represent the standard error of the mean (SEM). Significant difference was assessed using two-tailed t-

test analysis assuming unequal variance ($P < 0.05$). $n=3$ delineate that 3 different mice were examined per data point.

Chapter III Results

Generation of Mfn1 and Mfn2 conditional knockouts

It has been shown that Mfn2 plays a role in cerebellar development and that Purkinje cells require Mfn2 for dendrite outgrowth, spine formation and cell survival (Chen et al., 2007). As such, we hypothesized that Mfn1 and Mfn2 play a role in telencephalic development. Whole body Mfn1 and Mfn2 knockouts are embryonically lethal and die midgestation due to a defect in the placenta (Chen et al., 2003). To study the effect of Mfn1 and Mfn2 on the development of the telencephalon, we generated conditional Mfn1 and Mfn2 knockout mice in which each Mitofusin is deleted from the telencephalon. To create these mice, floxed Mfn1 (Mfn1^{f/f}) and floxed Mfn2 (Mfn2^{f/f}) (Chen et al., 2003; Chen et al., 2007), whereby a stop codon was engineered at the very beginning of the GTPase domain near the NH₂ terminus, were bred with mice in which Cre is knocked into the Foxg1 locus (Hebert and McConnell, 2000). Thus conditional Mfn1 knockout mice (Mfn1^{f/f} Foxg1 Cre^{+/-}) and conditional Mfn2 knockout mice (Mfn2^{f/f} Foxg1 Cre^{+/-}) were generated. Throughout the thesis, I will refer to Mfn1^{f/f} Foxg1 Cre^{+/-} as Mfn1^{ΔΔ} mice and Mfn2^{f/f} Foxg1 Cre^{+/-} as Mfn2^{ΔΔ} mice. Foxg1 is a transcription factor belonging to the fork head family (Tao and Lai, 1992) and is activated mid gestation at E8.5. By E10, Foxg1 is expressed in all of the telencephalon and in lower levels in the developing ear, olfactory epithelium, and foregut, and mid-hindbrain (isthmus) region (Hebert and McConnell, 2000). To confirm that conditional knockouts of Mfn1 and Mfn2 were obtained, PCR genotyping was performed using a similar protocol as used by Chen

et al. (Chen et al., 2003; Chen et al., 2007). Figure 3.1A shows the genomic DNA of Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} mice amplified by PCR using specific primers whereby distinct fragments of the wild type and loxp loci were amplified. To further confirm the absence of Mfn1 and Mfn2 in the telencephalon, protein was extracted from the cortex of embryos at E16.5. Western blot analysis showed the absence of Mfn1 protein at 86kDa in Mfn1^{Δ/Δ} mice (Fig3.1 B) and the absence of Mfn2 protein at 86kDa in Mfn2^{Δ/Δ} mice (Fig.3.1B). Thus these results confirm that Mfn1 and Mfn2 were absent from the telencephalon.

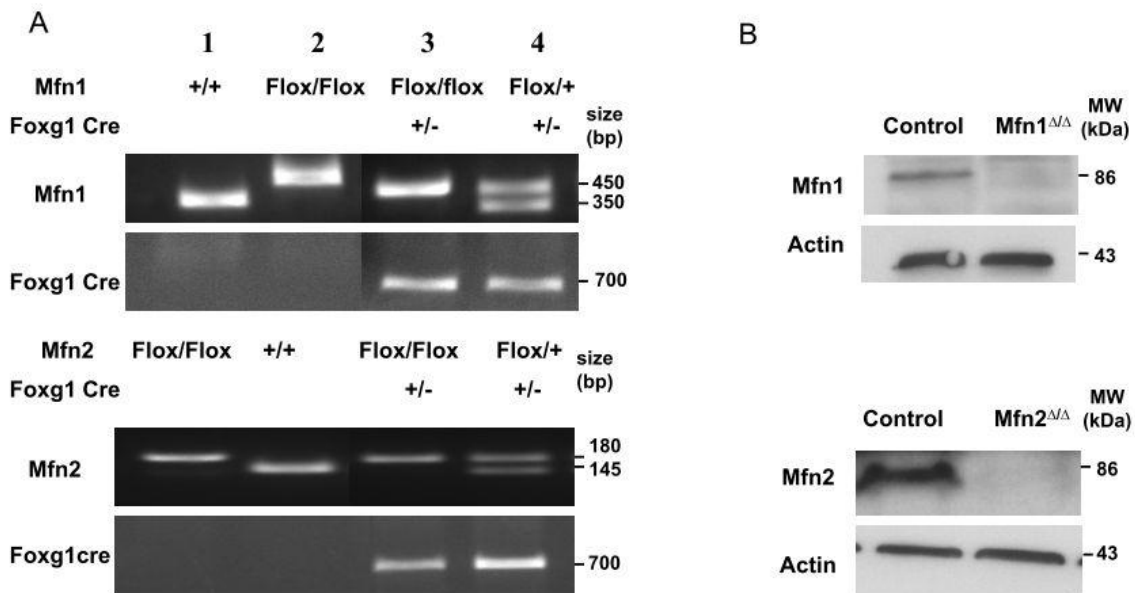


Figure 3.1 Generation of Mfn1 and Mfn2 conditional knockouts

A) Genotyping of Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} mice by PCR using specific primers that amplify distinct fragments at the wild type and loxp loci. For Mfn1, lane 1, 2, and 4 are controls representing wild type Mfn1 (350bp), floxed Mfn1 (450bp), and Mfn1^{Δ/+} (Mfn1f/+ Foxg1 Cre^{+/-}) respectively, and lane 3 represents Mfn1^{Δ/Δ} (Mfn1f/f Foxg1 Cre^{+/-}). For Mfn2, lane 1, 2, and 4 are controls representing floxed Mfn2 (180bp), wild type Mfn2 (145bp), and Mfn2^{Δ/+} (Mfn2f/+ Foxg1 Cre^{+/-}), and lane 3 represents Mfn2^{Δ/Δ} (Mfn2f/f Foxg1 Cre^{+/-}). (+) represents the wild type allele and flox represents the floxed Mfn1 and floxed Mfn2 allele.

B) Western blot analysis demonstrating the loss of Mfn1 in the cortex of Mfn1^{Δ/Δ} and the loss of Mfn2 in the cortex of Mfn2^{Δ/Δ}. Protein was extracted from the cortex of E16.5 embryos. Molecular weight of Mfn1 and Mfn2 is ~ 86 kDa. Actin is the loading control

Phenotype and viability of Mfn1 and Mfn2 conditional knockouts

Deletion of Mfn1 during telencephalic development with Foxg1 Cre showed no severe abnormalities and Mfn1^{Δ/Δ} mice were able to live over 6 months (Fig. 3.2A). Mfn1^{Δ/Δ} mice had a significantly reduced overall body weight. At postnatal day 30, the average weight of Mfn1^{Δ/Δ} animals was 14.34 ±1.9 g relative to its double heterozygous control Mfn1^{Δ/+} (Mfn1f/+Foxg1 Cre+/-) which was 17.8±1.3 g (n=4, P<0.05) (Fig 3.2B). Around postnatal day 22, we noticed that the Mfn1^{Δ/Δ} mice exhibited slow movement as well as a slight head tilt to the right.

In contrast to Mfn1^{Δ/Δ} mice, Mfn2^{Δ/Δ} pups die between postnatal day 9 and postnatal day 12. At postnatal day 9, the overall body weight was also significantly reduced where the average body weight for Mfn2 deficient pups was 1.9±0.26 g compared to 5.9±0.54 g for control littermates Mfn2^{Δ/+} (Mfn2f/+ Foxg1 Cre+/-) (n=3, P<0.001) (Fig. 3.2C). By postnatal day 9, no milk was observed in the stomach of the Mfn2 deficient pups suggesting that they are unable to feed.

Overall, these results demonstrate that the Mfn1 deficiency has a less severe effect on survival than that of Mfn2 mutant mice where Mfn1^{Δ/Δ} mice survive over 6 months while Mfn2^{Δ/Δ} pups die between P9-P12, and both Mfn1^{Δ/Δ} mice and Mfn2^{Δ/Δ} suffered a reduction in body weight.

To further characterize the phenotype of mice carrying a conditional null mutation for Mfn1 and Mfn2 in the developing telencephalon, morphological analysis was performed on Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} brains. Below I will describe the results of my analysis with each

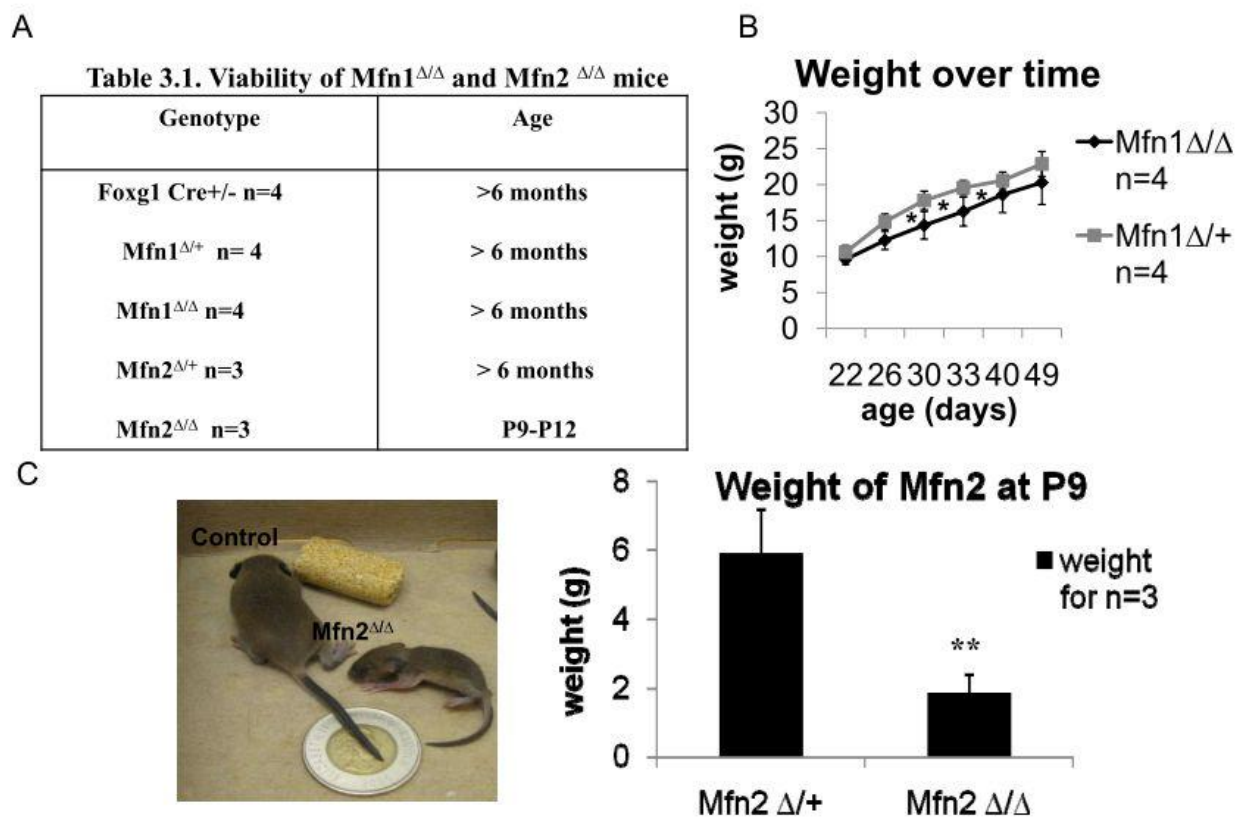


Figure 3.2 Phenotypic characterizations of Mfn1 and Mfn2 conditional knockouts

A. Table 3.1 represents the viability of Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} mice.

B. Graph representing the average weight of Mfn1^{Δ/Δ} mice and control littermates over time (n=4, two tailed t test indicates P<0.05)

C. Image of Mfn2^{Δ/Δ} pup at postnatal day 9 just before perfusion and control littermates. Note the difference in size. The bar graph represents the average weight of Mfn2^{Δ/Δ} mice at postnatal day 9 just before perfusion. Weights were measured in grams (n=3 two tailed t test, P<0.001).

(*) indicates statistical significance of P<0.05 and (**) indicates statistical significance of P<0.001.

of these mice independently and will conclude with a comparison between Mfn1 and Mfn2.

A) Mitofusin 1

Cresyl violet staining of Mfn1^{Δ/Δ} mice

In order to examine the role of Mfn1 during telencephalic development, Mfn1^{Δ/Δ} mice and control littermates Mfn1^{Δ/+} and Foxg1 Cre+/- alone were collected at postnatal day 9. Cresyl violet staining of coronal brain sections at postnatal day 9, suggests that a loss of the middle region of the corpus callosum may have resulted, however this would require further investigation (n=3) (Fig 3.3). The corpus callosum is the largest white matter of the brain and contains the axonal projections that connect the right and left hemispheres (Kandel and Schwartz, 2000). Thus, the loss of the corpus callosum at the midline suggests that Mfn1 may play a role in axonal maintenance. Furthermore, the overall gross morphology appeared normal. For example, the entire hippocampal CA1-CA3 regions, the dentate gyrus, the cortex and striatum were present and similar to controls. Thus, examination using cresyl violet staining showed-with the exception of the corpus callosum- no change in general morphology of the brain of Mfn1^{Δ/Δ} mice compared to controls suggesting that gross morphology was not altered.

Fewer progenitor cells postnatally in Mfn1 conditional knockouts

To further characterize the phenotype of Mfn1^{Δ/Δ} mice, we asked whether the loss of Mfn1 would affect the population of precursor cells during development and postnatally.

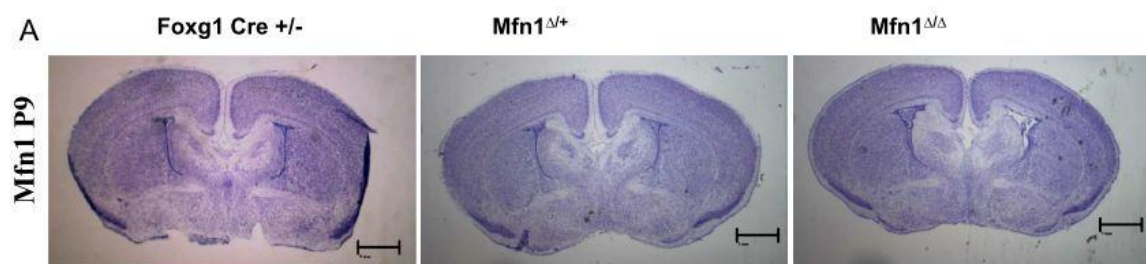


Figure 3.3 Cresyl Violet Staining of Mfn1^{Δ/Δ} mice

A. Cresyl violet staining of Mfn1^{Δ/Δ} mice and its controls Foxg1Cre^{+/-} alone and Mfn1^{Δ/+} mice at postnatal day 9. Brains were sectioned coronally and stained with cresyl violet. Scale bars represent 1mm (n=3).

Coronal sections of $Mfn1^{\Delta\Delta}$ brains and control littermates were stained with the proliferative marker ki67. Ki67 is a nuclear protein that is associated with cellular proliferation and is a marker for the growth of a given cellular population (Scholzen and Gerdes, 2000). At E16.5, quantification of the number of Ki67 positively labeled cells along the entire length of the dorsal ventricle surface showed no change between $Mfn1^{\Delta\Delta}$ mice and its control. Per dorsal ventricle length, $Mfn1^{\Delta\Delta}$ mice contained 282 ± 50 ki67 labeled cells and $Mfn1^{\Delta/+}$ mice contained 262 ± 44 ($n=3$, $P>0.05$) (Fig 3.4 A, C). In contrast, at postnatal day 9, quantification of the number of ki67 cells along the entire length of the dorsal ventricle demonstrated a significant decrease in $Mfn1^{\Delta\Delta}$ mice. Per dorsal ventricle, $Mfn1^{\Delta\Delta}$ mice contained an average of 89 ± 3 positively labeled ki67 cells and its controls $Mfn1^{\Delta/+}$ contained an average of 120 ± 3 ($n=3$, $P<0.05$) (Fig3.4 B, C). Overall these results demonstrated that the loss of Mfn1 in the telencephalon showed a significant decrease in neural precursor cells postnatally.

Assessment of apoptotic cell death in Mfn1 conditional knockouts

Since we observed a significant decrease in neuronal precursor cells postnatally, we asked whether this loss could be due to apoptotic cell death. $Mfn1^{\Delta\Delta}$ brains were stained for active Caspase 3, a marker for classical apoptosis. Staining with active Caspase 3 did not reveal any detectable cell death in $Mfn1^{\Delta\Delta}$ mice over levels found in the control at postnatal day 9 (Fig 3.5). Overall these results demonstrated that the loss of Mfn1 in the telencephalon did not result in increased apoptotic cell death.

No detectable change in number of cortical neurons in Mfn1 conditional knockouts

During cortical development progenitor cells in the ventricular zone undergo terminal mitosis and commit to migration into the cortical plate where they undergo differentiation

(Kandel and Schwartz, 2000). Since we did not detect any change in precursor cells during development, we did not expect to see a difference in number of cortical neurons.

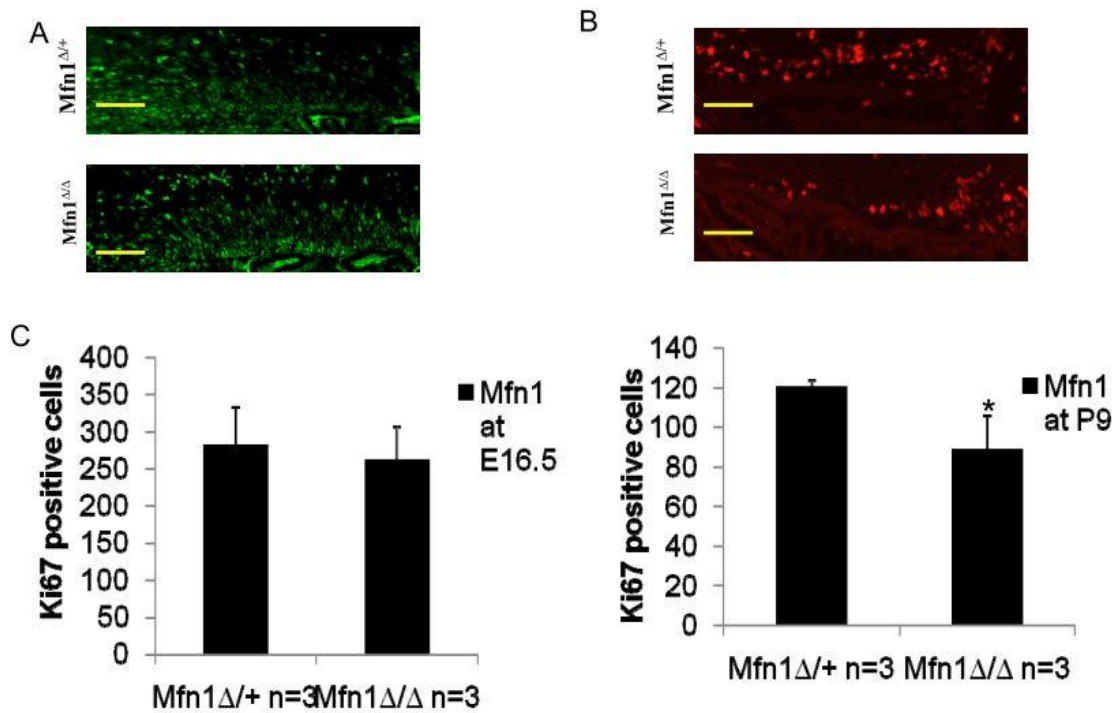


Figure 3.4 Less Ki67 labeled cells at P9 in Mfn1 conditional knockouts

- A. Coronal sections taken at embryonic day 16.5 showing the lateral ventricle of Mfn1^{Δ/Δ} and control littermate brains were stained with the proliferative marker Ki67. Scale bar represents 50 microns.
- B. Coronal sections of Mfn1^{Δ/Δ} and control littermate brains showing the lateral ventricle were stained with the proliferative marker Ki67 at postnatal day 9. Scale bar represents 50 microns.
- C. Graphs represent the quantification of Ki67 labeled cells along the ventricle at E16.5 and P9 for Mfn1^{Δ/Δ} mice and control littermates. Four different sections were taken per animal (n) and a total of n=3 were used for each genotype. Per genotype the average of 3 n was plotted and the error bars represent the standard error of the mean. (n=3, two tailed t-test, *P< 0.05)

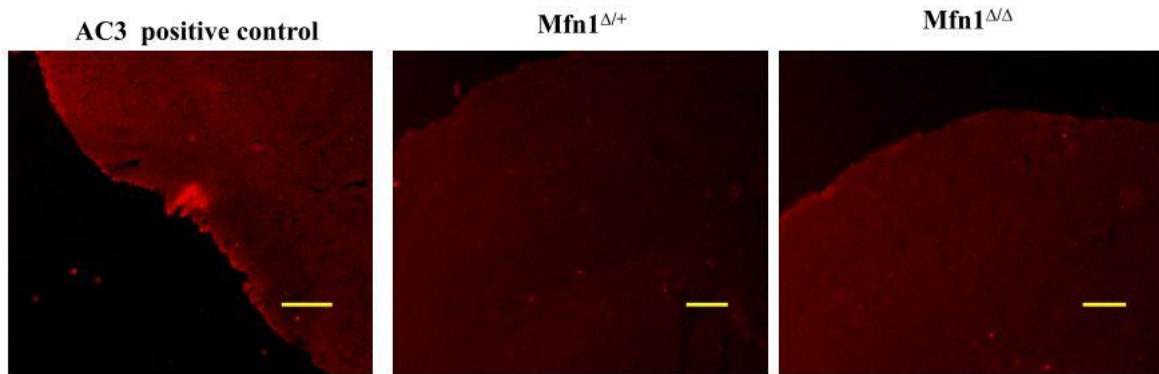


Figure 3.5 Active Caspase 3 in Mfn1 conditional knockouts

Coronal sections at postnatal day 9 of Mfn1^{Δ/Δ} and control littermate brains were stained with the apoptotic marker Active Caspase 3 (AC3). The positive control represents a region of a cortex after stroke where apoptosis occurred and is positive for active Caspase 3. Scale bar represents 125 microns

To further confirm this, we stained with NeuN, a neuron-specific nuclear protein expressed in the central nervous system (Mullen et al., 1992). NeuN labeled cells were quantified in the dorsal lateral boxed area of the cortex with respect to the length of the ventricle as shown in figure 3.6A. The cell counts indicated that there was no difference in neuronal number in the cortex of Mfn1^{Δ/Δ} mice compared to its control. Mfn1^{Δ/Δ} mice contained an average of 681± 96 NeuN positive cells and Mfn1^{Δ/+} mice had 778±100 (n=3, P>0.05) (Fig3.6 A, B). Therefore, our data demonstrated that the loss of Mfn1 in the telencephalon does not result in a detectable change in neuronal number at postnatal day 9.

In summary, the deletion of Mfn1 from the telencephalon does not affect the pool of progenitor cells during development and consequently no change is observed in post-mitotic cortical neurons. However, loss of Mfn1 causes a significant decrease in progenitor cells postnatally which was not due to apoptosis suggesting that Mfn1 may play a role in maintenance of the postnatal progenitor cells only.

Distribution of CuxI and Foxp2 in the deep and superficial cortical layers of Mfn1 conditional knockouts

Since there was no detectable difference in cortical neurons, we decided to evaluate whether Mfn1 might have an effect on cortical lamination. The superficial layers constitute cortical layers II/III and the deep layers are composed of cortical layers IV, V, VI. CuxI, the mammalian homologue of *Drosophila* Cut, is a transcriptional repressor that has been shown to be involved in cell cycle regulation and is expressed specifically in the pyramidal neurons of the superficial layers of the cortex (II-III) (Nieto et al., 2004). Therefore we used CuxI as a marker for the superficial cortical layers. Foxp2 is a member of the winged-helix/forkhead DNA-binding proteins and was found to act as a

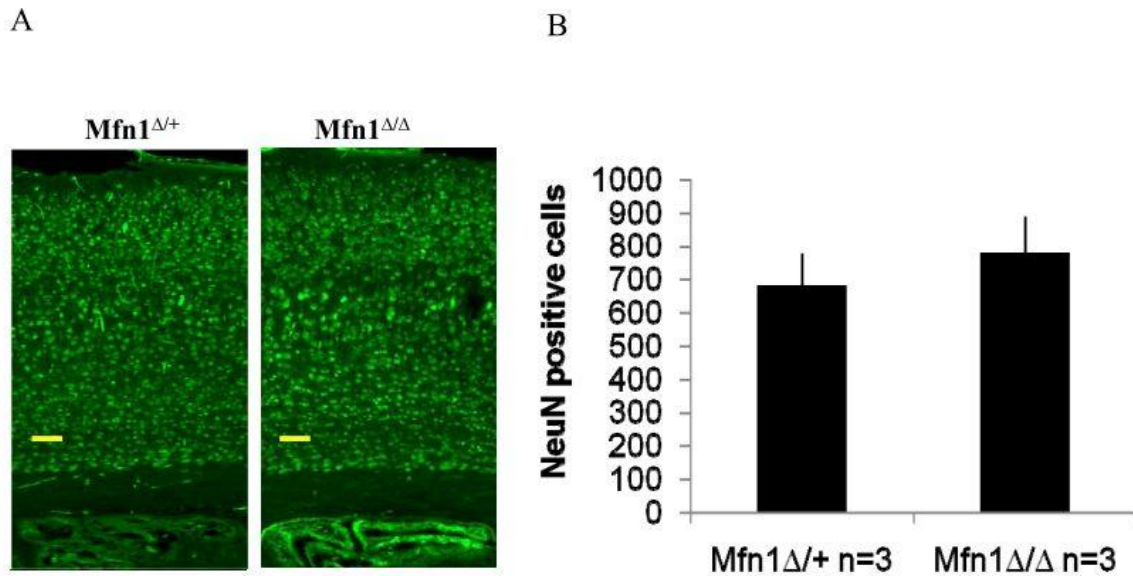


Figure 3.6 No detectable change in number of neurons in the cortex of Mfn1 conditional Knockouts

A. Coronal sections of Mfn1^{Δ/Δ} mice and control littermate brains were stained with the neuronal marker NeuN at postnatal day 9. Scale bar represents 125 microns.

B. Graph represents the quantification of NeuN labeled cells in the cortex of Mfn1^{Δ/Δ} mice and control littermates. Four different sections were taken per animal (n) and a total of n=3 were used for each genotype. Per genotype the average of 3 n was plotted and the error bars represent the standard error of the mean. (n=3, two tailed t-test, P> 0.05)

transcriptional repressor (Shu et al., 2001). Foxp2 is expressed in cortical layers IV, V, VI (Ferland et al., 2003), therefore Foxp2 was used as a marker for the deep layers of the cortex. Our results demonstrated that there was no difference in the distribution of CuxI (Fig3.7 B) and Foxp2 (Fig3.7 A) between Mfn1^{Δ/Δ} mice and its littermate control. This suggests that Mfn1 is not required for cortical lamination.

Absence of Tau1 expression in the middle region of the corpus callosum in Mfn1 conditional Knockouts

Since deletion of Mfn1 in the telencephalon appears to have resulted in defect the corpus callosum at the midline, and the corpus callosum contains axonal projections, we asked whether Mfn1 might have an effect on the axons. MAP2 is a microtubule associated protein that plays a role in microtubule assembly and is enriched in the dendrites where it stabilizes their shape during neural development (Goedert et al., 1991; Matus, 1994). Tau1 protein is a highly soluble microtubule-associated protein that is found to stabilize axonal microtubules and is not present in dendrites (Mercken et al., 1995). Therefore, we used MAP2 as a dendritic marker and Tau1 as a marker for axons. As I previously mentioned, cresyl violet analysis revealed a loss of the corpus callosum at the midline. Since the corpus callosum contains axons only, tau1 should be specifically expressed in this region. In Mfn1^{Δ/Δ} mice, tau1 was expressed in the anterior commissure and the corpus callosum, however tau1 staining was absent at the midline n=3 (Fig3.8). Out of the 3 animals examined for each genotype, 3/3 control littermates had a corpus callosum while none of the 3 (0/3) Mfn1^{Δ/Δ} mice had an intact corpus callosum. Thus, our results suggest that axons may be absent at the midline where the corpus callosum should cross to the opposite hemisphere in Mfn1 conditional knockouts, however further investigations should be conducted in future studies to substantiate this result.

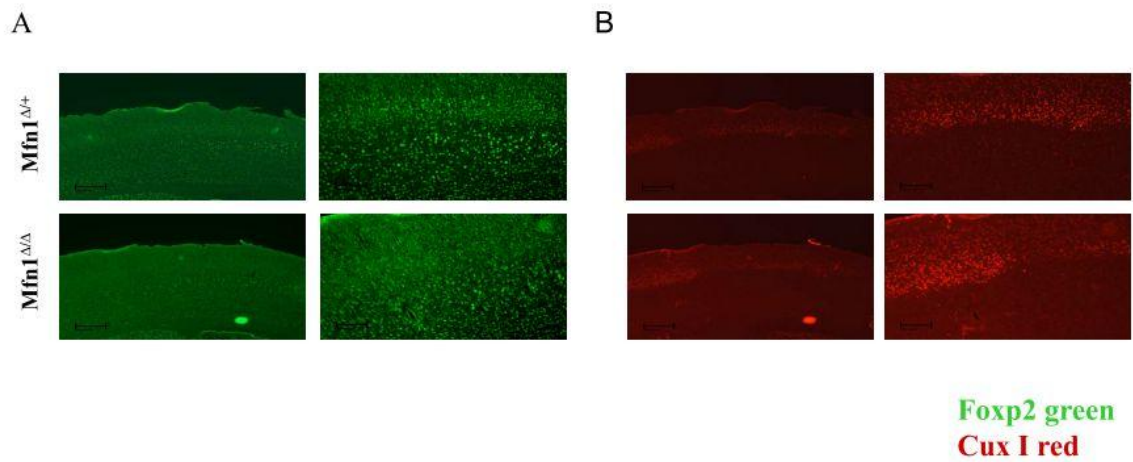


Figure 3.7 Distribution of CuxI and Foxp2 in the deep and superficial layers of $Mfn1$ conditional Knockouts

A. Representative images of the deep layers of the cortex in $Mfn1^{\Delta/\Delta}$ mice and control littermates. Coronal sections were stained at postnatal day 9 with the deep layer marker Foxp2. Scale bar represents 250 microns

B. Representative images of the superficial layers of the cortex in $Mfn1^{\Delta/\Delta}$ mice and control littermates. Coronal sections were stained at postnatal day 9 with the superficial layer marker CuxI. Scale bar represents 250 microns.

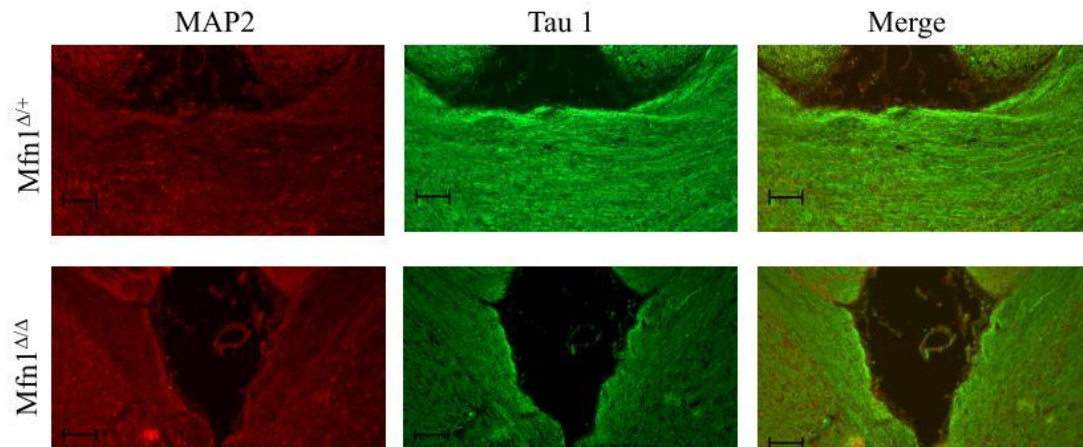


Figure 3.8 Absence of Tau1 expression in the middle region of the corpus callosum in Mfn1 conditional Knockouts

Representative images of the dendritic specific microtubule associated marker MAP2 and the axon specific microtubule associated marker Tau 1 and the merge of both staining at the middle corpus callosum. Coronal sections of Mfn1^{Δ/Δ} and control littermate brains were co-stained with α tau-1 and MAP2 at postnatal day 9. Scale bar represents 125 microns (n=3).

Mfn1^{Δ/Δ} mice still survive over 6 months, but with significantly reduced body weight. Moreover, Mfn1^{Δ/Δ} mice showed no change in ki67 labeling during development, and no change in post-mitotic cortical neurons. Also, distribution of CuxI and Foxp2 in Mfn1^{Δ/Δ} mice was similar to control indicating that loss of Mfn1 did not affect cortical lamination. In contrast, we found a significant decrease in ki67 labeling postnatally suggesting a decrease in the pool of postnatal progenitor cells. Although fewer progenitor cells were present, there was no significant increase in apoptotic cell death. Finally, our preliminary results demonstrated that the axons are absent at the middle region of the corpus callosum of Mfn1^{Δ/Δ} mice.

B) Mitofusin 2

Cresyl Violet Staining of Mfn2 conditional Knockouts

Cresyl Violet staining showed that the overall brain structures of Mfn2^{Δ/Δ} mice such as the cortex, the CA1-CA3 region of the hippocampus, and the striatum were present and similar to the control, but the brain size was significantly reduced (n=3) (Fig 3.9). In contrast to the disruption seen in the middle corpus callosum of Mfn1^{Δ/Δ} mice, the corpus callosum in Mfn2^{Δ/Δ} mice was present and similar to control.

Fewer progenitor cells postnatally in Mfn2 conditional knockouts

To further characterize the phenotype of Mfn2^{Δ/Δ} mice, we evaluated whether the absence of Mfn2 in the telencephalon would affect the number of precursor cells during development and postnatally. Similarly to Mfn1, we quantified the positively labeled



Figure 3.9 Cresyl Violet Staining of Mfn2 conditional knockouts

Cresyl Violet Staining of $Mfn2^{\Delta/\Delta}$ mice at postnatal day 9 with respect to its controls $Foxg1Cre^{+/-}$ and $Mfn2^{\Delta/+}$ mice. Brains were cut coronally and stained with cresyl violet. Scale bars represent 1mm (n=3).

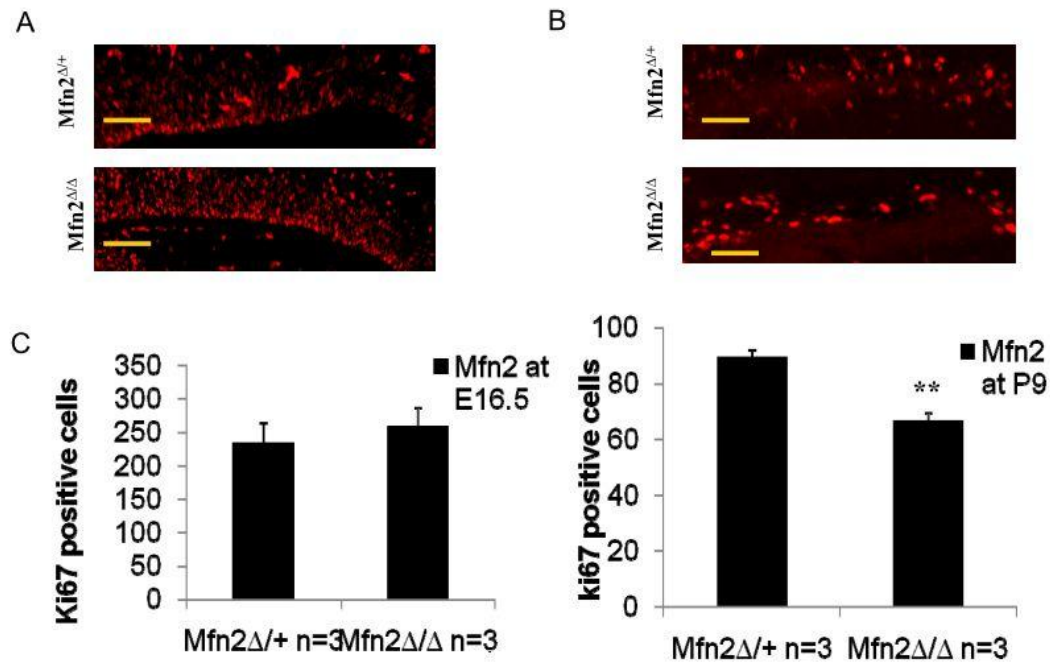


Figure 3.10 Less ki67 labeled cells in Mfn2 conditional knockouts at P9

- A. Coronal sections taken at embryonic day 16.5 of Mfn2 Δ/Δ mice and control littermates were stained with the proliferative marker Ki67. Scale bar represents 50 microns.
- B. Coronal sections of Mfn2 Δ/Δ mice and control littermates were stained with the proliferative marker Ki67 at postnatal day 9. Scale bar represents 50 microns.
- C. Graphs represent the quantification of Ki67 labeled cells along the ventricle at P9 and E16.5 for Mfn2 Δ/Δ mice and control littermates. Four different sections were taken per animal (n) and a total of n=3 were used for each genotype. Per genotype the average of 3 n was plotted and the error bars represent the standard error of the mean. (n=3, two tailed t- test, **P<0.001).

ki67 cells along the length of the dorsal ventricle. During development and at embryonic day E16.5, there was no significant change in ki67 positive cells in $Mfn2^{\Delta/\Delta}$ mice compared to controls. In $Mfn2^{\Delta/\Delta}$ mice, there was an average of 234 ± 29 ki67 labeled cells per dorsal ventricle length, and in $Mfn2^{\Delta/+}$ control mice there was an average of 260 ± 26 ($n=3$, $P>0.05$) (Fig 3.9 A, C). In contrast, at postnatal day 9, quantification of ki67 labeled cells along the dorsal surface of the lateral ventricle revealed a significant decrease in ki67 positive cells in $Mfn2^{\Delta/\Delta}$ mice compared to control (Fig 3.10 B, C). $Mfn2^{\Delta/\Delta}$ mice contained an average of 67 ± 3 ki67 positively labeled cells per dorsal ventricle compared to its controls $Mfn2^{\Delta/+}$ mice that contained 89 ± 3 ($n=3$, $P<0.001$) (Fig 3.10 C). Overall, our data demonstrated that the loss of Mfn2 in the telencephalon results in a significant decrease in progenitor cells postnatally.

Assessment of apoptotic cell death in Mfn2 conditional knockouts

Since Mfn2 has been shown to have an anti-apoptotic effect (Jahani-Asl et al., 2007; Neuspiel et al., 2005), and since we detected significantly less progenitor cells postnatally in $Mfn2^{\Delta/\Delta}$ mice, and a smaller brain size, we asked whether the loss of Mfn2 in the telencephalon would lead to cell death. $Mfn2^{\Delta/\Delta}$ brains were stained with the apoptotic marker active Caspase 3. Compared to its control, $Mfn2^{\Delta/\Delta}$ mice did not show any increased apoptotic cell death at postnatal day 9 (Fig 3.11). Therefore, our results reveal that similarly to what we saw in $Mfn1^{\Delta/\Delta}$ mice, the absence of Mfn2 from the telencephalon does not lead to increased apoptotic cell death.

Fewer cortical Neurons in Mfn2 conditional knockouts

Since Mfn2 brains do not exhibit a change in progenitor cell population during development, but have a significantly reduced brain size, we wanted to examine the role

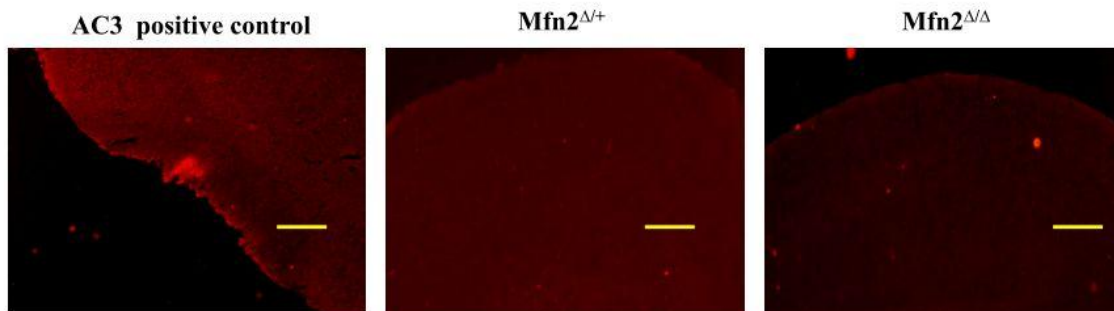


Figure 3.11 Active Caspase 3 in Mfn2 conditional knockouts

Coronal sections at postnatal day 9 of Mfn2^{Δ/Δ} mice and control littermates were stained with the apoptotic marker Active Caspase 3 (AC3). The positive control represents a region of a cortex after stroke where apoptosis occurred and is positive for Active Caspase 3. Scale bar represents 125 microns.

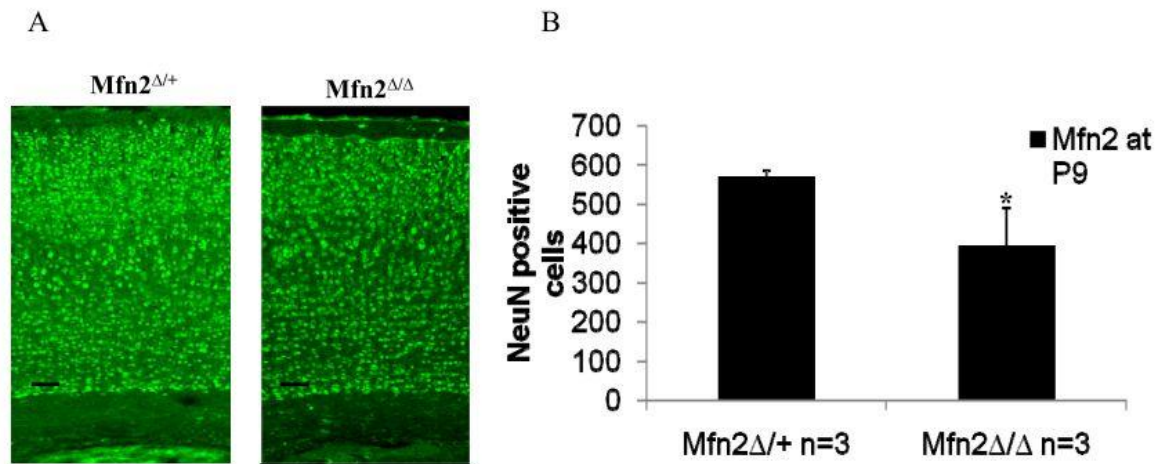


Figure 3.12 Decrease in number of neurons in the cortex of Mfn2 conditional knockouts

A. Coronal sections of Mfn2^{Δ/Δ} and control littermate brains were stained with the neuronal marker NeuN at postnatal day 9. Scale bar represents 250 microns

B. Graph represents the quantification of NeuN labeled cells in the cortex of Mfn2^{Δ/Δ} mice and control littermates. Four different sections were taken per animal (n) and a total of n=3 were used for each genotype. Per genotype the average of 3 n was plotted and the error bars represent the standard error of the mean. (n=3, two tailed t-test, *P<0.05)

of Mfn2 on the neuronal population in the cortex. Coronal sections from postnatal day 9 brains were stained with NeuN and the NeuN labeled cells were quantified with respect to the ventricle in the dorsal lateral boxed area of the cortex. Mfn2^{Δ/Δ} mice revealed a significant decrease in cortical neurons at postnatal day 9, whereby Mfn2^{Δ/Δ} mice contained an average of 395.3±95 NeuN positive cells compared to its control Mfn2^{Δ/+} mice that contained an average of 570±16 (n=3 P<0.05) (Fig3.12A, C). Thus, our data demonstrated that the loss of Mfn2 in the telencephalon results in a significant decrease in cortical neurons postnatally.

Distribution of CuxI and Foxp2 in the deep and superficial cortical layers of Mfn2 conditional knockouts

Since Mfn2^{Δ/Δ} mice showed a significant decrease in cortical neurons, we wanted to see if Mfn2 play a role in cortical lamination as well. To assess any changes in cortical lamination we stained with Foxp2 and CuxI (Ferland et al., 2003; Nieto et al., 2004). Our results showed no detectable difference in the distribution of CuxI and Foxp2 in the Mfn2^{Δ/Δ} mice compared to its controls (Fig 3.13 A, B) suggesting that Mfn2 is not required for cortical lamination.

Tau1 expression in the corpus callosum of Mfn2 conditional Knockouts

Mfn2 mutations have been associated with the CMT2A neurodegenerative disease, which results in axonal degeneration (Zuchner et al., 2004). For this reason, we wanted to investigate whether the loss of Mfn2 in the telencephalon might have an effect on the axons found in the corpus callosum. Co-staining with MAP2 and Tau1 revealed that both Mfn2^{Δ/Δ} and Mfn2^{Δ/+} mice express only Tau1 in the middle corpus callosum (n=3) (Fig3.14). Furthermore, the cresyl violet staining reveals that the connection between the left and

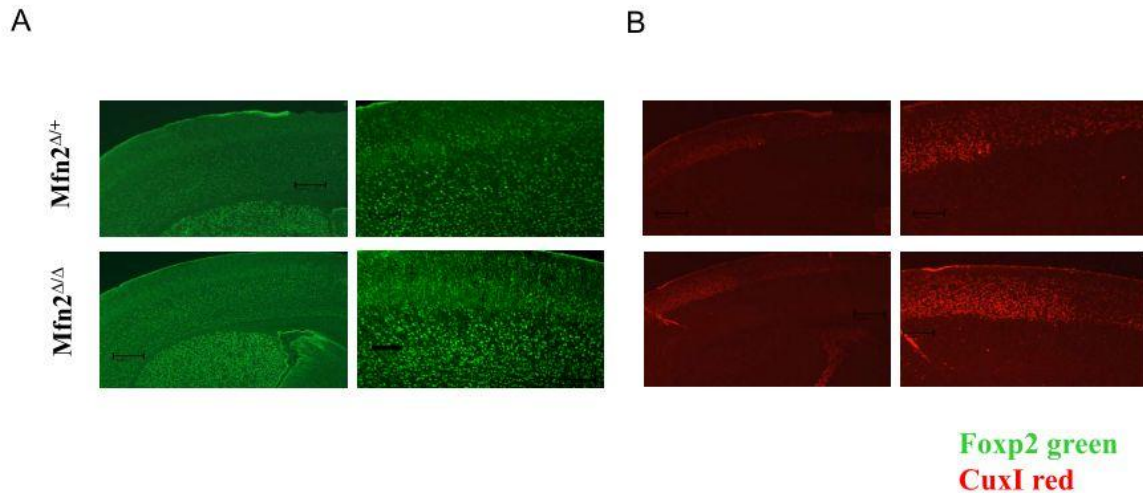


Figure 3.13 Distribution of CuxI and Foxp2 in the deep and superficial layers of Mfn2 conditional knockouts

A. Representative images of the deep layers of the cortex in $Mfn2^{\Delta/\Delta}$ mice and control littermates. Coronal sections were stained at postnatal day 9 with the deep layer marker Foxp2. Scale bar represents 250 microns.

B. Representative images of the superficial layers of the cortex in $Mfn2^{\Delta/\Delta}$ mice and control littermates. Coronal sections were stained at postnatal day 9 with the superficial layer marker CuxI. Scale bar represents 250 microns.

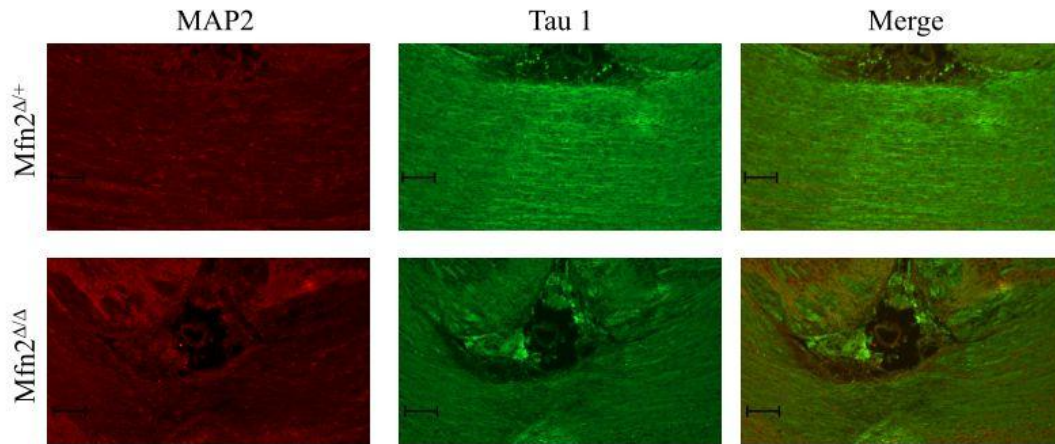


Figure 3.14 Tau1 was expressed in corpus callosum in Mfn2 conditional Knockouts

Representative images of the dendritic specific microtubule associated marker MAP2 and the axon specific microtubule associated marker Tau 1 and the merge of both staining at the middle corpus callosum. Coronal sections of Mfn2 Δ/Δ mice and control littermate brains were co-stained with α tau-1 and MAP2 at postnatal day 9. Scale bar represents 125 microns.

right hemisphere is still present in the $Mfn2^{\Delta/\Delta}$ mice. Thus our results show that axons are not disrupted in the corpus callosum in $Mfn2^{\Delta/\Delta}$ mice.

Overall, our results demonstrated that $Mfn2^{\Delta/\Delta}$ mice die between P9 and P12 and have a significantly reduced body weight. Their brain gross morphology appeared normal, albeit reduced in size. Quantifying ki67 labeled cells showed a significant decrease in progenitor cells postnatally. Even though there was no significant change in neural precursor cells during development, we did observe a significant decrease in cortical neurons. However, active Caspase 3 staining did not show any increase in apoptotic cell death. Furthermore, there was no detectable change in CuxI and Foxp2 distribution suggesting that $Mfn2$ does not play a role in cortical lamination. Finally, our data showed that the axons are not disrupted along the whole corpus callosum in $Mfn2^{\Delta/\Delta}$ mice.

Comparison of $Mfn1^{\Delta/\Delta}$ and $Mfn2^{\Delta/\Delta}$ mice

Our data has shown that both $Mfn1^{\Delta/\Delta}$ and $Mfn2^{\Delta/\Delta}$ mice have a significantly reduced body weight. $Mfn1^{\Delta/\Delta}$ mice survive over 6 months, but $Mfn2^{\Delta/\Delta}$ mice die between P9 and P12. When comparing morphology, our preliminary data of $Mfn1^{\Delta/\Delta}$ mice show a defect in the development of the corpus callosum at the midline whereas $Mfn2^{\Delta/\Delta}$ mice had relatively normal brain morphology although the brain was reduced in size. In both conditional knockouts the overall gross morphology and the different brain structures such as the cortex, hippocampus and striatum appear to be similar to their controls. Furthermore, our results have shown that both $Mfn1^{\Delta/\Delta}$ and $Mfn2^{\Delta/\Delta}$ mice have significantly less neural precursor cells postnatally, but not during development. Moreover, $Mfn2^{\Delta/\Delta}$ mice show a significant decrease in number of cortical neurons whereas no change was detected in $Mfn1^{\Delta/\Delta}$ mice. Also, no increase in apoptosis was

detected in both Mfn1^{Δ/Δ} mice and Mfn2^{Δ/Δ} mice. Finally, our results suggest that Mfn1 may play a role in axonal maintenance in the corpus callosum, whereas no such defect was detected with Mfn2 deficient brains.

Chapter IV. Discussion

Evidence has accumulated to demonstrate the role of Mfn1 and Mfn2 in regulating mitochondrial fusion and their importance during embryonic development (Chen et al., 2003). Furthermore, data implicating Mfn2 in cerebellar development has been presented where the absence of Mfn2 in the cerebellum has been associated with Purkinje cell degeneration (Chen et al., 2007). This led me to hypothesize that Mfn1 and Mfn2 will play a role in the development of other brain structures such as the telencephalon. The results presented in this thesis describe a requirement for Mfn1 and Mfn2 in survival and brain morphology as well as in the regulation of progenitor cell population and post mitotic cortical neurons. Through the generation of conditional knockouts for Mfn1 and Mfn2 in the telencephalon, I have described a novel requirement for Mfn1 in axonal maintenance in the corpus callosum and in the regulation of the population of postnatal neural progenitor cells. I have also described the requirement of Mfn2 for survival, regulation of postnatal progenitor cells and post-mitotic cortical neurons. The results presented in this thesis support the following conclusions: 1) Mfn1 may be required for maintaining axons in the middle region of the corpus callosum 2) deletion of Mfn1 from the telencephalon results in fewer postnatal progenitor cells; 3) Mfn2 is required for survival; 4) deletion of Mfn2 from the telencephalon results in fewer postnatal progenitor cells and post-mitotic cortical neurons. Thus, these findings highlight the importance of Mfn1 and Mfn2 in telencephalic development.

It has previously been shown that when Mfn1 and Mfn2 are conditionally knocked out in the cerebellum, one third of the Mfn2 deficient pups die at P1 (Chen et al., 2007). The

surviving pups show severe defects in movement, balance, as well as a smaller cerebellum with normal lobular organization and layer formation, while Mfn1 deficient pups are normal by these criteria (Chen et al., 2007). This clearly demonstrated that Mfn2 plays a role in the cerebellar development. Our results showed that when Mfn1 and Mfn2 were conditionally knocked out from the telencephalon, the Mfn2^{Δ/Δ} mice died between P9-P12 with a smaller overall brain structure while Mfn1^{Δ/Δ} mice survive over 6 months with a defect in the corpus callosum. Furthermore, around postnatal day 22, we noticed that the Mfn1^{Δ/Δ} mice exhibited slow movement as well as a slight head tilt to the right. Therefore, our results demonstrate the requirement of Mfn2 for survival and the role of Mfn1 and Mfn2 in telencephalic development.

Mitofusins in corpus callosum development

The corpus callosum is a large bundle of axons that transmits information between the left and right cerebral hemispheres. The axons of the corpus callosum cross from one hemisphere to another via a tissue bridge structure formed at the midline region (Wahlsten et al., 2006). The neurons in the cerebral cortex that have axons that pass through the corpus callosum are the neurons of the prefrontal cortex, motor cortex as well as somatosensory cortex (Lomber et al., 1994; Silver and Ogawa, 1983). In order for the axons of the corpus callosum to cross, the axons must arrive at the right time (Wahlsten et al., 2006). There are several possibilities that might cause a defect in the development of the corpus callosum at the midline. For instance, if there is a delay or alteration in axon growth or migration, or if the tissue bridge is not formed properly, then a defect in the axons or structure of the corpus callosum might occur (Wahlsten et al., 2006). During development, following telencephalic formation, midline patterning must take place

before the corpus callosum develops (Richards et al., 2004). Midline fusion happens around E14-E15 (Richards et al., 2004). This fusion process is important for the development of the corpus callosum as it is essential for axon growth and extension across the midline (Richards et al., 2004). Callosal development involves several steps. First, neurons within the cortical layers II, III, and V send their axonal projections ventrally toward the intermediate zone with the help of certain axon guidance factors such as Sem3A (Polleux et al., 1998). Then, these axons project in the direction of the midline instead of continuing through the lateral tract (Garcez et al., 2007). At the corticoseptal boundary, the axons move toward the midline in a sharp ventral pathway and rapidly turn as they cross the midline (Richards et al., 2004). At the corticoseptal boundary, there are certain glial structures identified as the glial wedges, and these structures are also involved in proper axon guidance of the callosal axons at the midline (Richards et al., 2004). As the axons reach the end of the midline, they come across another glial wedge on the other side, where they make a dorsal turn and go through the contra lateral cingulate cortex to finally reach the contralateral neocortex (Richards et al., 2004). At the contralateral neocortex, the axons locate their destination (Richards et al., 2004). One hypothesis is that in these axons the absence of Mfn1 decreases mitochondrial fusion and thus insufficient energy is supplied to these axons to complete their development. Our results provide preliminary evidence that, in the Mfn1 conditional knockouts, a loss of the corpus callosum was observed. However, to show that the axonal loss is specific to the corpus callosum, different regions of the brain should be studied. For instance, a comparison of the cerebral peduncle cross sectional area in the control and Mfn1^{Δ/Δ} of midbrain sections should be studied.

The absence of the axonal marker tau1 in the middle region of the corpus callosum suggested that axons may be absent in this region. Even though all three control littermates had a corpus callosum while none of the three Mfn1 conditional knockouts had an intact corpus callosum, however further evidence and in depth analysis should be added in order to confirm these preliminary findings. Therefore our results provide preliminary evidence that Mfn1 may play a role in axonal maintenance or callosal agenesis within the corpus callosum. It should be noted that it has been shown that in certain inbred strains such as the BALB/cWah1 and 129P1/Rej the corpus callosum is absent (Wahlsten et al., 2006). This suggests that genetic background could contribute to the defect seen in Mfn1 conditional knockouts.

In contrast, our results show that we still retain the corpus callosum in Mfn2 conditional knockouts. It should be noted that previously published data by Misko et al showed that Mfn2 is important for mitochondrial axonal transport and Mfn2 interacts with the Miro/Milton complex (Misko et al., 2010). The absence of Mfn2 from the dorsal root ganglion primary cultures caused a delay in axonal migration (Misko et al., 2010). Furthermore, this group showed that the loss of Mfn2 alone was sufficient to cause a delay in axonal migration, and the defect was restored by the expression of Mfn1 or Mfn2. Thus Mfn1 was able to compensate for the loss of Mfn2 in these primary cultures (Misko et al., 2010). However, our results demonstrated that in Mfn2^{ΔΔ} mice, the axons were still present in the corpus callosum. One explanation is that Mfn1 can be compensating for the absence of Mfn2, but in the corpus callosum Mfn2 is not able to compensate for Mfn1 suggesting an additional role for Mfn1 other than fusion in maintaining the axons of the corpus callosum and is not shared by Mfn2.

Mitofusins in regulation of postnatal progenitor cells

During development, progenitor cells in the ventricular zone commit to a neuronal fate, undergo terminal mitosis and migrate into the cortical plate where they undergo differentiation to become a mature neuron (Kandel and Schwartz, 2000; Tramontin et al., 2003). Furthermore, neurogenesis persists postnatally in the lateral ventricle and subgranule zone of the dentate gyrus in rodent brains (Altman and Das, 1965; Lois and Alvarez-Buylla, 1994). The postnatal precursor cells found in the subventricular zone generate immature neurons that migrate along the rostral migratory stream to the olfactory bulb where they differentiate into mature interneurons (Doetsch and Alvarez-Buylla, 1996; Vescovi et al., 2006). Moreover, the postnatal progenitor cells can also generate oligodendrocytes that migrate towards certain brain structures, such as the cortex, to complete the development of glial cells that had initially began during development (Levison and Goldman, 1993; Suzuki and Goldman, 2003). Our results show that the loss of both Mfn1 and Mfn2 from the telencephalon does not cause a change in the pool of progenitor cells during development (Fig3.4A, C and Fig 3.10A, C). In contrast, we see a decrease in the pool of progenitor cells postnatally (Fig3.4B, C and Fig 3.10B, C). Mfn1 and Mfn2 are fusion proteins and fusion can act as a quality control by allowing rapid mixing of membranes, mitochondrial DNA, and soluble contents that may have been damaged (Chan, 2006; Chen et al., 2005). Thus, when the fusion of two mitochondria occurs, each mitochondrion will complement the other to restore its normal function (Westermann, 2010). Therefore, our results suggest that the loss of Mfn1 and Mfn2 postnatally may affect the role fusion plays in quality control and this may affect the rate of cell division in neuronal progenitor cells leading to fewer progenitors postnatally.

Mitofusins in regulation of post-mitotic cortical neurons

During cortical development progenitor cells in the ventricular zone commit to a neuronal fate, and migrate into the cortical plate where they undergo differentiation to become neurons (Kandel and Schwartz, 2000; Tramontin et al., 2003). Since the pool of progenitor cells during development was not altered in Mfn1^{ΔΔ} mice and Mfn2^{ΔΔ} mice, we did not expect to see any difference in number of post-mitotic cortical neurons. Consistent with this, our results showed no difference in the number of post-mitotic cortical neurons in Mfn1^{ΔΔ} mice. However, quantification in Mfn2^{ΔΔ} mice showed a significant decrease in the number of cortical neurons. Thus, several explanations can arise. During development, neuronal progenitor cells commit to a neuronal fate, undergo terminal mitosis and migrate to their final destination where they undergo differentiation (Kandel and Schwartz, 2000; Tramontin et al., 2003). The pool of neuronal progenitor cells was the same during development, this implies that these cortical neurons are differentiating and becoming neurons, however they are not surviving. Surprisingly when we stained with the apoptotic marker active Caspase 3 we did not detect any increase in cell death with respect to the control. This could be explained in two ways: first, cell death may have occurred earlier than postnatal day 9, and was not detected in my studies. Secondly, post-mitotic cortical neurons may not be dying by apoptosis and may explain why cell death escaped detection by active Caspase3. Therefore, post-mitotic cortical neurons may have been dying by autophagy. Autophagy is a catabolic process that is activated when cells are starved in order to recycle nutrients that are necessary for survival (He and Klionsky, 2009; Mehrpour et al., 2010). Basal level autophagy is important for post-mitotic neurons to prevent the accumulation of damaged organelles and proteins (Germain and Slack, 2010). Furthermore, continued induction of autophagy

above basal levels could lead to cell death (Germain and Slack, 2010). Therefore, the loss of Mfn2 is affecting fusion, and consequently more mitochondria should be cleared by autophagy. Thus, autophagy is induced at higher levels leading to cell death. Furthermore, while our results showed a decrease in post-mitotic cortical neurons in Mfn2^{Δ/Δ} mice and not in Mfn1^{Δ/Δ} mice, this suggests that Mfn2 may be compensating for the loss of Mfn1 in Mfn1^{Δ/Δ} brains. Thus, our data suggests that Mfn2 may have an additional role in regulating post-mitotic cortical neurons.

Mfn1 and Mfn2 in cell death

When apoptosis is induced, Bax and Bak translocate to the outer membrane of the mitochondria and oligomerize (Saikumar et al., 1998; Wolter et al., 1997). It has been previously shown that Mfn2 co-localizes with Drp1 and Bax on mitochondrial foci sites (Karbowski et al., 2002), where Bax inhibited the activity of Mfn2 (Karbowski et al., 2002; Karbowski et al., 2006). This suggests that the decrease in Mfn2 expression should cause more apoptosis. Moreover, Mfn2 has been shown to have an anti-apoptotic effect in cerebellar granular neurons (Jahani-Asl et al., 2007; Neuspiel et al., 2005). When cerebellar granular neurons were cultured and infected with an Mfn2-antisense adenovirus, the loss of Mfn2 induced cell death (Jahani-Asl et al., 2007). In correlation with the *in vitro* studies performed by Jahani-Asl et al, our *in vivo* studies showed that the loss of Mfn2 from the telencephalon led to early death (P9-P12) of Mfn2^{Δ/Δ} pups. Furthermore, conditionally knocking out Mfn2 from the cerebellum led to degeneration of Purkinje cells and apoptosis (Chen et al., 2007). However, our results showed that staining with active Caspase 3 at postnatal day 9 did not reveal increased apoptotic cell death Mfn2^{Δ/Δ} mice. One possibility is that by postnatal day 9, Mfn2^{Δ/Δ} mice are already

dying; therefore apoptosis may have occurred earlier. To further explore this would be to look at apoptosis in $Mfn2^{\Delta/\Delta}$ mice before postnatal day 9.

Mitofusins and Mitochondrial function

Mitochondrial fusion plays a role in development and survival (Chan, 2006; Chen et al., 2005). Fusion of mitochondria serves as a protective mechanism to injury and prevents mitochondrial dysfunction by allowing rapid mixing of membranes, mitochondrial DNA, and soluble contents that may have been damaged during cellular stress (Chan, 2006; Chen et al., 2005). Furthermore, fusion is required for mtDNA stability. Experiments using double mutant $Mfn1^{-/-} Mfn2^{-/-}$ MEFs showed fewer copies of mtDNA per nuclear genome compared to their wild type control (Chen et al., 2010). The re-introduction of either $Mfn1$ or $Mfn2$ into $Mfn1^{-/-} Mfn2^{-/-}$ MEFs restored the mtDNA to wild type levels. This suggests that in the absence of fusion there is less mixing of mitochondrial contents which could explain the loss of mtDNA (Chen et al., 2010). Our results have demonstrated that the deletion of either $Mfn1$ and $Mfn2$ from the telencephalon led to a decrease in the pool of postnatal neuronal progenitor cells and the loss of $Mfn2$ resulted in a decrease in the number of post-mitotic cortical neurons. This suggests that the absence of $Mfn1$ or $Mfn2$ would result in less mitochondrial fusion resulting in increase in mitochondrial DNA instability. One consequence of this may be that neuronal progenitor cells and post-mitotic cortical neurons are not able to eliminate mtDNA mutations and these mutations and instabilities are retained. Moreover, the loss of either mitofusins can affect other mitochondrial functions such as energy production. The absence of mitochondrial fusion proteins would lead to less fusion and thus a decrease in the energy supply which is required for proper development. When fusion decreases,

mitochondrial length, and mitochondrial surface area decreases (Jahani-Asl et al., 2007), thus less oxygen, substrates and ADP are available for cells. Therefore, energy production decreases and less energy is available for cells to survive and perform the necessary biochemical processes. Furthermore, mitochondria undergo constant fission and fusion, and are in constant equilibrium. Therefore if fusion is decreased we would have more fragmented mitochondria due to an imbalance between fission and fusion which would cause alterations in mitochondrial functions and ultimately lead to cell death.

Implication of Mitofusins in neurodegeneration

A large amount of information has accumulated to demonstrate the association of mitochondrial fusion proteins with neurodegeneration. For example mutations in Mfn2 are associated with the CMT2A disease (Zuchner et al., 2004). Furthermore, Mfn2 has been shown to protect against injury induced neuronal cell death (Jahani-Asl et al., 2007). The results in this thesis describe the requirement of Mfn1 and Mfn2 in regulation of postnatal neuronal progenitor cells and the requirement of Mfn2 in the regulation of post-mitotic cortical neurons. Our data suggests that Mfn1 and Mfn2 play a role in telencephalic development. Thus, our results brought us a step forward in understanding the function of Mfn1 and Mfn2 and potentially use these mitochondrial fusion proteins as a therapeutic target for neurodegenerative disease. Although Mfn1 has not been directly associated with neurodegenerative diseases, understanding its role during development and comparing its function and role to Mfn2, makes Mfn1 an attractive candidate for pharmacological manipulation. Since Mfn1 is able to compensate for the loss of Mfn2, one approach would be to augment the expression of Mfn1 to compensate for the

mutations in Mfn2, and this could be a valid therapeutic strategy for treatment of neurodegenerative diseases.

Are Mfn1 and Mfn2 redundant or do they have separate roles?

Even though Mfn1 and Mfn2 are similar proteins with overlapping functions, differences exist. For instance, both Mfn1 and Mfn2 mediate fusion, but Mfn1 alone interacts with Opa1 (Cipolat et al., 2004). Moreover, Mfn2 has been shown to be located on the endoplasmic reticulum and can tether the ER and mitochondria (de Brito and Scorrano, 2008). The loss of Mfn2 in the dorsal root ganglion neurons caused mitochondrial axonal transport deficiency and consequently defects in axonal migration (Misko et al., 2010). The over expression of either Mfn1 or Mfn2 in dorsal root ganglion neurons deficient of Mfn2 was able to restore the defect (Misko et al., 2010). Furthermore, the loss of Mfn2 but not Mfn1 in the cerebellum led to Purkinje cell degeneration (Chen et al., 2007). However, the over expression of Mfn1 or Mfn2 in Mfn2 null Purkinje cells rescued these cells (Chen et al., 2007). The differences that exist could be explained by the possibility that Mfn1 and Mfn2 are expressed differently in different tissues, and it is the total complement of Mfn1 and Mfn2 that controls the level of mitochondrial fusion (Detmer and Chan, 2007). Another explanation is that Mfn1 and Mfn2 may have distinct functions. Our results show that the loss of Mfn2 in Mfn2^{ΔΔ} mice resulted in a decrease in cortical neurons, but the loss of Mfn1 in Mfn2^{ΔΔ} mice did not affect the number of cortical neurons. This suggests that Mfn2 in Mfn1^{ΔΔ} mice may be compensating for the loss of Mfn1 and that Mfn2 only plays a role in the regulation of post-mitotic cortical neurons. On the other hand, the loss of axons in the corpus callosum in Mfn1^{ΔΔ} mice only suggests that Mfn1 has an additional role other than fusion in maintaining the axons of

the corpus callosum at the midline and is not shared by Mfn2. In summary, Mfn1 and Mfn2 may be redundant in certain tissues, but essential in others and both mitofusins may have common functions as well as functions independent from each other.

Future directions

Our results have shown that Mfn1 and Mfn2 play a role in regulation of postnatal progenitor cells, and Mfn2 has been shown to maintain post-mitotic cortical neurons. In this thesis we generated Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} mice where Mfn1 and Mfn2 were deleted from the telencephalon by E8. Another *in vivo* approach would be to generate conditional knockouts, where Mfn1 and Mfn2 are deleted from the telencephalon postnatally. This approach will allow Mfn1 and Mfn2 to be expressed during development, and will help us better understand the role of Mfn1 and Mfn2 postnatally. Therefore, we can cross floxed Mfn1 and floxed Mfn2 mice with CAMII kinase Cre, a Cre expressed in the neurons of the cortex, hippocampus, and striatum by postnatal day 1 (Casanova et al., 2001). Mfn1 and Mfn2 will be deleted from the telencephalon postnatally. Another *in vivo* approach would be to cross floxed Mfn1 and floxed Mfn2 with transgenic mice carrying an inducible CreER. The inducible CreER has a Cre fused to mutated estrogen receptors and this receptor will not be able to bind the endogenous estrogen, but can bind synthetic tamoxifen ligands (Kuhn et al., 1995; Lewandoski, 2001). Therefore, treatment with tamoxifen injections will cause a dose-dependent excision of the floxed site, and this will inactivate Mfn1 or Mfn2. Thus, Mfn1 and Mfn2 can be inactivated during adulthood. Therefore, these two *in vivo* approaches will help further characterize the phenotype of Mfn1 and Mfn2 in the telencephalon postnatally.

Another approach would be to explore Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} *in vitro* to better understand the mechanism by which Mfn1 and Mfn2 function. Mfn1 and Mfn2 have been implicated to play a role in mitochondrial fusion. Thus, we can explore and study the mitochondrial morphology and the effect the complete loss of Mfn1 or Mfn2 may have on mitochondrial fusion in neurons. This can be done by culturing primary cortical neurons of Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} mice. Moreover, by re-infecting with an Mfn1 or Mfn2 adenovirus, we can investigate whether the mitochondrial morphology is restored, and how fusion might be affected. Furthermore, Mfn2 has been shown to be involved in tethering of mitochondria and ER (de Brito and Scorrano, 2008). Mfn2 is also located to the endoplasmic reticulum, and can tether the ER and mitochondria by homo or hetero dimers with Mfn2 or Mfn1 on the mitochondria (de Brito and Scorrano, 2008). One question that should be addressed is how Mfn2 at the ER and Mfn2 at the mitochondria affect mitochondrial function, and what their role is in neuronal survival. Finally, our results showed that the loss of Mfn1 and Mfn2 affected the pool of progenitor cells, and the loss of Mfn2 resulted in a decrease in post-mitotic cortical neurons. However, when we stained with active Caspase 3, we did not observe any increase in apoptotic cell death. Thus, another approach would be to look at Mfn1^{Δ/Δ} and Mfn2^{Δ/Δ} brains earlier than postnatal day 9 to see if cell death is occurring earlier.

Conclusion

The data presented in this thesis revealed a role for Mfn1 in the regulation of postnatal progenitor cells. Our data has also shown the requirement of Mfn2 for maintenance of postnatal progenitor cells as well as post-mitotic cortical neurons. In summary,

characterizing Mfn1 and Mfn2 conditional knockouts has revealed the requirement of both proteins for proper telencephalic development.

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