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Mcl-1 is a Key Regulator of Apoptosis in Neural Precursor Cells and Autophagy in Post-mitotic Neurons

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Mcl-1 is a Key Regulator of Apoptosis in Neural Precursor Cells and Autophagy in Post-mitotic Neurons

By: Jaclyn Nicole Le Grand

A report submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Master of Science

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Table of Contents

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Abstract

Unregulated neuronal cell death has been implicated in the pathogenesis of several neurological disorders and in acute neuronal injury such as stroke. Mcl-1, an anti-apoptotic member of the Bcl-2 family, is a known survival factor for hematopoietic cells, however little is known regarding its function in the nervous system. In this study, we examined the role of Mcl-1 in regulation of programmed cell death throughout nervous system development and in maintaining mature neurons. We found that both Nestin: Cre Mcl-1 and Foxg1: Cre Mcl-1 conditional knockout mice were embryonic lethal. Morphological analysis and immunohistochemical staining revealed severe deterioration of the cortices and an apoptotic phenotype, suggesting that Mcl-1 is required for embryonic neuronal survival. Both neural progenitors and newly differentiated neurons were affected, revealing that neurons were dying throughout the process of differentiation. Deletion of Mcl-1 in post mitotic neurons in postnatal mice with CamKIIα Cre also resulted in premature lethality. Cresyl Violet staining and NeuN immunohistochemistry revealed a rapid loss of neurons in the cortices of mutants. Electron micrographic imaging revealed double membraned vesicles within the cortical neurons, suggestive of an autophagic form of cell death. Consistent with this hypothesis, an upregulation of LC3 was observed in primary cortical neurons deficient for Mcl-1. Altogether our findings demonstrate that the loss of Mcl-1 in embryonic and post-mitotic neurons results in cell death. The two distinct forms of cell death activated indicate that Mcl-1 functions in multiple pathways to promote neuronal survival. In summary, we demonstrate that Mcl-1 is vital for the survival of neurons.

List of Tables

List of Figures

List of Abbreviations

A1	Bcl-2-related protein A1a
AC3	Active Caspase 3
Ad	Adenovirus
ADP	adenosine diphosphate
Akt	serine/threonin kinase
AMP	adenosine monophosphate
ANT	adenine nucleotide translocator
Apaf-1	Apoptotic protease activating factor 1
Atg	autophagy-related gene
ATP	adenosine triphosphate
AV	autophagic vacuole
Bad	Bcl-2/Bcl-xL-associated death promoter
Bak	Bcl-2-antagonist/killer-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-w	Bcl-weak
Bcl-x	Bcl-2-like X
Bcl-xL	Bcl-x Long
BF-1	Brain-Factor-1
BH domains	Bcl-2 homology domain
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting domain death agonist

Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator of cell death
Bmf	Bcl-2 modifying factor
Bok	Bcl-2-related ovarian killer
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CamKIIα	Calcium-Calmodulin Kinase IIα
Caspase	cysteine-aspartic acid protease
Cdk1	Cyclin-dependent kinase 1
CNS	central nervous system
Cp	cortical plate
Cre	Cre recombinase
cypD	cyclophilin D
cyt c	cytochrome c
Diva	(Bcl-2-like 10)
DNA	deoxyribonucleic acid
DP5	death protein 5
DRAM	damage-regulated autophagy modulator
E	embryonic day
E2F-1	Adenovirus E2 promoter binding factor-1
EDTA	ethylenediamine tetra-acetic acide
ERK	extracellular signal-regulated kinase
FADD	fas-associated death domain
FBS	fetal bovine sercum

FITC	Fluorescein isothiocyanate
fl	floxed
Foxg1	Forkhead box G1B
G2 phase	third subphase of interphase of cell cycle
GFP	green fluorescent protein
Hif-1	Hypoxia-inducible factor-1
HOPS	homotypic fusion and vacuolar protein sorting
HRE	hairpin response element
Hrk	Harakiri
hrs	hours
Iz	intermediate zone
JAK	Janus kinases
kD	kilo-dalton
LC3	microtubule-associated protein 1 light chain 3
M phase	mitosis phase of cell cycle
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia-1
min	minutes
mL	milliliters
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeability
MPTP	mitochondrial permeability transition pore
mRNA	mitochondrial ribonucleic acid
mTOR	mammalian target of rapamycin

MULE	Mcl-1 ubiquitin ligase E3
N-Bak	neuronal-Bcl-2-antagonist/killer-1
N-terminus	amino-terminus
NeuN	Neuronal Nuclei
NGF	nerve growth factor
Nip3	nineteen kD interacting protein-3
Noxa	(phorbol-12-myristate-13-acetate-induced protein 1)
P	postnatal day
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PE	phosphatidyl-ethanolamine
pen	penicillin
pH	potential of hydrogen
PH3	phosphohistone H3
PI3K	phosphoinositide 3-kinase
PNS	peripheral nervous system
PSA-NCAM	polysialic acid- neural cell adhesion molecule
PTP	permeability transition pore
Puma	Bcl-2 binding component 3
Rpm	rotations per minute
RT-PCR	reverse transcription-polymerase chain reaction
sec	seconds
SEM	standard error of mean

siRNA	small interfering ribonucleic acid
SNARE	soluble NSF attachment receptor
STAT	Signal Transducers and Activators of Transcription
strep	streptomycin
tBid	truncated Bid
TE	Tris-EDTA
TEM	transmission electron microscopy
TM	trans-membrane
TPA	12-O-tetradecanoylphorbol-13-acetate
µL	micro-liter
µm	micro-meter
µM	micro-molar
UV	ultraviolet
VDAC	voltage-dependent anion channel
VPS	vacuolar protein sorting
VZ	ventricular zone

Chapter I: Introduction

Regulation of programmed cell death is essential for the proper development and maintenance of tissue homeostasis. In the healthy mammalian nervous system, programmed cell death occurs throughout the developing embryo and in the adult nervous system. During embryonic development, apoptosis plays a major role in determining neuronal subpopulation sizes via elimination of excess neurons which fail to make synapses or synapse plays a roll in many neurodevelopmental pathologies such as schizophrenia (Jarskog et al. 2005) and Down's syndrome (Seidl et al. 1999, Helguera et al. 2005). Cell death is also important in the maintenance of the adult central nervous system, in which an increase in cell death is the underlining cause of an assortment of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease or excitotoxic death following stroke injury (Jellinger 2001, Lo et al. 2003).

Cell death can be classified into three different types based on their different morphological features; type 1, also termed apoptosis, characterized by chromatin condensation, cell rounding and the formation of membrane bound apoptotic bodies, type 2 autophagic cell death, characterized by the presence of autophagic vacuoles and type 3 programmed cell death or programmed necrosis which includes swelling of intracellular organelles without chromatin condensation (for review see Yuan et al. 2003). Of the three, the process of apoptosis has been firmly implicated in both neurodevelopmental cell death and in numerous cases of premature neuronal death of the adult central nervous system (De Zio et al. 2005). Given the prominent role of apoptosis in these processes, the molecules controlling this pathway, such as the Bcl-2 protein family, have been the center of intense scientific research.

One of these proteins, an antiapoptotic member of the Bcl-2 family termed Mcl-1 is of particular interest due to its apical role as a sensor for apoptotic stimuli and has therefore been the focus of this thesis. Throughout this thesis I will describe the role of Mcl-1 in the developing nervous system via the use of a nervous system-specific conditional knockout of Mcl-1 as well as provide evidence that Mcl-1

is required for neuronal survival both during development and in the mature central nervous system. In addition to its role in apoptosis, a novel role for Mcl-1 in the regulation of autophagy will also be examined.

This chapter will provide an overview of apoptosis with a detailed review of the Bcl-2 family and their implication in neuronal apoptosis. The process of autophagy, as it is implicated in neuronal cell death, and the crosstalk between these two pathways will be explored. Lastly, a review of the literature available on the proapoptotic Bcl-2 family protein, Mcl-1 will also be discussed.

1: Apoptosis

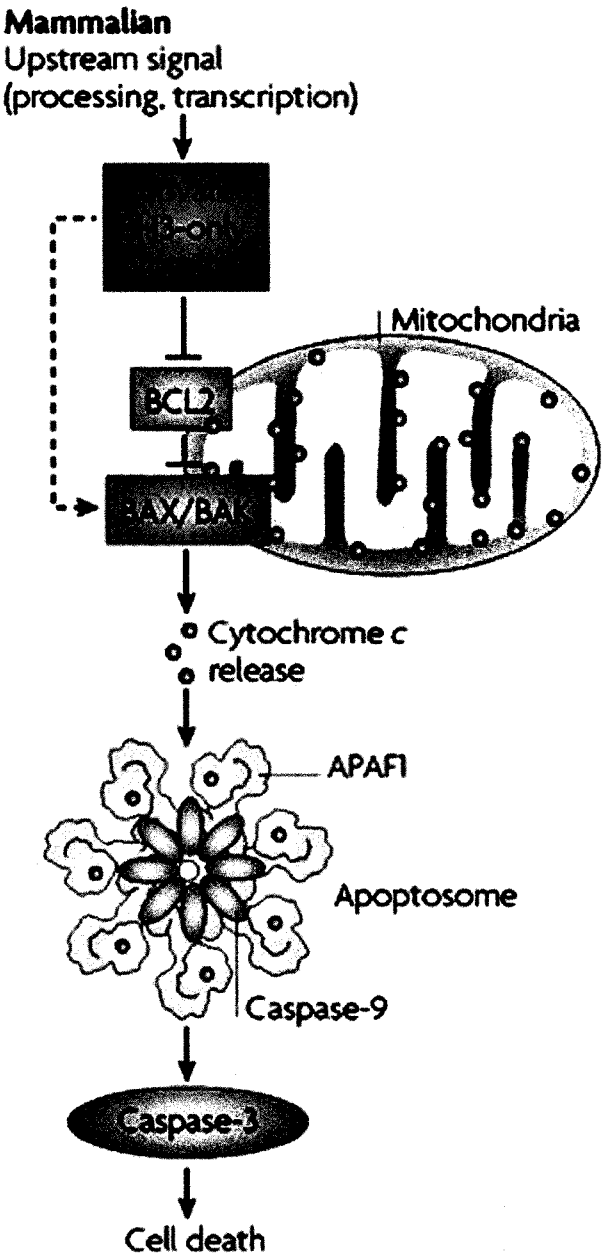
The term apoptosis, coined by Kerr in 1972 (Kerr et al. 1972), is used to describe a type of programmed cell death in which the cell undergoes a series of biochemical events leading to hallmark physical changes. These changes include cellular shrinkage, DNA fragmentation and chromatin condensation, membrane blebbing, the collapse of the nucleus and finally the formation of apoptotic bodies which are phagocytosed by macrophages or other neighboring cells to inhibit an inflammatory response (Häcker 2000).

The mammalian apoptotic pathway typically includes an upstream death initiation signal (such as UV damage or NGF deprivation in the case of sympathetic neurons), which causes a tip of the rheostat held closely in check by the Bcl-2 family in favor of programmed cell death. Signaling proteins such as cytochrome c are subsequently released from the mitochondria. These molecules ultimately activate the executioner proteins, the caspases, which are responsible for the physiological changes the apoptotic cell undergoes **(Figure 1-1)** (for a general review see Nika et al. 2004, Degterev et al. 2008).

Initiation of apoptosis occurs through two main pathways; the extrinsic pathway or the death receptor pathway and the intrinsic pathway or the mitochondrial pathway. These pathways are initiated

Figure 1-1: Overview of basic apoptotic machinery in mammalian cells.

An upstream signaling cascade activates B-cell lymphoma proteins-2 (Bcl-2) homology-3 (BH3)-only proteins to negatively regulate the antiapoptotic Bcl-2 proteins, releasing proapoptotic Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer-1 (Bak). Bax and Bak cause permeabilization of the outer mitochondrial membrane, releasing apoptogenic factors such as cytochrome c to form the apoptosome and activate the upstream caspases (such as caspase-9) which in turn activate the executioner caspases (such as caspase 3 or 7). Dotted line indicates the possible direct activation of Bax and Bak by the BH3-only proteins.



Adapted from Degterev et al. 2008. Nat Rev Mol Cell Biol. 9 (5): 378-390.

through death receptor activation at the cell surface (Ashkenazi and Dixit 1998) and stress factors such as DNA damage, endoplasmic reticulum stress, hypoxia, and growth factor deprivation (Jin and El-Deiry 2005), respectively. The focus of this thesis will be on the intrinsic apoptotic pathway and its regulation through the Bcl-2 family proteins.

The release of cytochrome c within the intrinsic apoptotic pathway is under the control of a family of proteins called the Bcl-2 protein family, of which each member contains at least one of the four described Bcl-2 homology (BH) domains (reviewed in Kelekar and Thompson 1998) **(Figure 1-2)**. The Bcl-2 family is comprised of two antagonistic groups of proteins: the multi-domain antiapoptotic proteins which include Bcl-2, Bcl-XL, Bcl-W, A1, Boo and Mcl-1 and the proapoptotic proteins: Bax, Bak, Bok and the BH3 only family which include Bim, Bid, Bad, DP5/Hrk, Bik, BMF, Noxa and Puma.

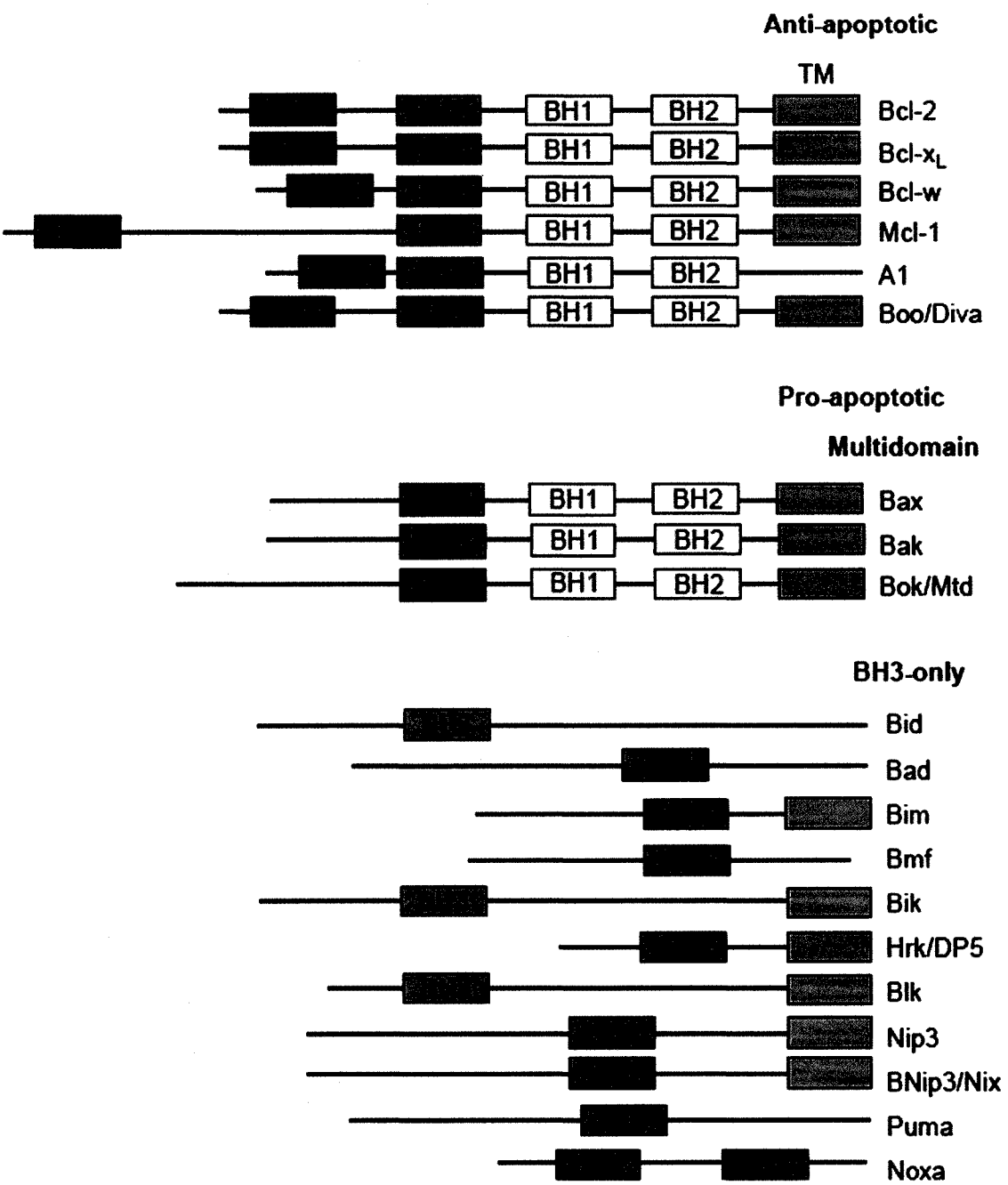
1.1 Antiapoptotic multidomain Bcl-2 proteins

The prototypical Bcl-2 protein was first cloned in part from chromosomal translocations observed in non-Hodgkin lymphomas (Tsujimoto et al. 1985). Less than a decade after, Bcl-2 overexpression was detected in many lymphocytic leukemias (Hanada et al. 1993) and Bcl-2 knockdown was shown to be lethal for leukemic cells (Reed et al. 1990). It was soon discovered that this family of proteins was highly conserved when Ced-9, the C. elegans orthologue of Bcl-2, was cloned (Hentartner & Horvitz 1994). In C. elegans, of the 1090 cells born, 131 undergo programmed cell death (Sulsten & Horvitz 1977). The simplicity of this model has allowed us to identify genes, elucidate a genetic pathway, and apply this knowledge to the research of more complicated systems such as the mammalian Bcl-2 pathway of apoptotic control.

In vivo murine models deficient for different Bcl-2 proteins demonstrate that these proteins have cell type specific roles in controlling apoptosis. For example, deficiency of the prototypic Bcl-2 protein revealed its role in regulating the development of renal epithelial and melanocyte progenitors as

Figure 1-2: Schematic of some members of the Bcl-2 protein family.

The Bcl-2 protein family regulates apoptosis through its ability to stimulate mitochondrial outer membrane permeability. The class of proteins is subdivided into two main categories: the multidomain antiapoptotic members and the proapoptotic members, including both multidomain and BH3-only proteins. Most Bcl-2 proteins have a carboxy-terminal transmembrane domain (TM) which aids in their localization to intracellular membranes. BH: Bcl-2 homology domain.



Kuwana et al. 2003. Curr Opin Cell Biol. 15(6): 691-699.

well as in the maintenance of mature T and B lymphocytes (Veis et al. 1993). Bcl-XL deficiency primarily affects fetal erythroid progenitors and certain neuronal subtypes (Motoyama et al. 1995). Bcl-w deficient mice, on the other hand, exhibit male sterility due to its role in spermatogonial survival (Print et al. 1998). The lack of Mcl-1 in the developing embryo results in peri-implantation lethality and conditional deletion of the protein has shed light on its role in B and T lymphocytes as well as in haemopoietic stem cells (Rinkenberger et al. 2000).

As important inhibitors of the apoptotic process, the antiapoptotic Bcl-2 proteins must be tightly regulated. Several members of the Bcl-2 family contain a hydrophobic carboxy terminal transmembrane spanning domain which can be inserted into the cytoplasmic face of intracellular membranes (Wattenberg et al. 2001). Accordingly, Bcl-2 is found at the endoplasmic reticulum, the nuclear envelope and the mitochondrial outer membrane (Krajewski et al. 1993). On the other hand, Bcl-xL, Bcl-W and Mcl-1 have all been shown to preferentially associate with the mitochondrial outer membrane (Kaufmann et al. 2003, O'Reilly et al. 2001, Yang et al. 1995). These subcellular locations prime the antiapoptotic family for their roles in apoptosis including the prevention of the release of cytochrome c from the mitochondrial intermembrane space and Ca^{2+} from the endoplasmic reticulum.

1.2 Proapoptotic Multidomain Bax and Bak

Bax deficiency results in viable mice with cell death defects which include; moderate lymphoid hyperplasia, some overgrowth in sympathetic and motor neurons and male sterility due to a severe defect in spermatogonia differentiation (Knudson et al. 1995). Paradoxically, female Bax -/- mice display an increase in oocyte numbers (Perez et al. 1999, Alton et al. 2007). In contrast to Bax deficiency, a lack of Bak produces a less severe phenotype. They are viable with no outwardly noticeable defect and have only recently been found to display a mild platelet hypertrophy due to Bak's role in platelet turnover (Lindsten et al. 2000, Mason et al. 2007). Bax/Bak double knockout mice, on

the other hand, did indeed exhibit a dramatic phenotype. These mice die during embryogenesis or perinatally, depending on the genetic background. Moreover, they display severe developmental defects including inter-digital webs and imperforate vaginas, as well as an increase in the number of lymphoid and myeloid cells (Lindsten et al. 2000, Rathmell et al. 2002). Interestingly, these double knockout mice develop normal heart, liver and lung tissues and do not display the exencephaly of apoptotic protease-activating factor-1 -/- (Apaf-1) and caspase-9 -/- mice (two proteins downstream in the apoptotic process). This suggests that there is yet another member of the Bcl-2 family, likely the less common member Bok, with functional redundancy to these two proteins or that the downstream apoptotic machinery is being activated by a mechanism other than the Bcl-2 proteins (Lindsten et al. 2000, Yoshida et al. 1998, Kuida et al. 1998).

In response to a plethora of death stimuli, the multi-domain proapoptotic proteins Bax and Bak are the last step in the ladder of regulation by the Bcl-2 protein family, ultimately causing the release of cytochrome c and committing the cell to an apoptotic form of cell death (for review see Antonosson 2001). These proteins also, however, function at the level of the endoplasmic reticulum and mitochondrial outer membrane to promote the release of Ca2+ stores and are therefore found at both the level of the endoplasmic reticulum and the mitochondria (Scorrano et al. 2003, Zong et al. 2003, Nutt et al. 2002, Germain et al. 2003). While Bak is consistently integrated into the mitochondrial outer membrane and to a lesser extent in the endoplasmic reticulum, Bax remains largely cytosolic until apoptotic signals stimulate its conformational change and integration into the membrane (Hsu et al. 1997, Wolter et al. 1997). It is thought to do so by retaining its transmembrane domain within itself through protein folding until prompted by an apoptotic stimulus to expose this region and allow for membrane insertion (Suzuki et al. 2000). Regardless of where these proteins act, Bax and Bak are critical regulators of the apoptotic cascade required to ultimately activate apoptosis.

1.3 Proapoptotic BH3-only proteins

Deletion studies of individual BH3-only proteins have revealed that different BH3 proteins respond to distinct stimuli in a variety of cell types typically by binding to their preferred antiapoptotic Bcl-2 protein to inhibit its function or by directly activating Bax or Bak (Kuwana et al. 2005). The first BH3 protein discovered to be essential for developmental apoptosis was Bim, which was found to play an essential role in many cell types such as lymphoid and myeloid cell regulation (Bouillet et al. 1999) and later in autoreactive thymocytes (Bouillet et al. 2002, Enders et al. 2003) and neuronal cell death (Putcha et al. 2001). Bid deficient mice were resistant to Fas-activated hepatocyte apoptosis, although other cell types were subsequently shown to remain sensitive to receptor induced cell death (Yin et al. 1999, Kaufmann et al. 2007). Both Noxa and Puma have been shown to play a role in p53-mediated apoptosis in several cell types (Villunger et al. 2003, Jeffers et al. 2003, Cregan et al. 2004). Puma is also critical for p53-independent cell death, including cytokine deprivation and treatment with glucocorticoids or phorbol esters (Villunger et al. 2003, Jeffers et al. 2003). Bad and DP5/Hrk deficiency display only mild resistance to growth factor withdrawal in different cell types (Ranger et al. 2003, Imaizumi et al. 2004) and no defect has yet to be discovered in mice deficient for Bik (Coultas et al. 2005).

While mice deficient for a single BH3 only protein can often display very mild phenotypes, animals lacking two demonstrate the complementary functions of many BH3 only proteins. For example, Bim/Bik double knockout mice display male infertility due to an abnormal accumulation of immature spermatogenic progenitors, despite the lack of any such defect in either of the individual knockouts (Coultas et al. 2005). Bim/Puma double knockout mice have shown that these genes play an integral role in haemopoietic apoptosis, displaying a pronounced level of protection against cell death higher than the loss of either individual protein (Erlacher et al. 2006).

As the 'guardians' of the gates to Bcl-2 regulation of apoptosis, the BH3-only protein family are under tight regulation both transcriptionally, in the case of Puma, Noxa, Hrk/Dp5 and Bim (Nakano et al. 2001, Oda et al. 2000, Imaizumi et al. 1999, Dijkers et al. 2000) and posttranslationally. For example, translocation of the BH3-only proteins in response to apoptotic stimuli can be initiated by several mechanisms including: dissociation from cytoskeletal structures (Bim and Bmf), and post-transcriptional processing such as dephosphorylation (Bad) or cleavage (Bid) (Puthalakath et al. 1999, Puthalakath et al. 2001, Zha et al. 1996, Li et al. 1998). BH3 only proteins have an essential role in developmental and stress-induced cell death as is clear through their tight regulation and apoptotic phenotypes.

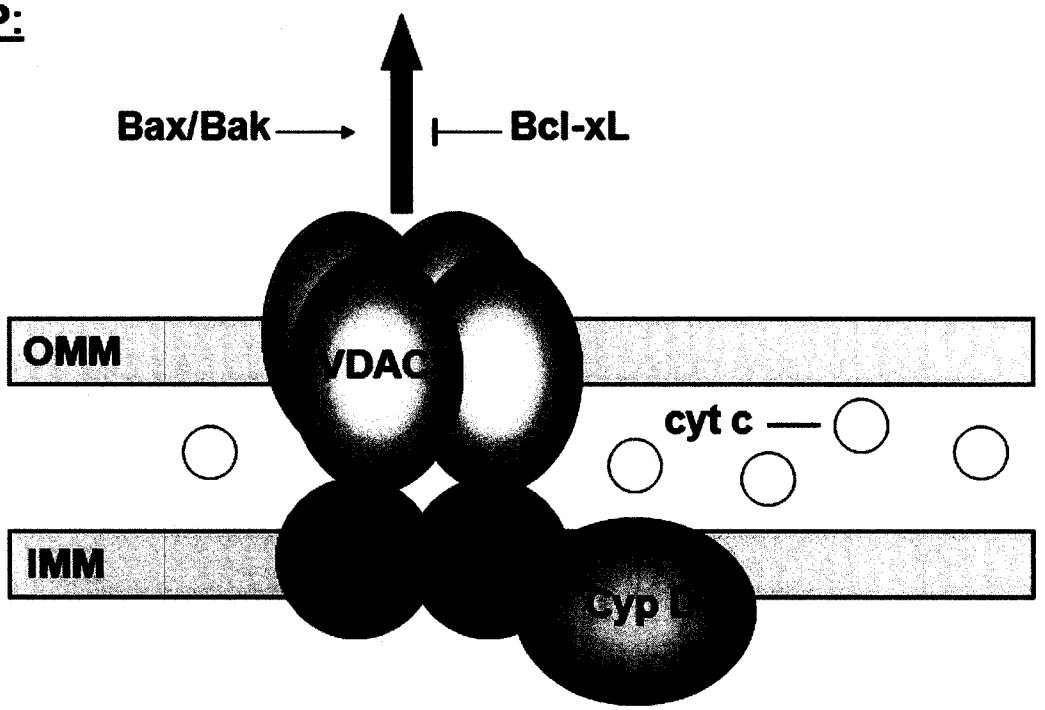
1.4 Permeabilization of the mitochondrial outer membrane

Release of cytochrome c from the mitochondrial intermembrane space into the cytosol marks the initiation of the caspase cascade which is ultimately responsible for the demise of a cell. There are two leading mechanisms for the permeabilization of the outer mitochondrial membrane: the permeability transition pore (PTP) and the mitochondrial outer membrane permeabilization **(Figure 1-3)**. The permeability transition pore is characterized by a mitochondrial permeability transition which causes mitochondrial proteins to 'escape' into the intermembrane space, changing the membrane potential and causing swelling of the mitochondria and rupture of the outer mitochondrial membrane. The mitochondrial permeability transition pore (MPTP) constituents are typically thought to include primarily the outer mitochondrial membrane resident, the voltage-dependent anion channel (VDAC), the inner membrane spanning protein, adenine nucleotide translocator (ANT), and cyclophilin D in the matrix (Crompton 1999, Galat and Metcalfe 1995, Halestrap and Brennerb 2003, Kroemer and Reed 2000, Tsujimoto et al. 2006, Tsujimoto and Shimizu 2006). The contribution of the MPTP components to apoptosis, however, is under much debate. For example, cyclophilin D -/- mice are still sensitive to

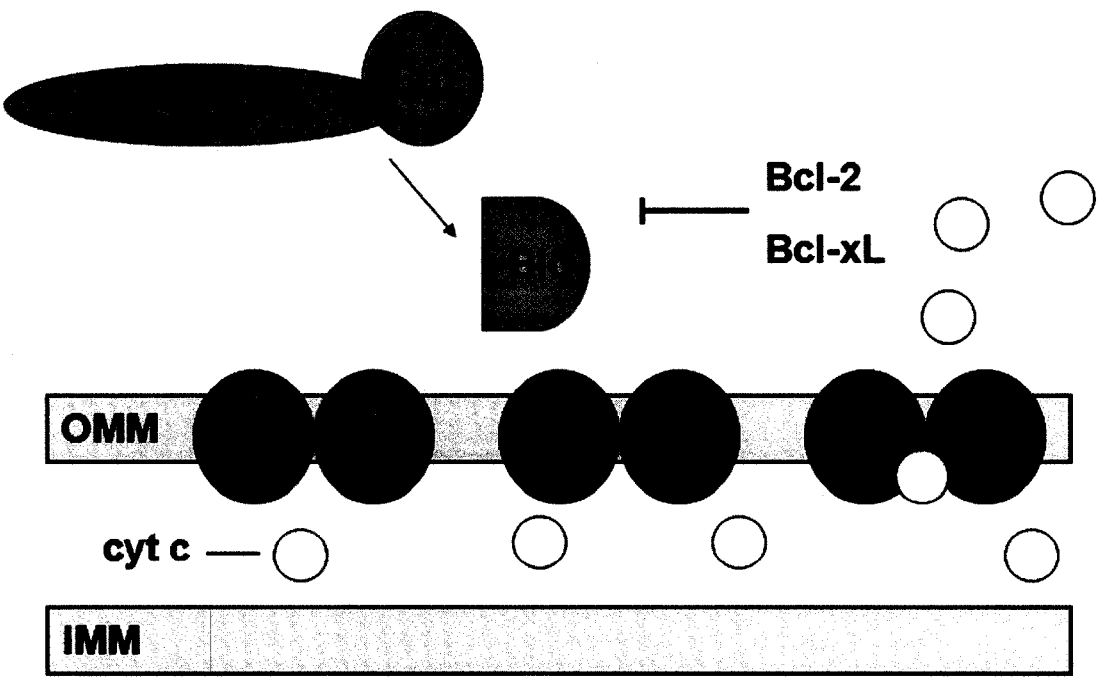
Figure 1-3: Permeabilization of the outer mitochondrial membrane.

The permeability transition pore (PTP) induces a collapse of membrane potential and ultimately allows the release of apoptotic mitochondrial molecules up to 1.5 kDa in size. Its structure has been proposed to include a pore protein called the voltage-dependent-anion-channel (VDAC) in the outer mitochondrial membrane and an ATP/ADP transporter named adenine-nucleotide-translocator (ANT) in the inner membrane. Cyclophilin D (cypD), a chaperone with peptidylprolyl isomerase activity, is directly associated to ANT in the mitochondrial matrix. Evidence for the existence of VDAC dimers and tetramers has been reported in brain mitochondria (Shoshan-Barmatz et al.2004). Involvement of the Bcl-2 family proteins in the PTP-mediated release of cytochrome c remains controversial. Mitochondrial outer membrane permeabilization (MOMP) involves the Bcl-2 proteins containing pore forming domains similar to those of diphtheria toxin which may include Bcl-xL, Bcl-2, Bax, Bak and the cleaved form of Bid, tBid. Caspase-8 cleaves the BH3-only protein, Bid to form truncated Bid (tBid). tBid can then translocate to the mitochondria where it induces the permeabilization process likely through promotion of homo- and/or hetero-oligomerization of Bax and Bak in the mitochondrial outer membrane to form pores.

PTP:



MOMP:



DNA damaging agents as well as Bax and tBid overexpression (Baines et al. 2005, Nakagawa et al. 2005, Schinzel et al. 2005) and overexpression of cyclophilin D can cause resistance to nitric oxide and staurosporine-induced apoptosis (Li et al. 2004), suggesting that cyclophilin D may not be required for the canonical Bcl-2 regulated apoptosis. Likewise, despite its ability to interact with Bax ([2]Marzo et al. 1998), studies have shown that ANT is not required for the formation of the permeability transition pore in the presence of a threefold increase in Ca2+ (Kokosza et al. 2004). Although Bcl-xL, Bax and Bak have also been suggested to cooperate with voltage-dependent anion channel ([1]Marzo et al. 1998, Shimizu et al. 1999), both Ca2+ and oxidative stress-induced MPTP cell death occurs in the absence of all three isoforms of VDAC (Baines et al. 2007). Moreover, the proapoptotic Bcl-2 family members Bax and Bid are still capable of inducing cytochrome c release in the absence of VDAC (Baines et al. 2007). Consequently, VDAC appears to be dispensable for MPTP formation and Bcl-2 dependent apoptosis. The uncertainty about the core components of the MPTP suggests that it is more likely involved in necrosis but not apoptosis. Although the possibility that MPTP may play a transient role in apoptosis still exists, these experiments suggest that it is the second method of permeabilization which is necessary for apoptosis and that MPTP is dispensable in this type of cell death.

The second hypothesis: the mitochondrial outer membrane permeabilization involves formation of mitochondrial pores by the Bcl-2 family, directly. This hypothesis originated from protein crystallography results displaying structural similarity between Bcl-xL and the pore forming domains of different bacterial toxins including diphtheria toxin and bacterial colicins (Muchmore et al. 1996). Since then, structural analysis of Bax (Antonsson et al. 1997), Bcl-2 (Schlesinger et al. 1997, Schendel et al. 1997) and the cleaved form of Bid, tBid (Schendel et al. 1999), has revealed that they are also competent of channel formation. During apoptosis, Bax and Bak have been shown to coalesce into clusters on the outer mitochondrial membrane surface, likely representing the site of pore formation (Nechustan et al. 2001). *In vitro* studies have revealed the ability of Bax to form membrane pores and

cause the release of intravesicular FITC-cytochrome c, although not larger proteins as those observed under physiological apoptotic conditions (Saito et al. 2000). More recently, the activated form of Bid, tBid, along with only Bax, has been shown to be sufficient to permeabilize the lipidic membranes of various vesicles, including isolated mitochondria, to allow translocation of extremely large macromolecules (Kuwana et al. 2002). Using atomic force microscopy, others have been able to visualize large Bax pores within lipid vesicles (Epand et al. 2002). All together, these data suggest that direct permeabilization of the outer mitochondrial membrane by the Bcl-2 proteins is the more likely culprit during an apoptotic cell death. Regardless of how these proteins act to permeabilize the mitochondrial outer membrane, they remain an integral part of the commitment step in apoptosis and their activation must therefore be tightly regulated.

1.5 Activation of Bax and Bak

BH3-only proteins alone, without Bax and Bak, cannot kill a cell since their function is upstream of the multi-domain proapoptotic proteins (Zong et al. 2001). Instead, they function as regulators of the proapoptotic proteins. Two models have prevailed in the regulation of pro-death proteins by the BH3-only class which will be described below: the direct and indirect activation models.

The direct model (also known as the hierarchical model), proposes that the BH3-only proteins are divided into two classes based on their proposed mode of action; the 'sensitizers' and the 'activators' (Letai et al. 2002, Kuwana et al. 2005, Certo et al. 2006). Members of the sensitizer subclass function by binding to the prosurvival Bcl-2 proteins and displacing the activator proteins and include Bad, Bik, Bmf, Noxa. Conversely, the 'activators', namely Bid, Bim and perhaps PUMA are able to directly bind to and induce activation of the proapoptotic proteins, Bax and Bak. Evidence for this model came from experiments that demonstrated that expression of either Bid or Bim can induce oligomerization of Bax and Bak and cytochrome c release from isolated mitochondria (Letai et al.

2002). Moreover, they have both been shown to permeabilize artificial liposomes in combination with Bax and in the absence of any other antiapoptotic protein (Kuwana et al. 2005). Although the direct interactions observed between BH3-only proteins and prosurvival Bcl-2 proteins are undisputed (Chen et al. 2005), evidence of binding between Bid/Bim/PUMA and Bax/Bak remains controversial. *In vitro* experiments have, however, revealed that all three activator proteins, Bid (Kuwana et al. 2002), Bim, and PUMA (Kim et al. 2006) can provoke cytochrome c release from isolated mitochondria.

The indirect activation model consists of interactions between members of a single group of BH3-only proteins and their preferred Bcl-2 antiapoptotic protein binding partner but lacks any interactions between BH3 proteins and the multidomain proapoptotic Bax and Bak (Adams and Cory 2007, Willis and Adams 2005, van Delft et al. 2006). In this model, each BH3 protein binds directly to and inhibits its prosurvival partner during apoptosis, replacing the proapoptotic proteins Bax and Bak from their hold. As previously mentioned, direct interactions between the antiapoptotic Bcl-2 family and BH3 family of proteins has been observed many times and is undisputed (Chen et al. 2005, Kuwana et al. 2005, Kim et al. 2006). This fact is not, however, hard evidence for the indirect model. Nor does it disprove the hierarchical model of Bax/Bak activation. Double knockout studies of Bim and Bid, the two main activator BH3-only proteins of the hierarchical model, in mice have, however, shed doubt onto the direct activation model. In these experiments, the absence of these two proteins was not sufficient to block a significant number of apoptotic pathways (Willis et al. 2007), suggesting that these proteins are not required for apoptotic cell death or that there are other factors involved yet to be discovered which play a role in Bax/Bak activation such as the BH3 only protein, PUMA. Whether Bax and Bak activation are caused by the direct or indirect model has yet to be definitively proven. What has been shown to be definite though, is the fact that BH3 only proteins are required to act upstream of Bax and Bak and induce mitochondrial permeabilization and thus the release of apoptogenic factors.

1.6 Caspases

The concluding step in the process of apoptosis is under the control of proteases termed caspases, short for cysteine aspartyl-specific proteases (Alnemri et al. 1996). There are fourteen members of the mammalian caspase family discovered to date, divided into two main subclasses: the initiator caspases, including caspase-2, 8, 9 and 10, and effector caspases, including caspase-3, 6, and 7 (Taylor et al. 2008, Thornberry et al. 1997). Studies of caspase deficient mice in which a deficiency of caspase-3, 8 or 9 can result in embryonic lethality and a loss of the apoptotic pathway, elucidate the importance of caspases in development and in the process of apoptosis (Hakem et al. 1998, Kuida et al. 1996, Kuida et al 1998, Varfolomeev et al. 1998, Woo et al. 1998).

The review above describes the general process an apoptotic cell undergoes and the controversies of how this process is regulated in the literature today. Although the central dogma of the apoptotic mechanism holds up from one system to another, there is also evidence which suggests that this process can be regulated differently, depending on the tissue in question. As such, a discussion of apoptosis in the context of neuronal apoptosis is necessary.

2: Neuronal Apoptosis

Apoptosis plays a critical role in the development of the mammalian nervous system, in which 20 to 80% of all neurons initially born are destined to die via developmental programmed cell death, depending on the specific neuronal regions in question (Oppenheim 1991). Precursor cells produce neurons in excess of what is required for the fully developed central nervous system. As such, cell numbers within the central nervous system are partially controlled by a programmed cell death, eliminating the excess neurons (Oppenheim 1991). In the mouse, programmed cell death comes in two main waves throughout neuronal development: during gastrulation (at embryonic day 6.5) within tissue destined to become the neural plate (Manova et al. 1998) and again throughout the formation of

the cerebral cortex (between embryonic day 14 and 16) within the ventricular zone (Blaschke et al. 1996, 1998, Thomaidou et al. 1997). These waves of cell death carry much weight in the proper development of the adult CNS. This can be clearly seen through examples of mice deficient in caspase 3 and 9 in which the reduction of apoptosis in areas of the CNS containing stem cells causes hyperplasia (Hakem et al. 1998, Kuida et al. 1996).

The Bcl-2 family proteins are essential players involved in the regulation of developmental apoptosis in the nervous system **(Table 1-1)**. Studies of individual apoptotic genes have revealed a large degree of context dependent function for each protein. For example, a neuronal specific splice variant of Bak, termed N-Bak, has been described in place of the full length Bak in many neuronal subtypes (Sun et al. 2001). N-Bak is a splice variant of the full length Bak which contains only the BH3 and a transmembrane domain of Bak. Interestingly, N-Bak appears to display both pro and antiapoptotic functions in neuronal subtypes and proapoptotic functions in non-neuronal cells (Sun et al. 2001, Uo et al. 2005). Its mechanism in neurons appears to be similar to the prototypical BH3 only proteins in that it binds to an antiapoptotic molecule, namely Bcl-x, to induce a Bax dependent apoptotic response (Uo et al. 2005). Given their specificity of function, much research has been focused on understanding their roles in an *in vivo* and tissue specific context. Below is a short review of the roles played by some of the key Bcl-2 family members involved in the regulation of neuronal apoptosis throughout the developing and mature nervous system.

2.1 Bcl-2 proteins in the early developing central nervous system

The long form of the cleaved Bcl-X protein, Bcl-XL, is found increasingly in immature postmitotic neuronal populations of the developing central nervous system, peaking at E12 and remaining high through postnatal development, with only a slight decline in the adult brain (Krajewska

	Expression in developing CNS	Areas affected by protein deficiency	References
Proapoptotic multidomain			
Bax	Detected at E6, peaks at E11 until E14.5 & declines to lower levels in adult CNS	Bax deficiency affects dorsal root ganglion, cerebellum and hippocampus of CNS *in vivo*; significantly affects cell death of sympathetic and motor neurons in context of excitotoxicity, DNA damage and growth factor deprivation; is essential for p53 mediated apoptosis of cerebellar granule and cortical neurons *in vitro*.	Cregan et al. 1999, Deckwerth et al. 1996, Knudson et al. 1995, Krajewska et al. 2002, Putcha et al. 2002, White et al. 1998, Xiang et al. 1998
Bak	Detected at E12, peaks at E14 & declines to lower levels in adult CNS	No CNS phenotype in Bak deficient mice.	Krajewska et al. 2002, Lindsten 2002
Proapoptotic BH3 only			
Bid	Detected as early as E6, peaks by E9 and remains high through development and adulthood; confined mainly to lower levels of CNS including spinal cord, brainstem, basal ganglia	No neuronal phenotype in germline deletion mouse, involved in cortical neuron degeneration following focal cerebral ischemia, Bid deficiency protects cortical neurons against oxygen and glucose deprivation *in vitro*	Krajewska et al. 2002, Plesnila et al. 2001, Yin et al. 1999, Yin et al. 2002
Bim	Expressed predominantly in neurons of CNS	No neuronal phenotype in germline deletion mouse, involved in survival factor withdrawal induced apoptosis in the dorsal root ganglion	Bouillet et al. 1999, O'Reilly et al. 2000, Putcha et al. 2001, Whitfield et al. 2001,
DP5/Hrk	Originally expressed in sympathetic neurons, also found in adult brain predominantly in the hippocampus and entorhinal cortex	No neuronal phenotype in germline deletion mouse, involved in sympathetic neuron and cortical ganglion neuronal apoptosis *in vitro*	Harris et al. 2001, Imaizumi et al. 1997, Imaizumi et al. 2004, Kanazawa et al. 1998
Bad	Expressed throughout embryonic nervous system during development, lower expression levels in postnatal neuronal tissue	No neuronal phenotype in germline deletion mouse, *in vitro* experiments delineate a role for Bad in neuronal apoptosis following apoptotic stimuli	Datta et al. 1997, Orike et al. 2001, Ranger et al. 2003, Shimohama et al. 1998

Noxa	Induced by p53, a tumor-suppressor protein shown to promote neuronal apoptosis	No neuronal phenotype in germline deletion mouse, important regulator for genotoxin-induced cell death of neural precursor cells of the telencephalon, implicated in axotomy-induced motor neuron death	Akhatar et al. 2006, Kiryu-Seo et al. 2005, Miller et al. 2000, Shibue et al. 2003
Puma	Induced by p53, a tumor-suppressor protein shown to promote neuronal apoptosis	No neuronal phenotype in germline deletion mouse, important regulator for genotoxin-induced cell death of neural precursor cells of the telencephalon, required for p53-mediated neuronal cell death	Akhatar et al. 2006, Cregan et al. 2004, Miller et al. 2000, Nakano et al. 2001, Villunger et al. 2003, Wyttenbach et al. 2006
Antiapoptotic multidomain			
Bcl-2	Highly expressed in developing nervous system, peaks at E11 until E14.5	No CNS phenotype in Bcl-2 deficient mice; profound loss of motoneurons, sympathetic and sensory neurons in PNS of post-natal animal	Abe-Dohmae et al. 1993, Krajewska et al. 2002, Merry et al. 1994, Michaelidis et al. 1996, Pinon et al. 1997
Bcl-xL	Detected increasingly in immature postmitotic neurons throughout development, peaks at E12 & remains highly expressed until a slight decline in the adult brain	*Embryonic lethality at E13.5, increased* neuronal caspase 3 activation and Bax-dependent apoptosis in the developing brain and spinal cord although ventricular zone unaffected *in vivo*, 33% decrease in catecholminergic neurons in conditionally deleted Bcl-xL mice	Krajewska et al. 2002, Motoyama et al. 1995, Roth et al. 2000, Savitt et al. 2005, Shindler et al. 1997
Mcl-1	Originally described to be absent from the brain, since then expression has been demonstrated in cerebellar granule neurons, neural precursor cells and hippocampal pyramidal neurons	Implicated in neural precursor cell survival, granule cell migration and differentiation and excitotoxicity-induced cell death	Krajewski et al. 1995, Mori et al. 2004, Oishi et al. 2004, Zhang et al. 2004
Bcl-w	Expression increases throughout development until it reaches highest levels in the adult brain, predominantly found in the mature neurons of the cortex, hippocampus and cerebellum of the adult CNS	Bcl-w deficient mice undergo twice as hippocampal neuronal death following induction of seizures	Hamnér et al. 1999, Murphy et al. 2007

Table 1-1: Bcl-2 protein family in the nervous system.

et al. 2002). Deletion of Bcl-XL from the whole embryo displays a neuronal phenotype, with increased caspase-3 and Bax-dependent apoptosis in the developing brain and spinal cord (Motoyama et al. 1995, Shindler et al. 1997, Roth et al. 2000). It is noted, however, that the ventricular zone, and thus progenitor cells of Bcl-xL deficient embryos, were unaffected. Moreover, conditional deletion of Bcl-X in catecholaminergic neurons in the substantia nigra resulted in only a 33% decline of this population of cells, demonstrating that Bcl-X is dispensible in some neuronal populations (Savitt et al. 2005).

The key proapoptotic Bcl-2 family protein expressed in the developing central nervous system in concert with Bcl-xL is Bax. Bax expression is detected in the central nervous system early on in development, at embryonic day 6 (E6) and peaks around E11 until E14.5 (Krajewska et al. 2002). Mice double deficient for Bax and Bcl-xL drastically reduces the neuronal apoptotic phenotype (Shindler et al. 1997). Interestingly, *in vitro* studies of Bax -/- Bcl-xL -/- E12 telencephalic cultures revealed no difference in the numbers of spontaneous apoptotic cells compared to wild type littermates (Shindler et al. 1997). Similarly, embryonic telencephalic cultures from Bax -/- mice also exhibited apoptotic activity similar to wild type cultures (Shindler et al. 1997). The Bax deficient mouse does not display any major central nervous system defect with only a few regions displaying decreased apoptosis including the dorsal root ganglia, cerebellum and the hippocampus (Knudson et al. 1995, White et al. 1998). Collectively, these results indicate that Bcl-xL and Bax play key opposing roles in the regulation of apoptosis within the developing central nervous system. However, the dispensability of Bcl-xL in some neuronal subtypes as well as the lack of a neuronal phenotype within Bax deficient mice and telencephalic cultures from Bax -/- Bcl-xL -/- and Bax -/- mice suggests that the requirement of these proteins are specific to certain neuronal subtypes and not universal to the whole developing central nervous system.

2.2 Bcl-2 proteins in the developing peripheral nervous system

While Bax deficiency does not display a neuronal phenotype within the central nervous system during development, it is required for cell death in sympathetic and motor neurons within the context of nerve growth factor deprivation (Deckwerth et al. 1996), excitotoxicity (Putcha et al. 2002) and DNA damage (Putcha et al. 2002). Countering its actions within the peripheral nervous system is the antiapoptotic protein, Bcl-2. Bcl-2 has been reported to be expressed at high levels in the developing nervous system (Abe-Dohmae et al. 1993, Merry et al. 1994, Krajewska et al. 2002). Although Bcl-2 expression levels are downregulated near birth in the central nervous system, they remain high within the peripheral nervous system through life (Merry et al. 1994). Deletion of the antiapoptotic protein displays no significant apoptotic defect in the central nervous system. A profound loss of motoneurons, sympathetic and sensory neurons, however, is observed in the post-natal animal (Michaelidis et al. 1996, Pinon et al. 1997). Cell death in these neurons demonstrates a role for Bcl-2 in the maintenance of postnatal neuronal populations. Accordingly, can be induced by growth factor signaling such as nerve growth factor (NGF) (Chiou et al. 2007), erythropoietin (EPO) (Klampfer et al. 1999), insulin-like growth factor I (IGF-I) and estrogen (Garcia-Segura et al. 1998, Fernandex et al. 1999).

2.3 Bcl-2 proteins in neural precursor cell survival

Within the central nervous system, neural precursor cells have been shown to be sensitive to the proapoptotic proteins Bax and Bak. Although the deficiency of either molecule alone does not display any major defect in the central nervous system (Deckwerth et al. 1996, Lindsten 2000), mice deficient for both Bax and Bak exhibit a pronounced phenotype. Bax-/- Bak-/- mice have also shed some light onto neural precursor cell maintenance during development as well as in the adult subventricular zone, an area of the brain in which neural precursor cells are thought to persist throughout adult life in mice. Telencephalic cultures from embryos of these double deficient mice

containing neuronal progenitor cells are resistant to different DNA damage-induced forms of apoptosis (D'Sa et al. 2003). In vivo, Lindsten and colleagues have shown an accumulation of neural precursor cells in the subventricular zone of Bax -/- Bak -/- mice such that the cross-sectional area of the subventricular zone was ten fold higher in the double deficient mice compared to wild-type brains (Lindsten et al. 2003).

2.4 BH3 only proteins in the nervous system

Functional redundancy of the BH3 only proteins within the nervous system is clearly demonstrated as none of the BH3 only deficient mice alone display any neuronal defect. BH3 only proteins do, nonetheless, display context dependency within the nervous system in that varying neuronal subpopulations are affected differently by the loss of any particular BH3 only protein, depending on how predominant its role was in that particular subgroup. For example, Bim deficiency has implicated it in survival factor withdrawal induced apoptosis in the neurons of the dorsal root ganglion (Whitfield et al. 2001, Putcha et al. 2001). Similarly, overexpression experiments have shed some light onto the role of DP5 in sympathetic and cortical ganglion neuronal apoptosis *in vitro* (Imaizumi et al. 1997, Harris et al. 2001). Both Noxa and Puma, on the other hand, have been identified as important regulators for genotoxin-induced cell death (such as camptothecin and bleomycin treatment) of neural precursor cells from the telencephalon (Akhtar et al. 2006). Puma alone has been shown to be required for p53-mediated neuronal cell death in several different studies (Cregan et al. 2004, Nakano et al. 2001, Wyttenbach et al. 2006), while Noxa has been implicated in axotomy-induced motor neuron death (Kiryu-Seo et al. 2005). Of the BH3-only protein sub family only Bid expression has been thoroughly studied in neurons and was found to be present in both the embryo and the adult central nervous system (Krajewska et al. 2002). When activated, caspase 8 can cleave Bid into its truncated form, tBid (Luo et al. 1998). tBid is present in the brain, where it has been

shown to interact with Bak to activate the release of cyt c from mitochondria (Brustovetsky et al. 2003). Although Bid does not seem to play a role in the naturally occurring neuronal death induced by nerve growth factor (NGF) withdrawal, it does appear to be involved in cortical neuron degeneration following focal cerebral ischemia (Plesnila et al. 2001, Yin et al. 2002). *In vitro,* the loss of Bid in cortical neurons can protect them from cell death induced by glucose and oxygen deprivation (Culmsee et al. 2005). Another BH3 only protein shown to display context dependency is Bad. Many *in vitro* experiments have delineated a role for Bad in neuronal apoptosis through overexpression of the protein as well as Bad activation following apoptotic stimuli in cerebellar granule neurons and adult sympathetic neurons (Datta et al. 1997, Orike et al. 2001).

The use of different knockout mice has identified a role for Bcl-2 protein family members in concert in the regulation of neuronal apoptosis with particular interest in the antiapoptotic protein Bcl-xL and proapoptotic Bax in the central nervous system and Bcl-2 within the peripheral nervous system. Individually, however, no Bcl-2 protein alone has been deemed essential to the development of the whole nervous system in the past. Central to this thesis is the discovery that Mcl-1 is essential for proper development of the central nervous system.

3: The antiapoptotic protein Mcl-1

Mcl-1 (myeloid cell leukaemia-1) was first identified in the ML-1 human myeloid leukemia cell line during phorbol ester-induced differentiation, where it was described as a member of the then emerging Bcl-2 family based on sequence similarity (Kozopas et al. 1993). Since its discovery, Mcl-1 expression has been shown to also be stimulated by several cytokines and growth factors including interleukin-3, -5, -6 and -7, granulocyte-macrophage colony-stimulating factor, stem cell factor, interferon-alpha and epidermal growth factor (Chao et al. 1998, Huang et al. 2000, Jourdan et al. 2000, Leu et al. 2000, Opferman et al. 2003, Yang et al. 1996). Our knowledge of its function as a Bcl-2

family member has also expanded to include a crucial role in apoptotic control. Mcl-1 overexpression has been shown to retard cell death induced by several stimulators including c-Myc overexpression, growth factor withdrawal, serum starvation and a multitude of cytotoxic agents (Austin et al. 2005, Chao et al. 1998, Piret et al. 2005, Reynolds et al. 1994, Yang et al. 2007, Zhou et al. 1997). Additionally, Mcl-1 expression is diminished in several cell systems undergoing apoptosis (Epling-Burnette et al. 2001, Leuenroth et al. 2000, Michels et al. 2004, Myklebust et al. 1999)

Mcl-1 is found predominantly in the outer mitochondrial membrane (Yang et al. 1995) where it acts as a prosurvival protein. The evidence described below suggests that Mcl-1 acts as a prosurvival protein by sequestering the pro-apoptotic protein Bak which may inhibit its oligomerization and thus ability to form pores in the outer mitochondrial membrane. Under apoptotic conditions, Bak is displaced by members of the BH3-only pro-apoptotic protein family including Bim, Bid, Puma and Noxa. Interactions between these proteins and Mcl-1 have been described previously in *in vitro* binding studies (Certo et al. 2006, Chen et al. 2005, [2]Willis et al. 2005). Moreover, Mcl-1-Bak interactions have been confirmed by immunoprecipitation from isolated mitochondria (Cuconati et al. 2003, Leu et al. 2004, Nguyen et al. 2007, [2]Willis et al. 2005)

The Mcl-1 germline knockout mouse was found to be lethal prior to implantation (before embryonic day 4.5), suggesting a prominent role for the molecule in early embryo development and implantation (Rinkenberger et al. 2000). Conditional knockout studies have since revealed a requirement for Mcl-1 in the development and survival of B and T lymphocytes (Opferman et al. 2003), as well as the maintenance of hematopoietic stem cells (Opferman et al. 2005).

3.1 Mcl-1 acts upstream of Bax signaling

Mcl-1 has recently been described as an 'apical sensor' for apoptotic stimuli owing to the fact that it has been shown to function upstream of the Bcl-xL-Bax signaling system which induces

apoptosis (Nijhawan et al. 2003). In these studies, both Mcl-1 and Bcl-xL were identified as the cytosolic inhibitors necessary to prevent the release of cytochrome c from the mitochondria and subsequent activation of the caspase family. Using a biochemical approach, the authors reported a series of sequential events leading to an apoptotic cell death in which the degradation of Mcl-1 is a primary step in the initiation of apoptosis and is essential to allow the translocation of Bax and Bcl-xL to the mitochondria in order to trigger the downstream apoptotic cascade (Cuconati et al. 2003, Nijhawan et al. 2003).

3.2 Mcl-1 expression is regulated at multiple levels

Regulation of Mcl-1 expression is carried out at multiple levels, involving transcriptional, post-transcriptional and posttranslational mechanisms (Croxton et al. 2002, Domina et al. 2000, Herrant et al. 2004, Michels et al. 2004, Yang et al. 1996). Understanding the systems which regulate this key antiapoptotic protein is of important pharmacological value and has therefore been the subject of many studies and these key mechanisms of regulation are described below.

3.2.1 Transcriptional

Transcription factors which control Mcl-1 expression have also been identified and include both hypoxia-inducible factor-1 (Hif-1) and E2F transcription factor 1 (E2F-1). The role of Hif-1 as a major transcription factor involved in the cellular response to oxygen deprivation has been established under several conditions (reviewed in Semenza 2003). Its role as an apoptotic response element, however, has only recently been suggested. Although several Bcl-2 proapoptotic members such as Nip3 and Noxa have been reported to be overexpressed under hypoxic conditions (Sowter et al. 2001, Bruick et al. 2000, Kim et al. 2004, Shoshani et al. 2002), other studies have described a protective effect of hypoxia under various conditions and cell types (Blagosklonny et al. 1998, Baek et al. 2000), and in

some cases, a downregulation of the proapoptotic proteins Bid and Bax (Erler et al. 2004). Hif-1 has been described as a positive regulator of Mcl-1 in that siRNA knockdown of Hif-1 largely inhibits Mcl-1 transcription (Piret et al. 2005, Liu et al. 2006). Moreover, Mcl-1 contains an active HRE (hypoxia-responsive element) site, recognized by Hif-1 (Piret et al. 2005). Consequently, Hif-1 would act to protect the cell from hypoxic conditions by upregulation of the antiapoptotic protein, Mcl-1.

In contrast to Hif-1 regulation of Mcl-1 which promotes cell survival, E2F-1 represses Mcl-1 transcription thus promoting apoptosis. Although E2F-1 commonly acts as a promoter of growth in cell cycle regulation (DeGregori et al. 1995, Johnson et al. 1993), its role in induction of apoptosis has also been ascertained (Kowalik et al. 1995, Qin et al. 1994, Tsai et al. 1998, Wu et al. 1994, Yamasaki et al. 1998). In the case of Mcl-1 regulation, studies have demonstrated that E2F-1 overexpression leads to a decrease in expression of antiapoptotic proteins including Mcl-1 (Yang et al. 2000). Later, it was determined that E2F-1 directly repressed the Mcl-1 promoter and that this function was specific to E2F-1 and dependent on its DNA-binding domain ([1]Croxton et al. 2002, [2]Croxton et al. 2002).

The complexity of Mcl-1 transcriptional regulation is further increased through the fact that several different downstream signaling pathways can mediate transcriptional regulation of its mRNA. For example, Mcl-1 mRNA levels are mediated by the MAPK/ERK signal transduction pathway (Townsend et al. 1998, Townsend et al. 1999), the MAPK/MEK and the PI3K/Akt pathways (Huang et al, 2000, Kuo et al. 2001), and the JAK/STAT pathway (Epling-Burnette et al. 2001, Isomoto et al. 2005, Puthier et al. 1999) in a cell type and stimulation factor dependent manner.

3.2.2 Post-transcriptional

Mcl-1 has also been shown to be regulated post-transcriptionally. A splice variant of Mcl-1, Mcl-1s, containing only its BH3 domain has been described (Bingle et al. 2000). Its function differs from that of the long form of Mcl-1 in that it acts as a BH3-only protein, promoting apoptosis, possibly

via interactions with full length Mcl-1 (Bingle et al. 2000, Bae et al. 2000). More recently, Mcl-1 mRNA has been shown to be downregulated by microRNAs, namely mir-29b, allowing the longevity of the transcript to be modulated as well (Mott et al. 2007).

3.2.3 Post-translational

At the level of post-translational modifications, Mcl-1 has a short half life due to its tight regulation by a variety of mechanisms including phosphorylation, ubiquitination and caspase cleavage. Mcl-1 is phosphorylated by several stimulators at its PEST domain and this phosphorylation is mediated via different pathways. For example, TPA, an agent which activates protein kinase C due to its structural similarity to the natural diacylglycerol activator, and agents that cause accumulation in G2/M phase of the cell cycle, such as taxol, induce phosphorylation of Mcl-1 in a Burkitt lymphoma cell line through both ERK-associated and ERK-independent mechanisms (Domina et al. 2000). IL3 withdrawal, on the other hand, causes phosphorylation of Mcl-1 via PI3K/Akt signaling in immature hematopoietic cells (Maurer et al. 2006).

Mcl-1 turnover is also regulated through proteasomal degradation via ubiquitination from the E3 ligase, MULE (Mcl-1 ubiquitin ligase E3) (Warr et al. 2005). MULE is a BH3 only Hect E3 ligase which binds Mcl-1 at its hydrophobic BH3 binding pocket as well as its N-terminus. This process has been shown to be required for the onset of apoptosis initiated by DNA damage (Nijhawan et al. 2003) and shRNA-mediated knockdown of MULE can protect cells from DNA damage induced apoptosis (Zhong et al. 2005). Moreover, proteasomal degradation of Mcl-1 appears to also be regulated by the BH3 only protein, Noxa ([1]Willis et al. 2005) which binds to Mcl-1 tightly, displacing Bak and that enforced Noxa expression caused proteasomal degradation of Mcl-1 that is dependent on the association of the two proteins. Mcl-1 protein levels can also be regulated through caspase cleavage of Mcl-1 at Aspartic acid residues 127 and 157 (Herrant et al. 2004, Weng et al. 2005). Mcl-1 is both

induced by several factors and regulated in a variety of ways at the level of transcription, post-transcriptionally, and post-translationally. In conjunction with its short half life, Mcl-1's multiple levels of regulation make it an attractive candidate for pharmacological manipulation.

3.3 Mcl-1 in the nervous system

Despite previous studies stating that Mcl-1 expression was insignificant or absent within the nervous system (Krajewski et al. 1995), recent work has nevertheless demonstrated that a role for Mcl-1 in neuronal survival does exists. Mcl-1's role in neuronal survival in response to injury in the nervous system has remained largely unexplored. In a study using mice heterozygous for Mcl-1, Mori and colleagues have described a role for Mcl-1 in excitotoxicity-induced cell death (Mori et al. 2004). In these experiments, mice heterozygous for Mcl-1 displayed an increase in the amount of DNA damage and caspase dependent cell death in response to seizures when compared to their wildtype littermates. While these studies demonstrate that Mcl-1 plays a role in the survival of neurons in response to injury, its function in neuronal development remains largely unexamined. Preliminary data do exist, however, suggesting that Mcl-1 may be involved in neuronal survival during development. First, expression of Mcl-1 in the developing nervous system has been demonstrated in cerebellar granule neurons in the developing post-natal brain (Zhang et al. 2004). Later studies suggested a role for Mcl-1 in neural precursor cell survival mediated by Notch-1, dependent on its RAM domain, a ligand binding molecule which regulates gene transcription (Oishi et al. 2004). In these experiments, Notch-1 signaling increased survival of mouse embryonic neural precursor cells *in vitro* and this effect was accompanied by an upregulation of the antiapoptotic proteins, Bcl-2 and Mcl-1. Moreover, when these proteins were knocked down by RNA interference, Notch-1 mediated cell survival was suppressed.

Mcl-1 is a highly attractive molecule to study in the regulation of apoptosis due to its apical role in the apoptotic cascade. Without the loss of Mcl-1 function, the proapoptotic proteins, Bax and Bak

are unable to translocate to the mitochondria to initiate the cell death pathway. Additionally, Mcl-1 is tightly regulated and expression of this protein can be quickly up- or downregulated, making it an attractive candidate for pharmacological manipulation. The following studies reported throughout my thesis explore the role of Mcl-1 in neuronal development and maintenance of the mature nervous system for the first time.

4: Autophagy

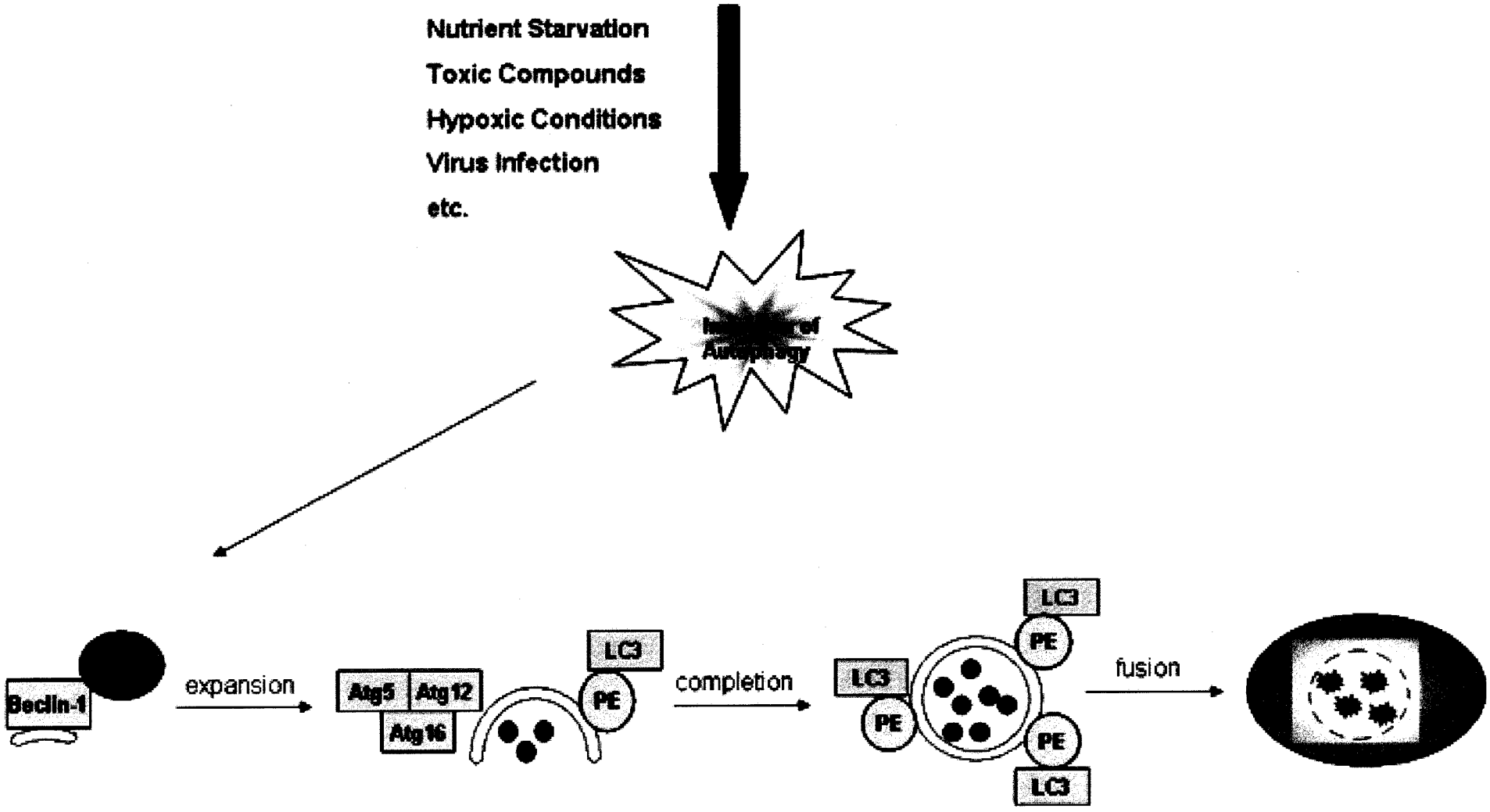
Autophagy (from the Greek "to eat oneself') is a catabolic process which is responsible for both bulk degradation and more precise recycling of cytoplasmic components including proteins and whole organelles via delivery to the lysosome **(Figure 1-4)**. This phenomenon, which has been identified in a wide array of eukaryotic cells and is conserved from yeast to humans (Klionsky et al. 2000), is induced in response to an array of both extracellular and intracellular stresses. These events can include nutrient starvation, hypoxia and infection by a pathogenic bacterium or virus, or an accumulation of damaged organelles or proteins. While its exact role in both healthy and diseased states is yet to be determined in mammalian cells, the autophagic process has been implicated in cellular processes such as lifespan extension and cellular development and differentiation (Levine et al. 2004) as well as in several diseases including cancer, Huntington's, Alzheimer's and Parkinson's disease (Cuervo 2004, Shintani et al. 2004).

4.1 Forms of autophagy

The word autophagy is a general term which can be subdivided into three different catabolic processes: chaperone-mediated autophagy, microautophagy and macroautophagy. Chaperone-mediated autophagy is a degradative process for specifically targeted proteins and thus has a more

Figure 1-4: The autophagic process.

Autophagy can be induced by a plethora of stimuli. Subsequent steps include: nucleation (the first steps of formation) of a double membraned vesicle, expansion, completion of the autophagosome, fusion with the lysosome and degredation of the inner membrane and cytoplasmic components contained within its walls. This process is tightly regulated by the autophagy-related genes (Atg's) which use two distinct ubiquitin-like conjugation systems to produce the two Atg complexes which associate with the forming double membraned vacuole: Atg5-Atg12-Atg16 and LC3-PE. Atg3, Atg7 and Atg10 contain ligase activity and are responsible for formation of the complexes, while Atg4 mediates proteolysis of LC3 before it is conjugated to PE. Between the two complexes, only LC3-PE remains associated with the vacuole throughout the autophagic process.
LC3:microtubule-associated protein 1 light chain 3, PE: phosphatidyl-ethanolamine.



limited degradation capacity. It entails the delivery of proteins with the signature sequence KFERQ to the lysosome through lysosomal transporters for degradation (Dice 2007, Majeski et al. 2004). Conversely, microautophagy and macroautophagy are considered to be bulk degradative processes. Microautophagy involves the invagination or protrusion of the membrane belonging to the degradative organelle in order to engulf the cytoplasmic material destined to be digested (Muller et al. 2000). Macroautophagy refers to a process in which a double-membrane vacuole, termed an autophagic vacuole (AV) or autophagosome, is formed de novo and subsequently fuses with the lysosome where its contents are degraded along with its inner membrane (Shintani et al. 2004). Macroautophagy is the largest contributor to bulk degradation of proteins and organelles. Consequently, macroautophagy, hereafter referred to simply as autophagy, is the most studied of the three processes and will be the focus of this short review.

4.2 Autophagic regulation

4.2.1 Regulation of induction

The morphological markers of this phenomenon were originally described in electron micrographic images of mammalian cells in the 1960's (de Duve 1963). Since then, the mechanism of the autophagic process has been described in much more detail. In lower organisms, autophagy is induced under conditions of nutrient starvation as a means to maintain metabolic homeostasis. As such, autophagy is, at least in part, regulated by the nutrient sensing pathways which affects the phosphorylation state of the serine/threonine protein kinase TOR (Target Of Rapamycin) (Petiot et al. 2000). Phosphorylated TOR acts to negatively regulate autophagy primarily through regulation of transcription and translation but also via hyperphosphorylation of Atg13, an autophagic protein which when phosphorylated displays a decreased binding affinity for other autophagic proteins (Kamada et al. 2000, Shmelzle et al. 2000). Also regulating autophagic induction, are the phosphatidylinositol 3-

kinase complexes (PI3Ks), a family of enzymes which ultimately regulate glucose uptake through phosphorylation of inositol phospholipids. PI3Ks are divided into three classes, class I, II and III, based on their structural differences, lipidic substrate specificity, and regulatory components. The class I and class III PI3Ks have been associated with earlier steps of autophagosomal formation (Petiot et al. 2000). Class III PI3Ks, in particular, are believed to induce autophagy through interaction with the yeast Atg6 or the human homologue, Beclin-1.

4.2.2 The autophagic vacuole

Initiation of autophagy begins with the nucleation of a double membraned vacuole which has been speculated to be derived from several organelles including the endoplasmic reticulum (Dunn 1990) and the phagophore, a double membraned autophagosomal precursor formed de novo (Seglen et al. 1996). A subsequent vesicle expansion step follows in which autophagic proteins encoded by the autophagy-related (Atg) genes (Klionsky et al. 2003) guide the elongation and fusion of the double membrane structure to form the autophagosome. Atg genes use two different ubiquitin-like conjugation systems to form complexes which associate with the double membrane and aid in the formation of the final vesicular structure (Ohsumi 2001). The first involves the ligase activity of both Atg7 and Atg10 in the formation of the Atg5-Atg12-Atg16 complex and the second conjugation system involves proteolytic processing of Atg8 by Atg4 and sequential conjugation to the lipid phosphatidyl-ethanolamine (PE) of the autophagic vacuole with help from the ligase-like proteins Atg7 and Atg3 (Kabeya et al. 2003). In the mammalian system, the homologue of Atg8 is microtubule-associated protein 1 light chain 3 (LC3) which undergoes similar proteolytic processing and conjugation to PE to produce LC3-II (Kabeya et al. 2003). Of all the Atg genes, LC3-II is the only protein which remains associated to the vesicle and is thus often used as an autophagic marker (Klionsky et al. 2007).

4.2.3 Fusion with the lysosome

Following the completion of autophagosome formation, the vesicles are targeted to the lysosomal membrane, where the outer membrane fuses to the lysosome, releasing the inner membrane and all its contents into the acidic environment of the lysosome. Vesicle fusion involves several of the SNARE proteins as well as the class C VPS/HOPS complex (Darsow et al. 1997, Fischer von Mollard et al. 1999, Sato et al. 1998, Kim et al. 1999, Wang et al. 2002, Sato et al. 2000, Wurmser et al. 2000). Finally, the inner autophagosome membrane is degraded by lysosomal enzymes and the proteins and organelles contained within it can be broken down into their base components ready to be recycled.

4.3 Autophagic cell death

Albeit the general acceptance for the roles of autophagy in response to cellular stresses as a cellular protectant, its role in programmed cell death is less definite. Earlier experimentations have only described cell death associated with autophagy instead of demonstrating a requirement for the autophagic process for cell death.

The term "type 2 cell death" was originally coined in 1973 by Schweichel and Merker to describe a physiological cell death associated with formation of intracellular vacuoles (Schweichel et al. 1973). Since then, autophagy has been implicated in many forms of physiological cell death including the elimination of obsolete larval tissues during the metamorphosis of Drosophila (Gorski et al. 2003), the developmental cell death of neurons in chick isthmo-optic nucleus (Clarke 1990), and cell death associated with growth factor withdrawal from sympathetic neuronal cells as well as serum deprivation from PC12 cells *in vitro* (Ohsawa et al. 1998, Xue et al. 1999).

However, the necessity of autophagy in programmed cell death type 2 has been observed. For example, haploinsufficient animals for beclin-1 were found to be predisposed to tumors, suggesting

that autophagy is a tumor suppressor (Qu et al. 2003). Mouse embryonic fibroblasts deficient of Bax and Bak can be induced to undergo autophagic cell death when treated with staurosporine or etoposide and this cell death was dependent on the autophagic genes Atg5 and Beclin-1 and could be suppressed by inhibitors of autophagy, including 3-methyl adenine (3MA), a PI3K inhibitor which inhibits endogenous protein degradation and autophagosomal formation (Seglen & Gordon 1981, Shimizu et al. 2004). Programmed autophagic cell death has also been observed in L929 cells when apoptosis is inhibited via a general caspase inhibitor (Yu et al. 2004). Despite recent advances, further studies are still required to ascertain the role of autophagy as a type of programmed cell death *in vivo* during development.

4.4 Autophagy in neurodegeneration

The autophagic process is particularly important in quiescent cells such as neurons. In fact, the loss of this process by genetic ablation of either Atg5 or Atg7 (genes whose protein products are required for proper autophagic function) causes severe neurodegeneration in the murine central nervous system and death within the first day of birth (Hara et al. 2006, Komatsu et al. 2006). In these studies the authors show that autophagy can act as a normal cellular function for protein turnover and that without it, an accumulation of abnormal protein aggregates can lead to neurodegeneration. Autophagy has also been implicated in several neurodegenerative diseases due to observations of an accumulation of autophagic vacuoles in affected neurons (Boland et al. 2006, McCray et al. 2008). In Alzheimer's disease, numerous autophagic vacuoles can be found in the neocortical and hippocampal pyramidal neurons affected by the disease (Nixon et al. 2005). Likewise, these autophagosomes have been described in the substantia nigra of Parkinsonian brains (Anglade et al. 1997). Autophagic vacuoles have also been described in patients with polyglutamine diseases such as Huntington's disease (Kegel et al. 2000). Moreover, autophagic vacuoles are observed in the axons of

degenerating neurons in the brains of patients with prion diseases such as Creutzfeldt-Jakob disease (Sikorska et al. 2004). Although the presence of autophagy has been thoroughly described in many neurodegenerative disorders, the information obtained thus far does not allow us to draw conclusions concerning its role in the neurodegenerative process. It may be that the accumulation of these vacuoles is meant to be beneficial in order to rid the cell of unwanted protein aggregates. Conversely, an accumulation of autophagic vacuoles may signify an autophagic cell death. In other words, autophagy in neurodegeneration may be a mechanism of survival, attempting to inhibit cell death such as apoptosis or it may be in itself another form of programmed cell death.

4.5 Cross-talk between apoptosis and autophagy

Given the connections made between autophagy and apoptosis in which autophagy can act as both an antiapoptotic mechanism as well as another form of programmed cell death, it is not surprising that recent evidence suggests that there is cross-talk between the two pathways. The cross-talk between autophagy and apoptosis begins at the level of regulation, upstream of the machinery governing either process. Due to the discovery of a new target gene named DRAM (damage-regulated autophagy modulator), p53, a major regulator of apoptosis, became implicated in the regulation of autophagy (Crighton et al. 2006). In these studies, the authors showed that p53 induced autophagy in a DRAM-dependent manner. Likewise, autophagy has been shown to be negatively regulated via the PI3 kinase/Akt pathway, similar to the regulation of apoptosis (Arico et al. 2001). Moreover, FADD, a key regulator of the extrinsic death receptor pathway of apoptosis, has also been shown to induce a programmed cell death involving the formation of autophagic vacuoles (Thornburn et al. 2005) and is required for Atg5-induced cell death (Pyo et al. 2005).

4.5.1 Bcl-2 proteins in autophagy

The cross-talk between these two pathways has also been seen further downstream, at the level of their regulatory proteins in which Bcl-2 proteins can aid in the regulation of autophagy and the Atg proteins function to regulate apoptosis. Interestingly, Beclin-1 was originally discovered as a Bcl-2-interacting protein, suggesting that this protein plays a role in the cross-talk between apoptosis and autophagy (Liang et al. 1999). More recently, structural and biochemical evidence has demonstrated that Beclin-1 binds to Bcl-2, Bcl-x, Bcl-w and to a lesser extent Mcl-1 and it does so via its BH3 binding domain (Erlich et al. 2007, Feng et al. 2007, Maiuri et al. 2007, Oberstein et al. 2007). Both Bcl-2 and Bcl-x can inhibit autophagy by binding to Beclin-1 and interfering with its actions (Pattingre et al. 2005). As such, disrupting the interaction between Bcl-2 and Beclin-1 has been shown to upregulate the autophagy process (Maiuri et al. 2007). Interestingly, the pool of Bcl-2 proteins which can control autophagy are those at the endoplasmic reticulum and not those located at the mitochondria since mitochondrial-targeted Bcl-2 was not able to inhibit autophagy induced by starvation (Pattingre et al. 2005). As previously mentioned, Bcl-2 can act at the endoplasmic reticulum to control the release of Ca^{2+} during regulation of apoptosis. This function, however, also has implications in autophagy regulation in that an increase of cytosolic Ca^{2+} results in activation of Ca^{2+}/calmodulin-dependent kinase β and AMP-activated protein kinase which phosphorylate mTOR and increase its activity as a negative regulator of autophagy, resulting in an accumulation of autophagosomes (Høyer-Hansen et al. 2007). Contrary to their role in Beclin-1 mediated regulation of autophagy, Bcl-2 and Bcl-x have also been shown to have a proautophagic function. In mouse embryonic fibroblasts lacking both proapoptotic proteins Bax and Bak, Bcl-2 and Bcl-x overexpression induced an autophagic form of cell death and Bcl-x knockdown reduces autophagy (Shimizu et al. 2004), reminding us that the exact role of Bcl-2 proteins in autophagy regulation has yet to be elucidated.

4.5.2 Atg proteins in apoptosis

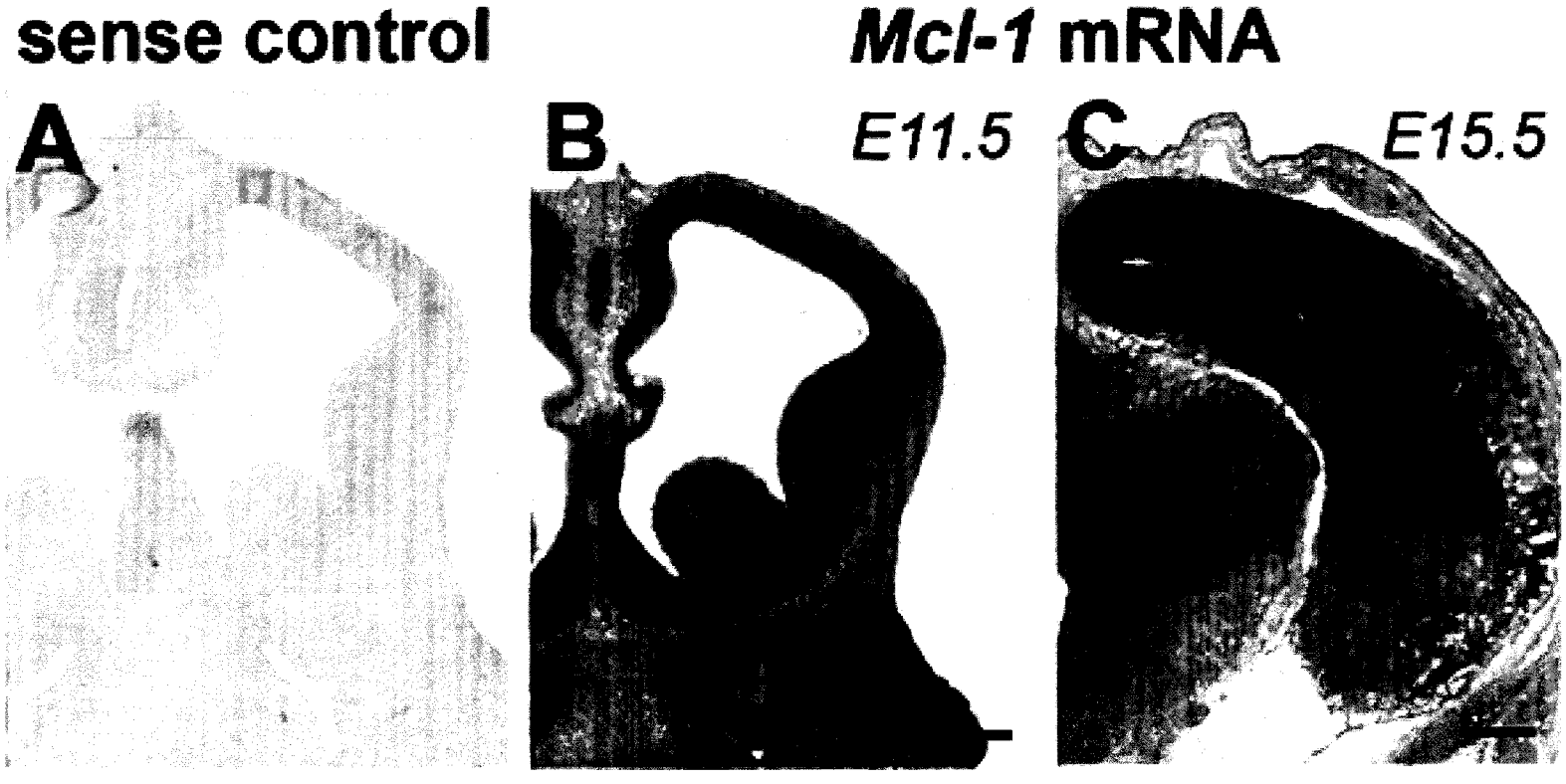
Not only do players of apoptosis aide in the regulation of autophagy, but there is evidence to support a case for the converse as well, that is that autophagic genes play a role in the regulation of apoptosis. Atg5 is of particular interest as it has been identified as a regulator of apoptosis by two different groups. Pyo and colleagues first suggested that Atg5 was involved an interferon-gamma-induced cell death by interacting with FADD (Pyo et al. 2005). This cell death was not affected by inhibiting autophagy and caspase inhibition did block cell death without affecting the number of autophagosomes present. A 24kD truncated form of Atg5 was then identified to be associated with apoptosis by Yousefi and colleagues (Yousefi et al. 2006). Atg5 was found to undergo calpain-mediated cleavage followed by translocation to the mitochondria, where the truncated form interacted with Bcl-xL, ultimately leading to cytochrome c release. Similarly, Atg5 knockdown can inhibit staurosporine-induced apoptosis. Lastly, knockdown of Beclin-1 by siRNA has also shown to inhibit DISC formation and caspase 3/8 activation (Kim et al. 2008). These studies clearly describe a role for Bcl-2 and Atg proteins in other mechanisms of cell death than their primary roles, expanding our views on apoptosis and autophagy regulation and describing a link between these two cell death pathways.

5: Previous work in our laboratory

Although its role as an antiapoptotic Bcl-2 protein has been clearly delineated, little is known regarding the role of Mcl-1 in the nervous system. However, previous work in our laboratory, examining the role of the antiapoptotic Bcl-2 protein family in neuronal apoptosis have shed some light on the importance of Mcl-1 in neuronal programmed cell death (Arbour et al. 2008). These studies have shown that Mcl-1 is expressed in neural precursor cells and post mitotic neurons of the developing murine telencephalon **(Figure 1-5)**. A role for Mcl-1 in the regulation of apoptosis in cortical neurons *in vitro* was also demonstrated. DNA damage induced cell death with camptothecin resulted in a rapid loss of Mcl-1 in cortical neurons *in vitro* **(Figure 1-6)**. In these experiments, Mcl-1 was

Figure 1-5: Mcl-1 expression in the developing telencephalon.

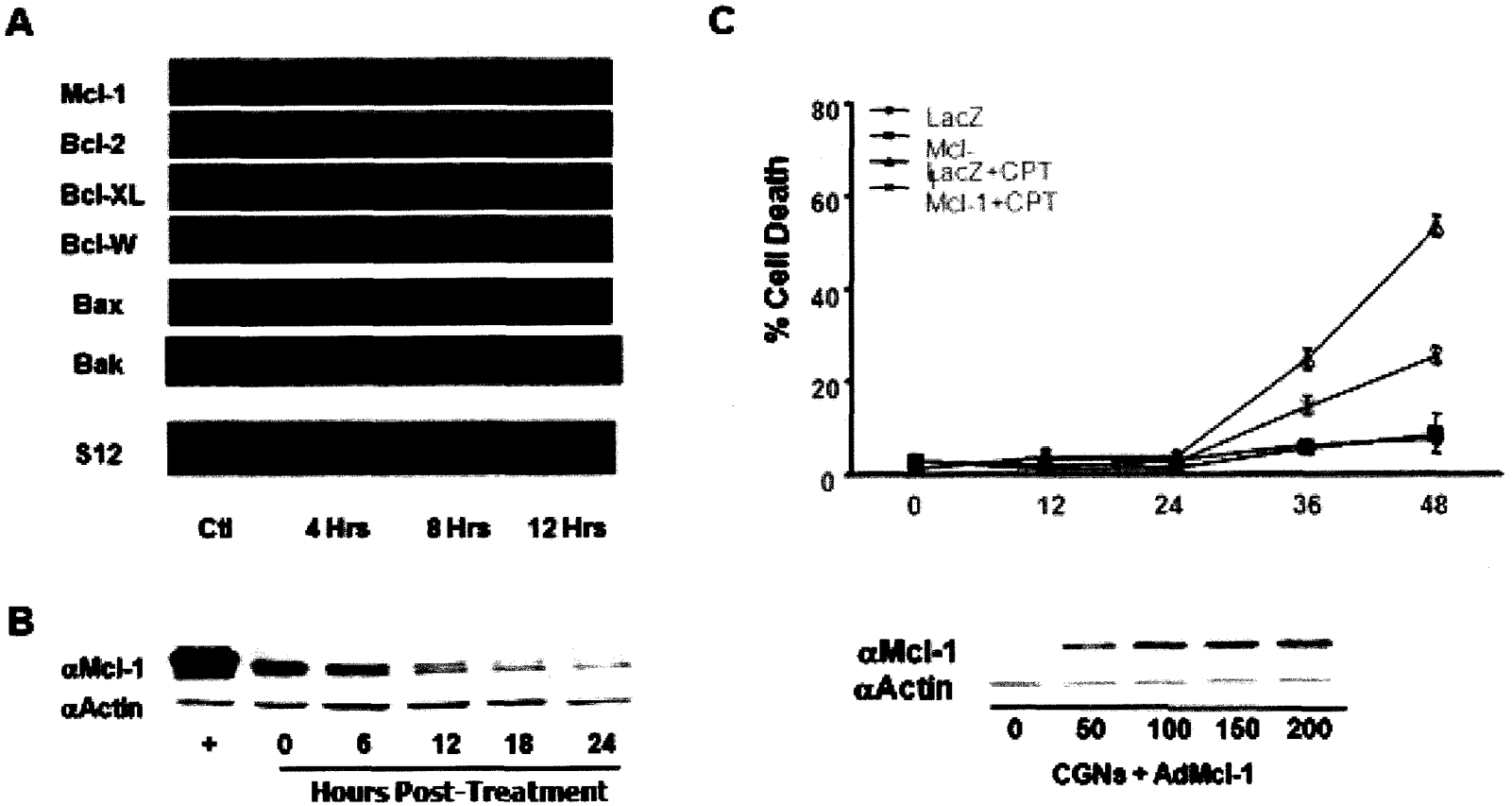
In situ hybridization analysis of Mcl-1 mRNA expression in telencephalic coronal sections revealed that Mcl-1 is expressed in the developing mouse telencephalon within neural precursors and postmitotic neurons. (A) Sense riboprobe to Mcl-1 was utilized as a negative control. (B, C) *In situ* hybridization with a riboprobe to Mcl-1 mRNA was performed on coronal section of mouse telencephalon at E11.5 and E15.5. Scale bar= 250 μm.



Arbour et al. 2008. J Neurosci. 28(24): 6068-6078.

Figure 1-6: A role for Mcl-1 in DNA damage-induced cell death in cortical neurons.

Mcl-1 is the only Bcl-2 family member protein downregulated during DNA damage induced cell death. (A) Semiquantitative RT-PCR of cortical neurons analyzed after treatment with 10 μM camptothecin. (B) Western blot analysis of Mcl-1 protein expression after treatment with camptothecin reveals levels of Mcl-1 decrease after treatment. Sustained levels of Mcl-1 can protect neurons from p53-induced apoptosis. (C) Cerebellar granule neurons were infected with AdMcl-1. Mcl-1 expression was confirmed by western immunoblot. LIVE/DEAD viability/cytotoxicity assays were performed to measure % cell death after a 10 μM camptothecin treatment.



Arbour et al. 2008. J Neurosci. 28(24): 6068-6078.

singled out as the only Bcl family protein found to be downregulated at the initiation of cell death and sustaining Mcl-1 expression through adenovirus mediated gene delivery could protect neurons against p53-induced apoptosis. Clearly, Mcl-1 is functioning at a critical point in the regulation of apoptosis and increasing its expression can enhance the ability of a neuron to persist.

6: Hypothesis

With these data as a basis, I have proposed the following hypothesis: ***Mcl-1 plays an important role in programmed cell death throughout nervous system development and in maintaining mature neurons.*** To examine this hypothesis, my research objective goals are three fold and include: 1) identification of the role of Mcl-1 *in vivo* in neuronal development 2) identification of the role of Mcl-1 in the survival of mature differentiated neurons *in vivo* and 3) examination of the mechanism of Mcl-1 regulation of neuronal cell death *in vitro*.

Chapter II: Materials and Methods

Mice

Floxed Mcl-1 (Mcl-1 fl/fl) mice previously described (Opferman et al. 2003) were bred on a C57Bl/6 background. Telencepalon-specific Mcl-1 deficient mice were generated by crossing Mcl-1 fl/fl mice with mice expressing Cre recombinase from the Foxg1 promoter bred on a C57Bl/6 and FVBN mixed background (Foxg1: Cre) (Hébert and Mcconnell 2000) to produce the Foxg1 $^{cre/+}$: Mcl-1 $^{flox/flox}$ mice. The generation of neural progenitor cell-specific Mcl-1 deficient mice was accomplished by breeding Mcl-1 fl/fl mice with mice expressing Cre from the Nestin promoter (Nestin: Cre) bred on a pure FVBN background (Bérube et al. 2005) to create Nestin $^{cre/+}$: Mcl-1 $^{flox/flox}$ mice. Generation of mice deficient for Mcl-1 in post-mitotic cortical neurons was attained by breeding the Mcl-1 fl/fl mice with CamKIIα: Cre mice created on a C57Bl/6 and FVBN mixed background (Casanova et al. 2001) to produce CamKIIα $^{cre/+}$: Mcl-1 $^{flox/flox}$ mice. The controls for the *in vivo* experiments were double heterozygote littermate (Cre/+ Mcl-1 flox/+) mice. For embryonic time points, the time of plug discovery is designated as embryonic day (E) 0.5. Experiments were approved by the University of Ottawa Animal Care Ethics Committee which adheres to the Canadian Council on Animal Care Guidelines.

DNA extraction

Tail clippings from the adult and embryonic mice were digested with 10ng of proteinase K (Gibco, 25530-031) diluted in 500uL of extraction buffer (100mM Tris, 5mM EDTA, 0.2% SDS, 200mM NaCl dissolved in dH2O, pH8.0) overnight at 55 °C. The phenol/chloroform extraction method was used as follows: 500uL of phenol:chloroform was added to each sample which was then shaken and incubated for 10 minutes at room temperature. Samples were then centrifuged at 12000 rpm for 5 minutes at room temperature and the upper aqueous layer was removed and put into an eppendorf tube and 500uL of ice cold isopropanol was subsequently added. After samples were mixed by inversion, they

were centrifuged at 12000 rpm for 5 minutes. The aqueous layer was removed, the samples were washed with 70% ethanol three times and dried on a 55 °C heat block set until just dry. The resulting pellets are then dissolved in 50uL of TE buffer (10mM Tris, 1.0mM EDTA, dH2O, pH8.0) and stored at -20 °C.

Polymerase Chain Reaction (PCR)

Genotyping for Cre was carried out using the following primers and PCR protocol: 5'-TGACCAGAGTCATCCTTAGCG-3' and 5'-AATGCTTCTGTCCGTTTGCC-3'; 94 °C for 2min, followed by 30 cycles of 94 °C for 1min to denature the template DNA, 56 °C for 1min for annealing of primers, and 72 °C for 1min 30sec for primer extension, and finally 72 °C for 5min. Each reaction contained 1X Reaction buffer (Invitrogen) containing 2.5μM of each primer, 0.2mM dNTP's, 1.75mM $MgCl_2$, Taq Polymerase (Invitrogen, 10342-020) and 1ug of template DNA. Genotyping for iCre was carried out using the following primers and PCR protocol: 5'- GACAGGCAGGCCTTCTCTGAA-3' and 5'-CTTCTCCACACCAGCTGTGGA -3'; 94 °C for 4min, followed by 30 cycles of 94 °C for 1min, 58 °C for 1min, and 72 °C for 1min, and finally 72 °C for 5min. Each reaction contained 1X Reaction buffer containing 2.5μM of each primer, 0.2mM dNTP's, 1.25mM $MgCl_2$, Taq Polymerase and 1ug of template DNA. Genotyping for floxed Mcl-1 was carried out using the following primers and PCR protocol: 5'- GCA GTA CAG GTT CAA GCC GAT G-3' and 5'- CTG AGA GTT GTA CCG GAC AA-3'; 94 °C for 5min, followed by 35 cycles of 94 °C for 1min, 55°C for 1min, and 72 °C for 1min, and finally 72 °C for 1min. Each reaction contained 1X Reaction buffer containing 2.5μM of each primer, 0.2mM dNTP's, 1.5mM $MgCl_2$, Taq Polymerase and 1ug of template DNA. The Cre and iCre PCR products were run on a 1.5% agarose gels and the Mcl-1 product was run on a 3.5% gel with ethidium bromide (Sigma, E1510-10ML). The resulting DNA bands were visualized at 700bp for Cre, 522bp for iCre, 400bp for floxed Mcl-1 and 360bp for wildtype Mcl-1 with an Alphalmager 2200 (Alpha Innotech

Corporation).

Tissue fixation and cryoprotection

Animals were sacrificed by injection of sodium pentobarbital (900mg/kg body weight). Dissected embryos were fixed in a 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at pH 7.4 overnight and cryoprotected by saturation in a sucrose gradient of 12, 16 and 22% sucrose solutions in PBS at 4 °C. Tissues were frozen on dry ice in OCT (Tissue Tek 4583), sectioned into 14 um sections at -20 °C using a cryostat (Microm, HM500) and placed on Superfrost Plus (R) slides (Fisher Scientific, 12-550-15) and stored at -80 °C.

Cresyl Violet staining

Tissue was stained with a 0.1% Cresyl Violet solution in H_2O for 15 minutes at room temperature and subsequently washed in water, followed by dehydration through ethanol gradient baths ranging from 60 to 100% ethanol for 30 seconds each. Sections were ultimately immersed in xylene solution, mounted and coversliped with permount.

Immunohistochemistry

For immunohistochemical staining, slides were prepared by rehydrating in 1X PBS for 10 minutes followed by post-fixation in acetone for 3 minutes and rinsed three times with 1X PBS for 5 minutes at room temperature. Tissue was subsequently incubated with the appropriate primary antibody dissolved in 1X PBS overnight at room temperature. Primary antibodies used include: Active Caspase 3 (AC3) (rabbit, BD Biosciences, 559565, 1:500), Phospho-Histone H3 (PH3) (rabbit polyclonal, Upstate Biotechnology, 06-570, 1:500), Nestin (mouse monoclonal; RDI, 21714, 1:200), βIII tubulin (Tuj1) (mouse monoclonal, a generous gift from Dr. David Brown, 1:100) , PSA-NCAM (mouse

monoclonal, Chemicon International Inc., MAB5324NESTINabm1:1000), Cre recombinase (mouse monoclonal, BAbCo MMS-106P, 1:500), and light chain 3 (LC3) (rabbit polyclonal, Novus Biologicals, NB 100-2220, 1:400). Slides were then washed three times with 1X PBS for 10 minutes each at room temperature before being incubated with the appropriate secondary antibody for 1 hour at room temperature. Secondary antibodies used included goat anti-rabbit 594 Alexa (Molecular Probes A-11005, 1:1000), and goat anti-mouse 488 Alexa (Molecular Probes, A-11001, 1:1000), secondary antibodies. Finally, the slides were washed with 1X PBS three times and cover slips were mounted with 1:3 glycerol:PBS and sealed with a clear nail polish.

Western blot analysis

50µg of Mcl-1 and Actin proteins were resolved on a 10% polyacrylamide gel. Gels were run in 1X SDS-PAGE running buffer (25µM Tris base, 200µM glycine and 0.1% SDS), at 200 volts, using a Biorad Power Pack 1000. Proteins were transferred onto nitrocellulose membranes for 1 hr at 4 °C at 100 volts in 1X Western Blot transfer buffer (48 µM Tris base, 386µM glycine, 0.1% SDS and 20% methanol). Membranes were subsequently blocked in 1XTPBS (NaH_2PO_4, Na_2HPO_4, NaCl, Tween-20) with 10% skim mild powder. Membranes were blotted with antibodies diluted in 1XTPBS/5% skim milk against Mcl-1 (rabbit polyclonal, Rockland Immunochemicals Inc., 600-401-394, 1:10000) and Actin (mouse monoclonal, Sigma, A-5316, 1:5000) as a loading control. The secondary antibodies, α rabbit IgG and α mouse IgG, were prepared in 1X TPBS containing 10% skim milk powder. Binding of antibodies to membranes was detected by enhanced chemi-luminescence (ECL) by mixing equal volumes of freshly made Solutions A (0.1M Tris (pH 8.5), 390µM Coumaric Acid, and 2.46mM Luminol) and B (0.1M Tris (pH 8.5) and 0.02% H_2O_2) and applying them to the membranes for 2 minutes. Membranes were then exposed to Hyperfilm x-ray film (Amersham Biosciences) and developed.

Microscopy

Sections were examined with one of two microscopes: a Zeiss Axioskop 2 microscope (Carl Zeiss MicroImaging, Inc.) or a Zeiss 510 meta confocal microscope (Carl Zeiss MicroImaging, Inc.). Immunohistochemistry or Cresyl Violet images captured with the Zeiss Axioskop 2 microscope involved standard fluorescence and brightfield/darkfield light settings at 1X 0.25NA, 5X 0.25NA, 10X 0.3NA, 20X 0.5NA or 40X 0.75NA objectives. Images were taken by a Sony Power HAD 3CCD camera with Northern Eclipse software. Images obtained on the Zeiss 510 meta confocal microscope were done on an inverted Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) with the manufacturer's integrated digital imaging software. All figures were assembled using Adobe Photoshop CS2 software.

Electron Microscopy

Postnatal mice were euthanized and subsequently perfused transcardially initially with ice-cold 1X PBS (pH 7.4) followed by a fixative solution of 2.5% glutaraldehyde and 0.5% paraformaldehyde in phosphate buffer (pH 7.4). Removed brains were then cut into 1.5mm blocks and post-fixed in 2.5% glutaraldehyde overnight before tissue was embedded in Epon. Copper grids were used to collect ultra-thin sections. Sections were then stained with a 2% uranyl acetate/lead citrate solution and viewed on a transmission electron microscope. Sections for images were cut using a Leica Ultracut E ultramicrotome and counterstained with lead citrate and uranyl acetate. Images were produced using a JEOL 1230 TEM at 60kV adapted with a 2K X 2K bottom mount CCD digital camera (Hamamatsu, Japan) with AMT software.

Quantification of labeled cells and measuring software

Measurements of ventricular zone and intermediate zone/cortical plate were done using Northern

Eclipse software. Active caspase-3 labeled cells and active caspase-3 and Nestin or β-Tubulin double labeled cells were quantified by counting in both dorsal and ventral-lateral boxed areas with respect to the ventricle. Phosphohistone H3 labeled cells were counted over a 1000μm length along the ventricle. All data is expressed as the mean of all embryos examined. At least three sections per animal were examined.

Primary neuronal cultures

Primary neurons were collected from individual cortices dissected from E 17.5 embryos. Cortices were dissected out into 1X HBSS to remove meninges at 4 °C. Cortices were then transferred into solution A (a stock solution containing 124 mM NaCl, 5.37 mM KCl, 1 mM NaH_2PO_4, 14.5 mM D-(+)-glucose, 25 mM HEPES, 3 mg/ml BSA, pH 7.4 with 1.2 mM $MgSO_4$ added) to triturate and break up the tissue. Solution and tissue pieces are then transferred to a 50mL tube containing 30mL of solution A and spun at 3000 rpm for 2 minutes. Supernatant is subsequently removed and 10mL of solution B is added (stock solution with 1.2 mM $MgSO_4$ and 0.425mg/mL Trypsin (Sigma, T-4549)). The mixture is shaken for 18-22 minutes at 200 rpm and 37 °C to further break up tissue pieces. 10mL of solution C (stock solution with 1.2 mM $MgSO_4$, 0.164mg/mL trypsin inhibitor and 0.25mg/mL DNAse 1 (Boehringer & Mannheim, 1284-932)) are then added and the tube is mixed for 2 minutes to stop the trypsin reaction. Tissue pieces are spun down by centrifugation at 3000 rpm for 5 minutes, supernatant is removed, 2mL of solution D (stock solution with 2.7 mM $MgSO_4$, 1.04mg/mL trypsin inhibitor and 0.75mg/mL DNAse 1) is added and mixture is transferred to a 15mL tube. The solution is triturated with a flame polished pasture pipette to break up tissue to single cells and left to settle for 5 minutes and supernatant subsequently transferred to a new tube and 0.3mL of solution E (stock solution with 2.5 mM $MgSO_4$, 0.1 mM $CaCl_2$) per mL of supernatant is added. The solution is mixed and left to settle for 10 minutes. The supernatant is then spun at 3000 rpm for 5 minutes and then the supernatant from

the spin is removed and fresh neurobasal media (Invitrogen, 21103-049) containing N2 (Invitrogen, 17502-048) and B27 (Invitrogen, 17502-048) supplements and Pen/Strep (Invitrogen, 15140-122) is added to triturate and dilute cells to 1.0 million cells per mL to be plated. Neurons were platted on poly-D-lysine (VWR, 40210) coated culture dishes.

Vector production

The Cre recombinase gene was obtained via polymerase chain reaction from a pAdTrack plasmid with Cre cloned into it (a generous gift from Dr. Peter Greer) using the following primers and protocol: 5'-ACTGCTCGAGATGTCCAATTTAC-3' and 5'-ATCGGAATTCATCGCCATCTTCCAG-3'; 94 °C for 5min, followed by 30 cycles of 94 °C for 1min to denature the template DNA, 55 °C for 1min 30 sec for annealing of primers, and 72 °C for 1min 30sec for primer extension, and finally 77 °C for 5min. The reaction contained 1X Reaction buffer containing 2.5μM of each primer, 0.2mM dNTP's, 1.25mM $MgCl_2$, 1ug of template DNA and High Fidelity Platinum Taq Polymerase (Invitrogen, 11304-011). Results were run on a 2% agarose gel and visualized through UV exposure to excise the gel piece. DNA was purified from the gel with the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, 28-9034-70). To ligate Cre into pEGFP (Clontech, 6080-1), the Cre DNA and vector were both digested with EcoRI (NEB, R0146S) and XhoI (NEB, R0101S). DNA was then run on a 2% gel, visualized with UV light and purified from the gel using the illustra GFX PCR DNA and Gel Band Purification Kit. The resulting pieces were then ligated together with T4 DNA ligase (NEB, M0202S) at 4°C overnight. Resulting DNA was then transformed into Stbl3 *E. coli* cells, plated onto 10 cm dishes containing LB growth media and 100μg/ml kanamycin, and incubated overnight at 37°C. From overnight plates, 10 colonies were picked to inoculate 5ml of LB broth containing 100μg/ml kanamycin. Cells were grown at 37°C overnight with shaking at 225rpm. DNA was then isolated from each colony using a QIAprep Spin Miniprep Kit (Qiagen, 27104). Vectors were analyzed for Cre insert by restriction

digest with BamHI (NEB, R0136S) and NheI (NEB, R0131S). The correct plasmid was then selected to inoculate 250ml LB broth containing 100µg/ml kanamycin and grown overnight at 37°C while shaking. DNA was isolated using a QIAprep Plasmid Maxi Kit (Qiagen, 12162). To ligate Cre-EGFP into the final gene delivery vector: pWPXLd (Trono Lab, École Polytechnique Fédérale de Lausanne) was then digested with BglII (NEB, R0144) and XbaI (NEB, R0145T) and purified with a GFX column between digestions. pEGFP-Cre was digested with BamHI (NEB, R0136S) and SpeI (R0133S) and run on a 2% gel to excise the plasmid using the illustra GFX PCR DNA and Gel Band Purification Kit. DNA pieces were ligated together with T4 DNA ligase overnight at 4°C. The final plasmid was selected for using the same protocol as Cre-EGFP selection with the following change: kanamycin was replaced with ampicillin.

Lentivirus production

Lentivirus particles were produced using 293T cells maintained in 0.45µm filtrated DMEM, 10% non-heat inactivated serum, 1%pen/strep. Cells were platted to be 70% confluent around time of transfection. 2 hrs before transfection, medium was replaced with 15ml of fresh medium preheated to 37°C. To prepare for transfection, all three lentivirus plasmids (vector pWXLd, envelope pMDG.2, and packaging psPAX2) (Trono Lab, École Polytechnique Fédérale de Lausanne) were incubated in DMEM with 0.15M NaCl at a molar ratio of 1:1:1 and a final volume of 7.5ml. A separate tube was incubated with DMEM (0.15M NaCl, 2.75ml of PEI) to a final volume of 7.5ml. Tubes were incubated for 5 min at room temperature and then combined, mixed by vortexing and incubated for another 20 to 30 minutes at room temperature. 3ml of mixture was subsequently added to each 15cm plate. 14-16 hrs post-transfection, medium was removed and replaced with fresh medium. Medium containing lentiviral vectors was collected 24 and 48 hrs post-transfection. Medium was then centrifuged at 2000rpm for 5 min to remove cell debris and filtered through a 0.45µm filter. Supernatants were

pooled together and 23ml of viral supernatant was transferred to a Beckman ultra clear centrifuge tube (Beckman, 344058) with a 20% sucrose cushion and concentrated by ultracentrifugation at 20,000 rpm for 2 hrs at 6°C in a Beckman SW28 Ti swinging rotor. Pellets are resuspended in 200µl 1XPBS/1%BSA.

Lentivirus titering

For a live titer, 293T cells (2.5 x 10^5 cells/ml) were seeded into a 24-well plate using complete media (DMEM/10%FBS/1%pen/strep). Virus stock was diluted with un-supplemented media to a final concentration of 10^{-3} to 10^{-10}. 0.5ml of each diluted virus was added to paired wells and plates were incubated at 37°C in 5%CO_2 for 48 hrs. Media was then replaced with pre-warmed 1X PBS and the number of positive cells was counted under 20X or 32X objective. Final titer for pWPXLd-Cre-EGFP was 3.5 x 10^7 IVP/ml. Final titer for pWPXLd was 1.5 x 10^9 IVP/ml.

Transduction of neuronal cultures

Primary neuronal cultures were transduced with either pWPXLd-Cre or pWPXLd lentiviruses by addition of virus to the cultured media at an MOI of 1.

Statistical analysis

Error bars in all figures represent the standard error of the mean (SEM). Significant difference between numbers were assessed using two-tailed t-test analysis assuming unequal variance ($P<0.05$). N=3 delineates that 3 different mice were examined per data point.

Chapter III: Results

1: The role of Mcl-1 in neuronal development *in vivo*

1.1 Neuronal Mcl-1 is required for embryonic development and cortical neurogenesis

We have previously shown that Mcl-1 is expressed in neural precursor cells and post-mitotic neurons of the developing murine telencephalon and that Mcl-1 expression is downregulated at the initiation of cell death *in vitro* (Arbour et al. 2008). As such, we hypothesized that Mcl-1 plays an important role in programmed cell death of the developing nervous system *in vivo*. Germline Mcl-1 deficiency in mice results in pre-implantation lethality (Rinkenberger et al. 2000), thus to determine the role of Mcl-1 in the developing nervous system, we used neural specific conditional knockouts for Mcl-1. To create these mice, Mcl-1-floxed mice (Opferman et al. 2003) (Mcl-1 fl/fl) were bred with mice expressing Cre-recombinase driven from the Nestin (Nestin:Cre) (Bérubé et al. 2005) or Foxg1 (Foxg1:Cre) (Hébert et al. 2000) promoters. Nestin is an intermediate filament gene expressed in neural stem and progenitor cells, first appearing at embryonic day (E) 7.75 but becomes widespread throughout the whole nervous system by E10.5 (Dahlstrand et al. 1995 & Mignone et al. 2004). Foxg1, (also known as Brain Factor-1 [BF-1]), is a transcription factor belonging to the Drosophila homeotic gene fork head family (Tao et al. 1992). Foxg1 expression is detected in the murine brain as early as E8.5 and is found predominantly in the telencephalon, with lower levels in the developing ear, olfactory epithelium and foregut (Hébert et al. 2000, Tao et al. 1992 & Shimamura et al. 1997). Interbreeding the Mcl-1 floxed mouse with two different mice driving Cre expression through two distinct neural specific promoters (Nestin and Foxg1), provides a system with which to selectively examine the role of Mcl-1 in the developing nervous system.

Deletion of Mcl-1 during nervous system development with either Nestin:Cre or Foxg1:Cre resulted in embryonic lethality around E 12.5 and 16.5, respectively **(Table 3-1)**. Cortical development

		E9.5	E10.5	E12.5	E15.5	E16.5	E17.5	E19.5
Nestin Cre: Mcl-1	# embryos collected	9	30	74	20			
	# knockouts (%)	2 (22%)	8 (27%)	9 (12%)	0 (0%)			
	# expected Mendelian ratio (%)	2 (25%)	8 (27%)	17 (24%)	5 (25%)			
Foxg1 Cre: Mcl-1	# embryos collected			20	32	11	11	4
	# knockouts (%)			3 (15%)	8 (25%)	1 (9%)	0 (0%)	0 (0%)
	# expected Mendelian ratio (%)			4 (20%)	7 (21%)	2 (25%)	2 (25%)	1 (25%)

Adapted from Table 2 of Arbour et al. 2008. J Neurosci. 28(24):6068-6078.

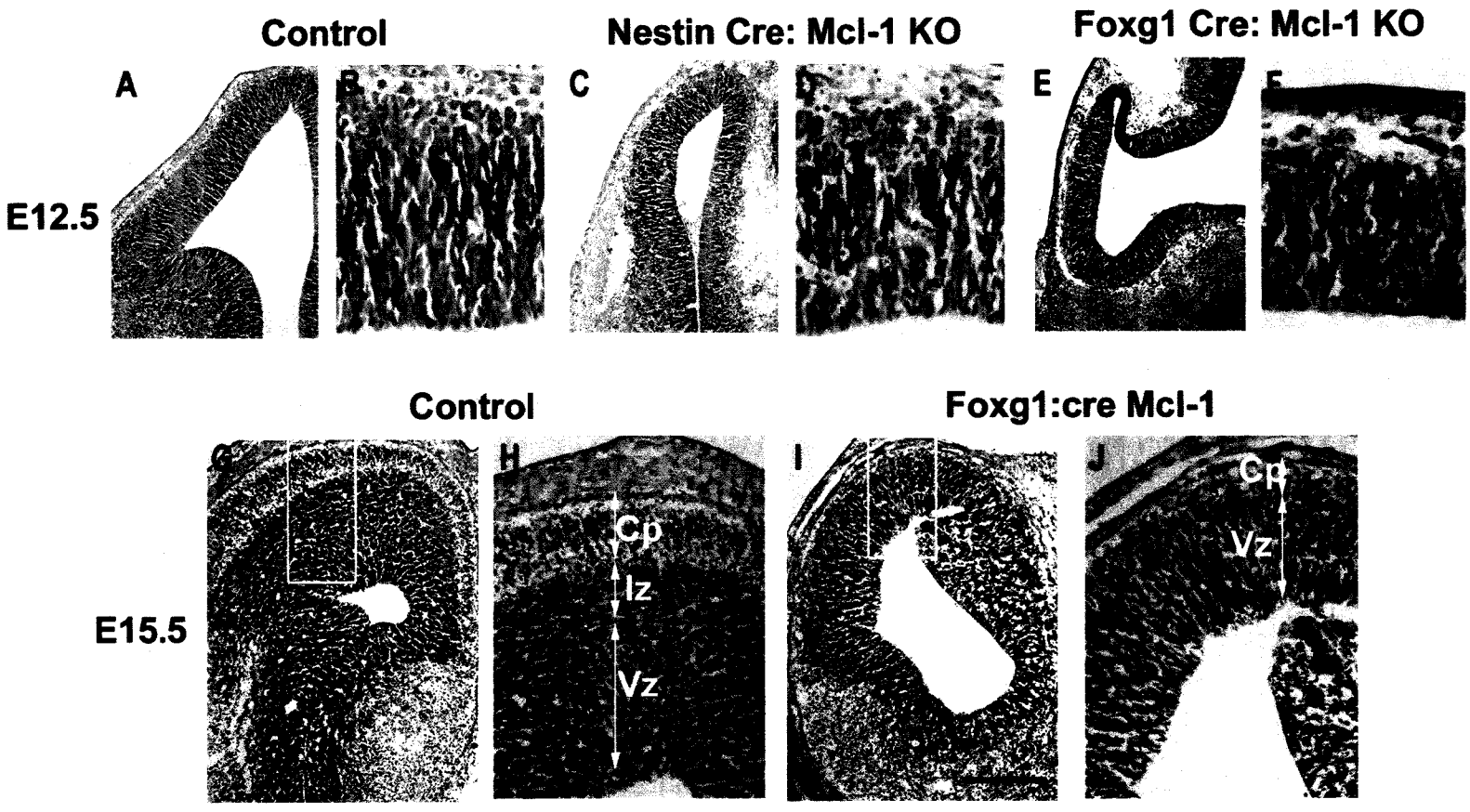
Table 3-1. Conditional deletion of Mcl-1 in the developing brain results in embryonic lethality. Mcl-1 fl/fl female mice were bred with either Nestin Cre: Mcl-1 fl/+ or Foxg1 Cre: Mcl-1 fl/+ males and sacrificed at embryonic days specified. Data is presented as a percentage of the number of knockout embryos per total embryos recovered from the Mcl-1 conditional mutants versus the expected number of knockouts based on a Mendelian ratio. Note the drop in number of embryos collected compared to the expected Mendelian ratio at E12.5 for the Nestin: Cre Mcl-1 fl/fl mice and at E16.5 for the Foxg1: Cre Mcl-1 fl/fl mice, suggesting that the mutants are dying at the respective ages.

takes place in the mouse embryo between embryonic day 10.5 and 17.5. As such, brains from the Nestin: Cre +/- Mcl-1 fl/fl and Foxg1: Cre +/- Mcl-1 fl/fl mutants and their heterozygote control littermates: Nestin: Cre +/- Mcl-1 fl/+ and Foxg1: Cre +/- Mcl-1 fl/+ were collected at E12.5 and E15.5 (Foxg1: Cre Mcl-1 mice only) corresponding to early neurogenesis and mid-neurogenesis, respectively, in order to examine the role of Mcl-1 during cortical development. Morphological analysis using Cresyl Violet staining revealed a severe defect in cortical development of both conditionally deficient mice **(Figure 3-1)**. A reduction in the size of the intermediate zone and cortical plate was also observed, in which both areas combined measured at 86.3 ± 12.2 microns on average in the mutant brains compared to 253.7 ± 35.0 microns in the control brains. In contrast, ventricular zone thickness remained unchanged between knockouts and controls with 169.9 ± 19.0 and 196.5 ± 8.8 microns in width for the mutants and controls, respectively. Moreover, the distinction between intermediate zone and cortical plate of the mutant brains was not clearly delineated from one another as would be the case in wild type animals **(Figure 3-2)**. Overall, these results demonstrate that Mcl-1 is required for cell survival during forebrain development. In particular, the loss of Mcl-1 affects the composition of the intermediate zone and cortical plate, regions that comprise largely newly committed neurons, suggesting that Mcl-1 could be required for the survival of post-mitotic cells that have migrated out of the ventricular zone.

1.2 Neural progenitors lacking Mcl-1 die during differentiation and migration via an apoptotic mechanism

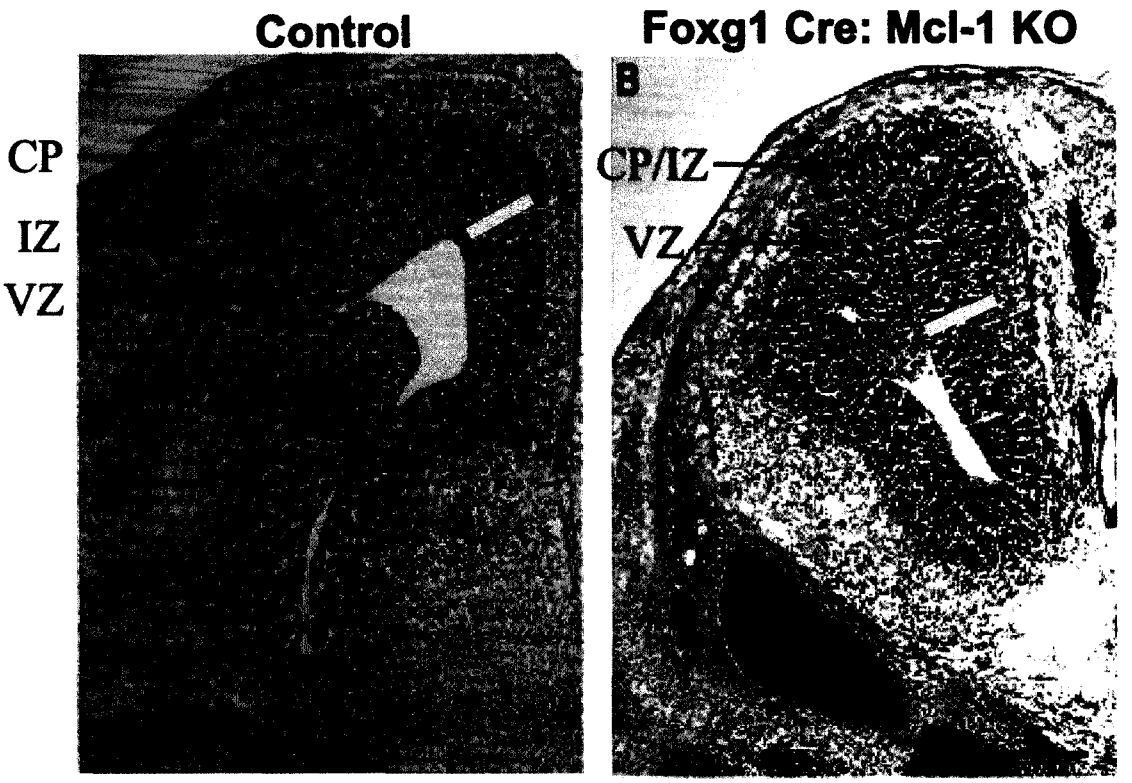
The drastic reduction in neurons in both models of Mcl-1 deficiency suggests that Mcl-1 is required for the survival of neural progenitor cells and committed neuroblasts within the developing brain. Given its prominent role in apoptotic regulation, acting upstream of the BclxL-Bax signaling cascade (Nijhawan et al. 2003), we hypothesized that the loss of Mcl-1 would affect neuronal

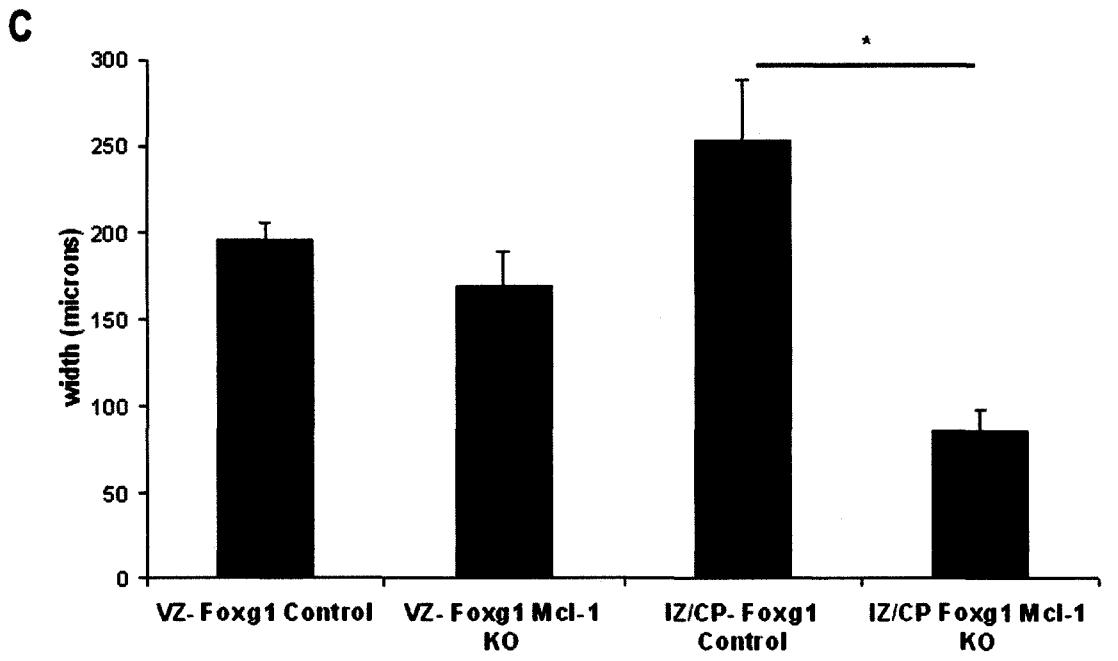
Figure 3-1. Conditional deletion of Mcl-1 in neuronal progenitors disrupts cortical development. (A-J) Coronal sections of E12.5 and E15.5 (Foxg1 Cre: Mcl-1 mice only) embryos were collected from control (A-B & G-H) as well as Nestin Cre: Mcl-1 (C-D) and Foxg1 Cre: Mcl-1 (E-F & I-J) conditional knockout mice and stained with cresyl violet. Cp= cortical plate, Iz= intermediate zone, Vz= ventricular zone. Scale bars represent 250 μm.



Adapted from Figure 2 of Arbour et al. 2008. J Neurosci. 28(24): 6068-6078.

Figure 3-2. Foxg1 Cre: Mcl-1 mutants display significantly thinner cortices. (A-B) Coronal sections from E15.5 control and Foxg1:Cre Mcl-1 mutants were stained with cresyl violet and the thickness of cortical plate (CP), intermediate zone (IZ) and ventricular zone (VZ) were measured (C). Scale bar represents 250 μm. Unlike the controls, mutant brains do not have a clearly delineated border between IZ and CP and these regions are dramatically reduced in size compared to littermate controls (P= 0.046). Error bars represent standard error of the mean. n=3

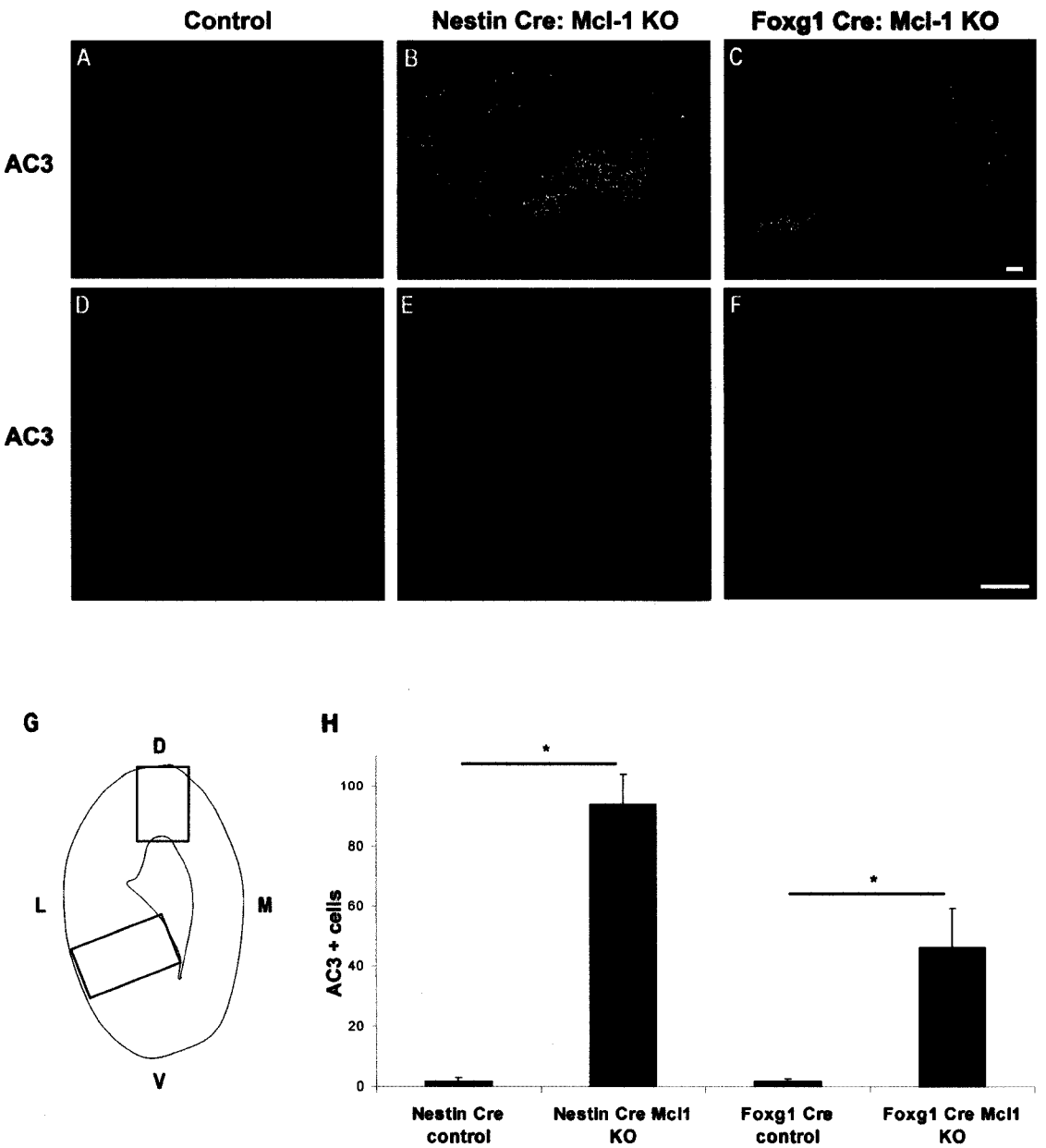




apoptosis. To assess whether this loss in cellularity is the consequence of increased apoptosis, the mutant brains were stained for a hallmark of classical apoptosis, active caspase 3 (AC3). Active caspase 3 immunostaining performed on both mutant lines at E12.5 revealed a significant increase in the number of apoptotic cells throughout the developing ventricular and subventricular zone of the mutant mice compared to their control littermates **(Figure 3-3)**. The number of positively labeled active caspase 3 cells within the developing cortex was quantified by counting within defined areas of the somatosensory cortex and motor cortex as delineated by the dorsal and ventral-lateral boxed areas diagram. In the regions examined, Nestin: Cre and Foxg1: Cre Mcl-1 mutants contained an average of 93.9 ± 28.8 positively labeled cells compared to 1.6 ± 1.5 in the littermate control and 46.3 ± 12.9 versus 1.6 ± 0.8 positively labeled cells, respectively. These results indicate that there is an increase in neural progenitor apoptosis in the developing forebrain when Mcl-1 is removed, demonstrating that Mcl-1 is an important regulator in neural precursor apoptosis during cortical development.

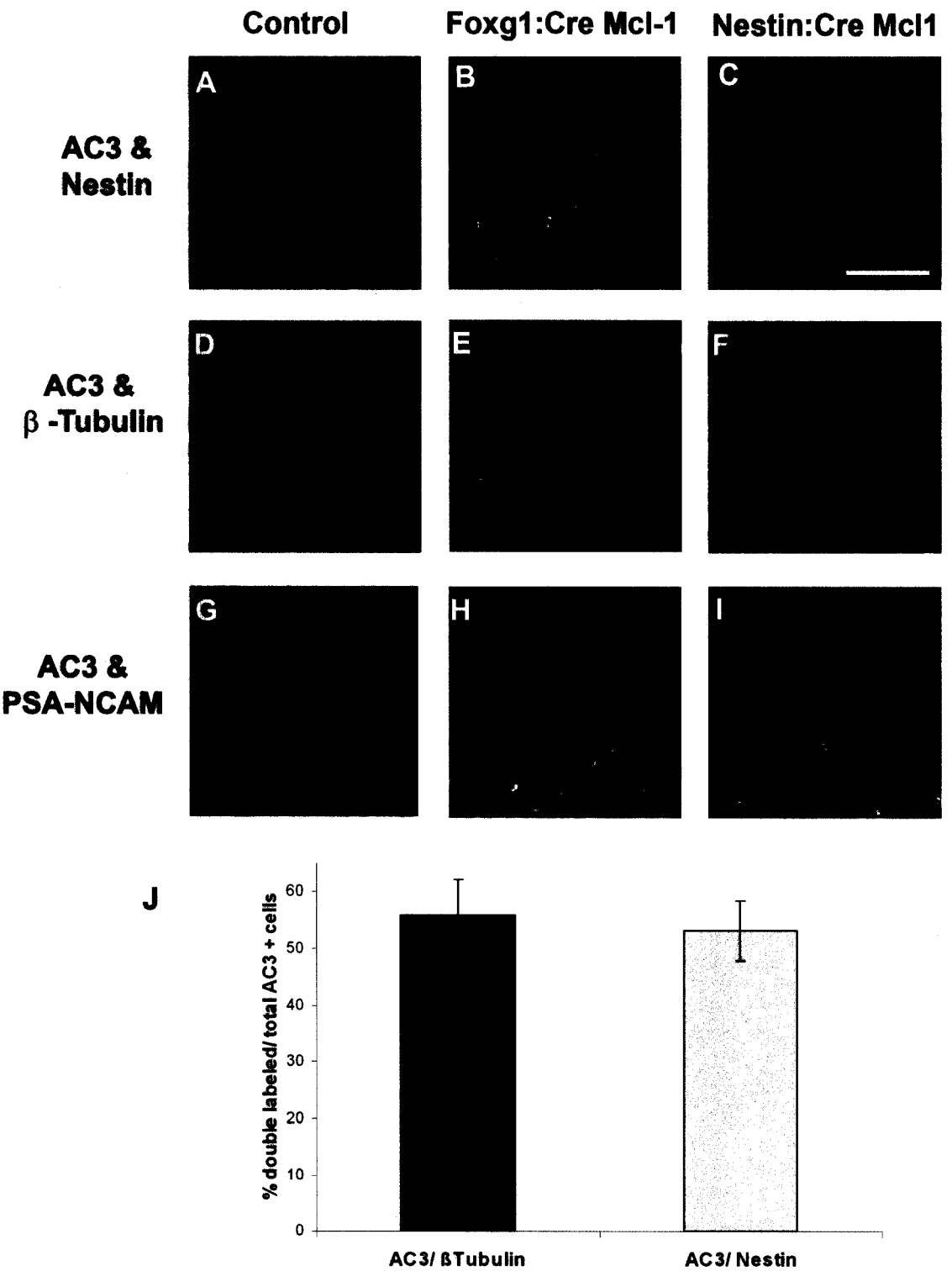
The apoptotic cells observed appeared to span the width of the forebrain from the ventricular zone to the cortical plate. Consequently, further investigation was necessary to determine at what developmental time point these Mcl-1 deficient neural precursors are undergoing apoptosis. To determine which subpopulations of neurons are dying, cells with double staining of antibodies against active caspase 3 with a marker of neural progenitor cells (Nestin), a marker of newly differentiated neurons (β-Tubulin), or a marker of migrating immature neurons (PSA-NCAM) were quantified **(Figure 3-4)**. Double labeled neurons can be observed with all three markers suggesting that neurons are dying before and after commitment to a neuronal fate. Quantification of the percentage of apoptotic neurons which are co-labeled with Nestin or β-Tubulin revealed that approximately half of the apoptotic neurons were labeled with either marker (55.8% ± 6.3 of AC3 positive cells expressing β-Tubulin versus 53.1% ± 5.3 of AC3 positive cells expressing Nestin), suggesting that the apoptotic neurons consisted of both uncommitted neural precursors and newly committed neurons. In conjunction with

Figure 3-3. Mcl-1 deficient neural progenitors die via an apoptotic mechanism. (A-F) Foxg 1: Cre and Nestin: Cre Mcl-1 conditional knockout and control littermate brains were collected at E12.5, sectioned coronally and stained with Active Caspase 3 (AC3). Scale bars represent 100 μm (A-C) and 50 μm (D-F). (G-H) AC3 positively labeled neurons were counted in both dorsal and ventral-lateral boxed areas and found to be significantly higher in the conditional mutants (P=0.040 and 0.012 for Foxg I Cre: Mcl-1 and Nestin Cre: Mcl-1). Error bars represent standard error of the mean. n=4 for Foxg1 Cre: Mcl-1 data and 3 for Nestin Cre: Mcl-1 data



Adapted from Figure 4 of Arbour et al. 2008. J Neurosci. 28(24): 6068-6078.

Figure 3-4. Mcl-1 deficient neural progenitors are dying during differentiation. (A-I) E12.5 Foxg1: Cre Mcl-1 and Nestin: Cre Mcl-1 conditional knockout and control littermate brains were stained with AC3 (red) and either Nestin, β-Tubulin or PSA-NCAM (green). Double labeled cells appear as yellow. Scale bar represents 50 μm. (J) Double labeled cells were counted in Nestin and β -Tubulin stained sections of Nestin: Cre Mcl-1 conditional knockout and control littermate brains. Results are displayed as % double labeled cells/ total AC3 positive cells. Error bars represent standard error of the mean. n=3



the considerable co-labeling observed with the intermediate marker between these two time points of neuronal differentiation, PSA-NCAM, the data presented above provides evidence that the antiapoptotic protein, Mcl-1, functions to regulate apoptosis in neural progenitor cells during the process of neural precursor commitment and migration away from the ventricular zone.

1.3 The loss of Mcl-1 does not significantly affect proliferation

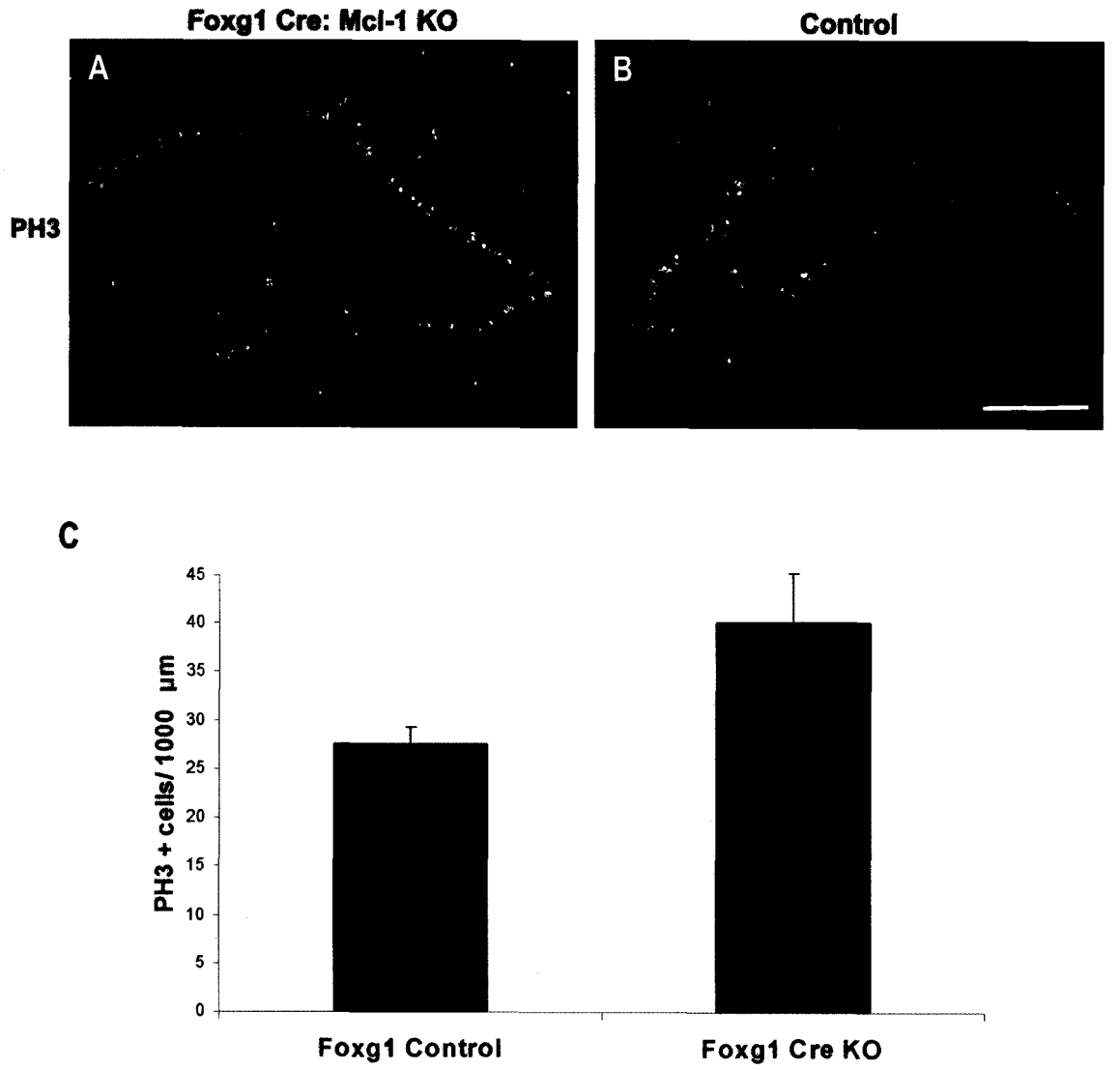
Mcl-1 has been shown to have implications in the regulation of cell cycle progression. In two separate studies, overexpression of Mcl-1 in cell lines produced an antiproliferative effect (Fujise et al. 2000 & Jamil et al. 2005). While the increase in cell death we observe could account for the decrease in cortical volume, a role for Mcl-1 in regulation of cell cycle progression may also contribute to the observed reduction in thickness of the mutant cortices. To examine this hypothesis, E15.5 coronal sections of the Foxg1: Cre Mcl-1 mutant and control mice were labeled with an antibody against phosphohistone H3 (PHH3), a marker of M-phase in the cell cycle (Hendzel et al. 1997). Quantification of the number of cycling cells of the subventricular zone did not display a significant difference between mutants and control littermates with an average of 40.2 ± 5.1 and 27.5 ± 1.7 cells expressing phosphohistone 3, respectively **(Figure 3-5)**. Thus Mcl-1's role in cell cycle progression is not a factor in the loss of cellularity of these Mcl-1 deficient mice. The results above indicate that Mcl-1 is required for proper cortical development and for the survival of neural progenitors as it is a critical antiapoptotic regulating protein, demonstrating a novel role for Mcl-1 in neuronal development.

2: The role of Mcl-1 in mature post-mitotic neurons *in vivo*

2.1 The loss of Mcl-1 in post-mitotic neurons results in cortical degeneration

Given the novel role for Mcl-1 in cell survival during neuronal development, we hypothesized that a loss of Mcl-1 would also affect the survival of the mature nervous system. To examine the role

Figure 3-5. The loss of Mcl-1 does not significantly affect proliferation. (A-B) E15.5 Foxg1: Cre Mcl-1 sections were stained with phosphohistone-3 (PHH3) antibody. Scale bar represents100μm. (C) Quantification of PHH3 positive cells revealed no significant difference between the number of positively labeled PHH3 cells in the conditional mutant compared to control littermate. (P= 0.141) Counts are expressed as number of positively labeled cells per a fixed distance of 1000 μm along the ventricles. Error bars represent standard error of the mean. n=3



Adapted from Figure 3 of Arbour et al. 2008. J Neurosci. 28(24): 6068-6078.

of Mcl-1 in the survival of mature, differentiated neurons, conditional knockouts of Mcl-1 were created by crossing Mcl-1-floxed mice with mice expressing Cre from the CamKIIα promoter (Casanova et al. 2001). CamKIIα: Cre is expressed around birth between embryonic day 18 and postnatal day 1 and is found predominantly in the cortex and hippocampus but is also present in the amygdala, olfactory bulb, striatum, thalamus and hypothalamus (Casanova et al. 2001).

Similar to the embryonic Mcl-1 deficient mice, deletion of Mcl-1 in mature neurons of postnatal mice with CamKIIα: Cre resulted in premature lethality around postnatal day 30 (P30) (Arbour & Slack, unpublished data). To examine the role of Mcl-1 in maintenance of cortical neurons, mutant mice were collected at the onset of deletion of Mcl-1 by CamKIIα: Cre at birth (P1), throughout the maturation of the transgenic mice at one (P7) and two weeks (P14) of age and prior to their demise at one month of age (P30). Cresyl violet staining revealed a rapid loss of neurons in the cortices of the conditional knockouts between P7 and P30, resulting in a near complete loss of the cortex by one month of age. Magnification of the mutant cortex reveals a decrease in cellularity between the mutant mice relative to their control littermates at each time point following P7 **(Figure 3-6)**. Loss of mature neuronal cells was confirmed by staining with a mature neuronal marker NeuN, in which a drastic loss of mature neurons is apparent at P14 **(Figure 3-7 a-b)**. At higher magnification, morphological markers of cellular distress such as swollen nuclei and the formation of intracellular vacuoles were observed **(Figure 3-7 c-d)**. Many Bcl-2 protein family members have been implicated in maintaining cellular homeostasis apart from their antiapoptotic roles (Hetz et al. 2008). As such, these data may indicate that cellular homeostasis such as metabolism is affected in the absence of Mcl-1.

The unusual morphology of the neurons observed in the Cresyl Violet stained sections of the CamKIIα: Cre mice prompted us to examine them using transmission electron microscopy (TEM). This technique revealed multiple vacuoles containing cytoplasmic components including organelles such as mitochondria within the mutant cortical neurons **(Figure 3-8)**. Included among these vacuoles were

Figure 3-6. The loss of Mcl-1 in post-mitotic cortical neurons results in cortical deterioration. (A-P) CamKII α Cre: Mcl-1 conditional knockout and control littermate brains were collected at P1, P7, P14 and 1 month of age. Brains were sectioned coronally and stained with cresyl violet. (E-G & M-P) Higher magnification shown below each representative section spans from cortical plate (top of panel) towards ventricular zone (bottom). Scale bars represent 100 μm.

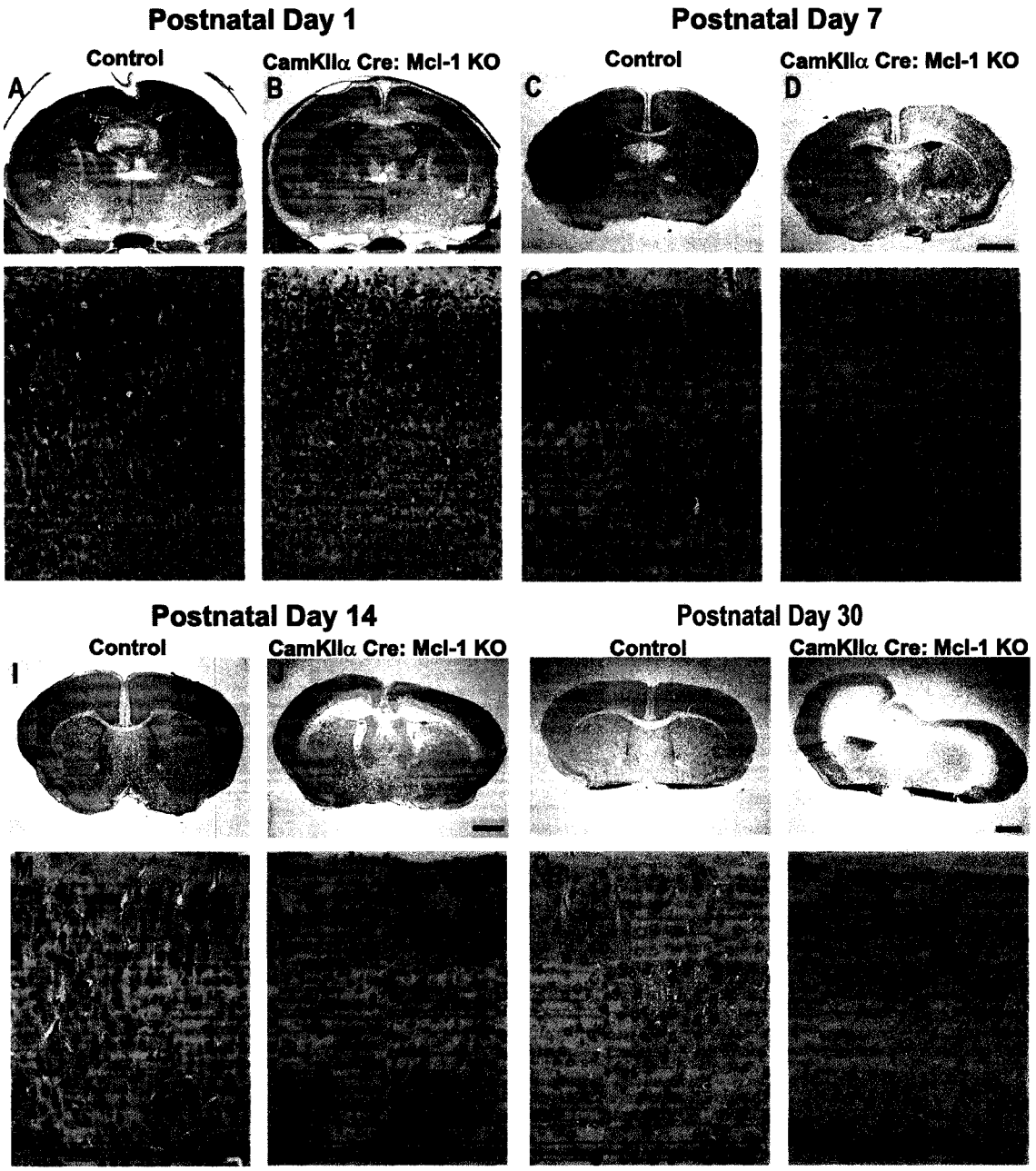


Figure 3-7. The loss of Mcl-1 in cortical neurons reduces the mature neuronal population and causes cellular vacuolation. (A-B) Coronal sections of CamKII α: Cre Mcl-1 conditional knockout and control littermate brains at postnatal day 14 were stained with a marker of mature neurons, NeuN. (C-D) High magnification photomicrographs of cresyl violet stained cortical neurons reveals abnormal nuclear morphology such as swollen nuclei and the formation of intracellular vacuoles (arrows). Scale bar represents 100 μm.

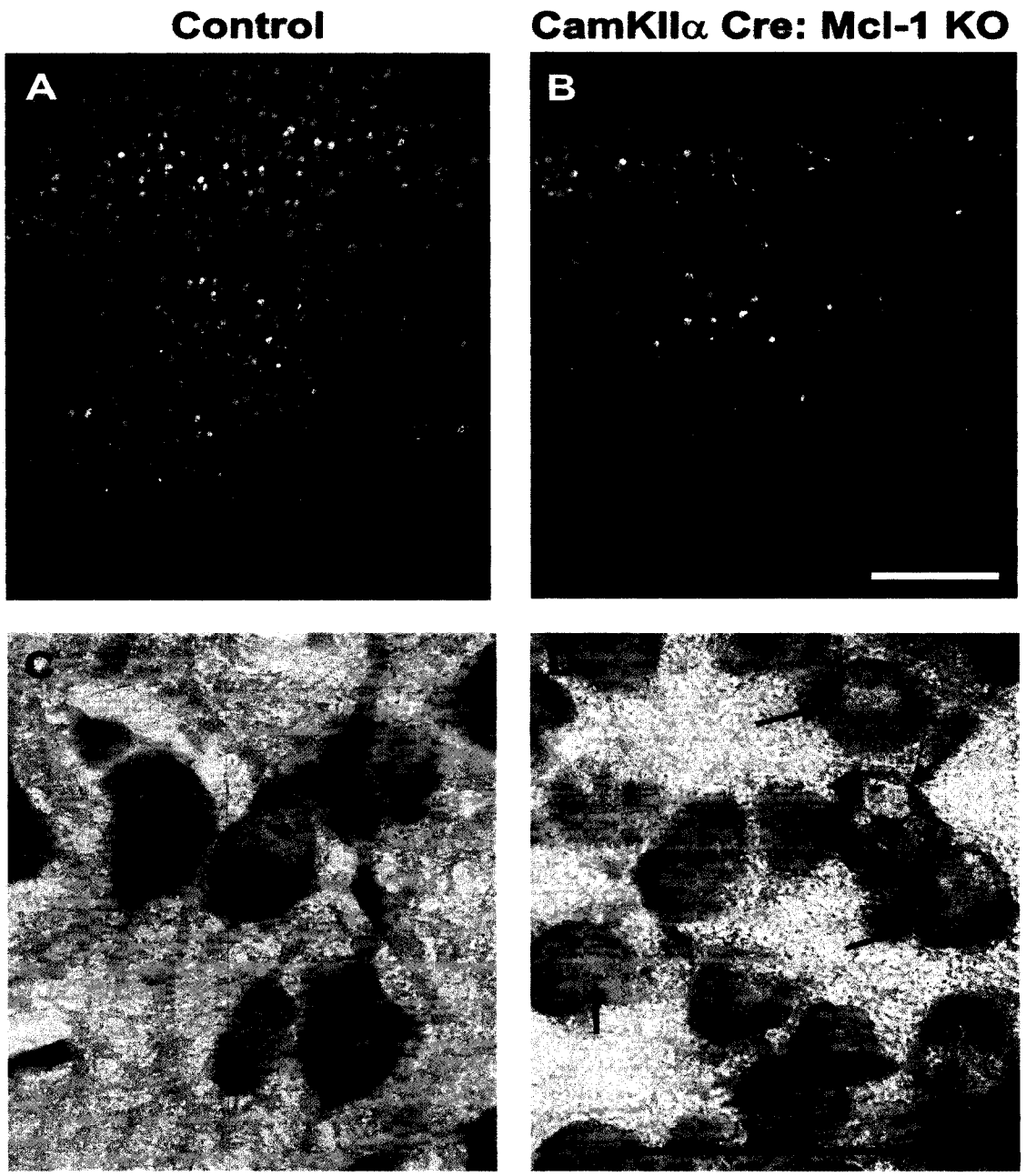
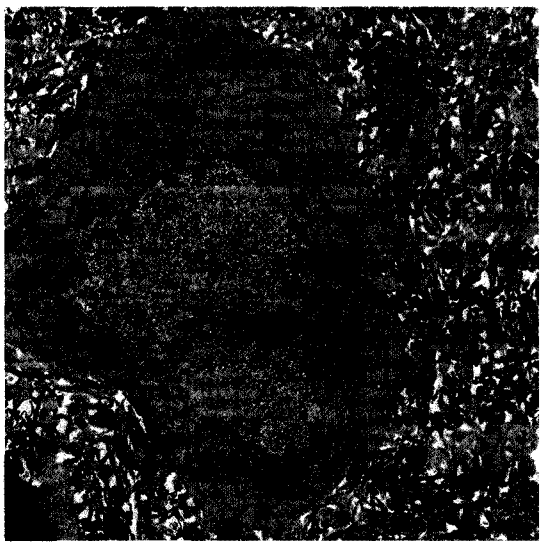
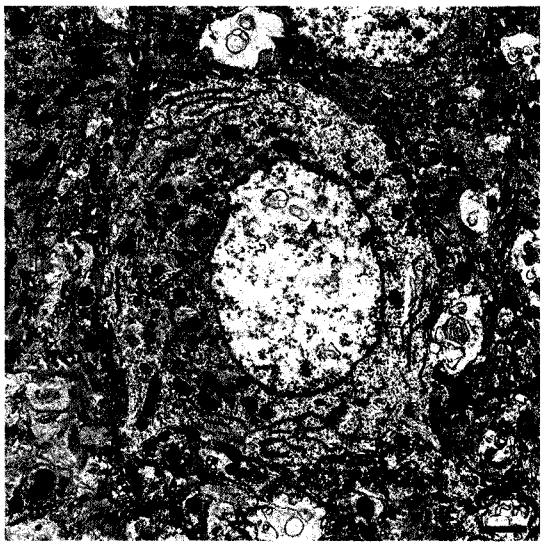


Figure 3-8. Post-mitotic neurons lacking Mcl-1 are dying via an autophagic mechanism in vivo. (A-D) Electron micrographic imaging of CamKII α: Cre Mcl-1 conditional knockout mice and control littermate brains. Black arrows point to mitochondria within vacuoles. Black arrow heads point to a double membrane enclosing a vacuole. Scale bars represent 10 μm (A), 2 μm (B-C), and 500 nm (D).

Control



CamKII: Cre Mcl-1



CamKII: Cre Mcl-1

CamKII: Cre Mcl-1

double membraned vesicles, a hallmark of autophagy (Mizushima 2004), suggesting that these neurons are dying via an autophagic mechanism. The studies outlined above have demonstrated a requirement for Mcl-1 in the maintenance of post-mitotic neurons of the cortex and postnatal survival of the animal. The loss of Mcl-1 in maturing neurons results in a dramatic loss of mature neurons between postnatal day 7 and 1 month of age. Collectively these studies demonstrate that Mcl-1 is universally required for neuronal survival from development through maturation and suggests that Mcl-1 plays a role in the regulation of autophagy.

3: The role of Mcl-1 in post-mitotic neurons *in vitro*

3.1 Generation of a Cre recombinase expressing lentivirus system

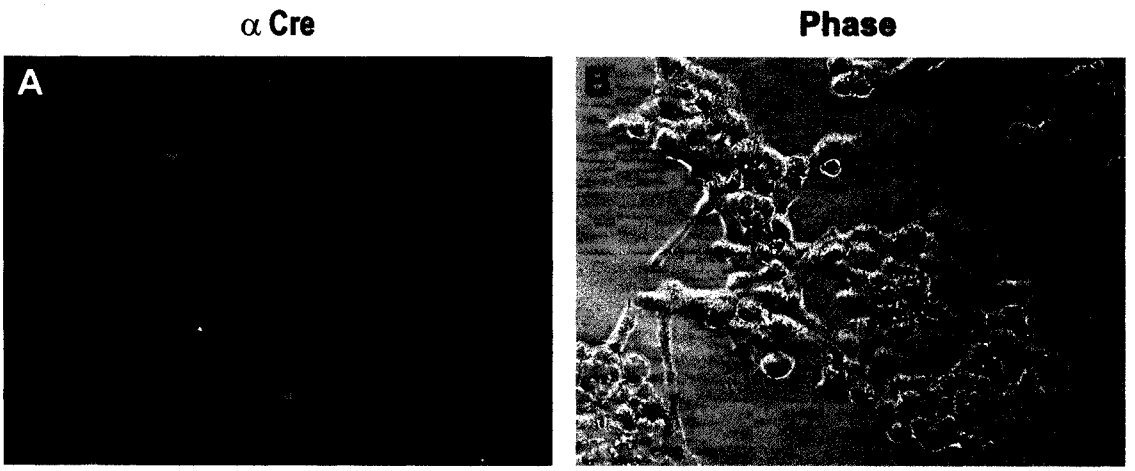
In order to more closely examine the role of Mcl-1 in maintaining the mature neuronal population and in the regulation of autophagy, we moved to an *in vitro* system of primary cortical cultures from mice nearing the end of their embryonic development at E17.5. Collecting neurons from the previously generated embryonic deficient mice (Foxg1: Cre Mcl-1 fl/fl or Nestin: Cre Mcl-1 fl/fl) was not possible at this time point due to their embryonic lethality and the fact that minimal cortical neurons remain in these mutants prior to their demise. As such, we obtained neurons from Mcl-1 fl/fl mice to introduce Cre recombinase into the system virally. Moreover, following the Mcl-1 deficient cells over an extended period of time required the use of a lentivirus system to introduce Cre expression due to the non-toxic nature of lentiviruses even after extended periods of time post-transduction (Bensadoun et al. 2000, Blömer et al. 1997, Déglon et al. 2007, Kordower et al. 1999). Production of the Cre recombinase lentivirus using a third generation lentivirus packaging system (described in methods) (Trono lab, École Polytechnique Fédérale de Lausanne, Switzerland), produced lentiviral titers in the order of 10^8 IVP/mL (infectious viral particles per milliliter). Immunohistochemical labeling revealed successful expression of the Cre recombinase protein within 293T cells infected with the Cre lentivirus

at 1 MOI (multiplicity of infection) **(Figure 3-9)**. Similarly, western blot analysis of primary cortical cultures of Mcl-1 floxed neurons transduced with the Cre recombinase lentivirus displayed efficient expression of the Cre enzyme as well as the absence of the Mcl-1 protein product. Successful generation of a Cre recombinase lentivirus provided us a useful tool with which to express the Cre protein in non-dividing cells such as neurons.

3.2 Autophagic programmed cell death in post-mitotic neurons deficient for Mcl-1

To explore the hypothesis that Mcl-1 is involved in the regulation of autophagy within cortical neurons, I used an *in vitro* system in which Mcl-1 floxed primary cortical neurons were cultured near the endpoint of cortical development at E17.5 and treated with a lentivirus carrying the Cre recombinase gene. Cortical cultures were treated with the Cre recombinase lentivirus (1 MOI). Autophagy is revealed by immunofluorescence labeling using an antibody against the autophagic marker light chain 3 (LC3). LC3 is diffused within a cell as its full length version under normal conditions. When a cell is subjected to stress, LC3 is cleaved to its short form and translocates to the outer membrane of the forming autophagic vacuoles. A translocation of this protein to distinct small or punctuate clusters within the cell marks the formation of these vesicles (Klionsky et al. 2007). Indeed, by 10 days after Cre recombinase treatment, neurons deficient for Mcl-1 displayed a minimum of a three fold increase in punctuate LC3 staining compared to the three controls **(Figure 3-10)**. More specifically, Mcl-1 fl/fl neurons treated with Cre lentivirus averaged 31% ± 6 LC3 positive cells compared to Mcl-1 fl/fl neurons treated with GFP lentivirus at 8% ± 4, and Mcl-1 fl/+ heterozygote mice treated with a GFP or Cre expressing lentivirus at 7% ± 3 and 12% ± 4, respectively. These results confirm that Mcl-1 deficient neurons display a higher rate of autophagy compared to controls. Altogether, an appearance of double membraned vesicles enclosing large cytoplasmic organelles *in vivo* and an accumulation of punctuate LC3 staining *in vitro* in neurons lacking Mcl-1 demonstrates that

Figure 3-9. Efficient *in vitro* knockout of Mcl-1 using a Cre Lentivirus. (A) Immunofluorescence of Cre recombinase in 293T cells transduced with a lentivirus carrying Cre recombinase (B) Phase contrast of cells represented in A. Scale bar represents 100 μm. (C) Western blot analysis of Mcl-1 floxed primary cortical neurons transduced with GFP lentivirus or Cre recombinase lentivirus.



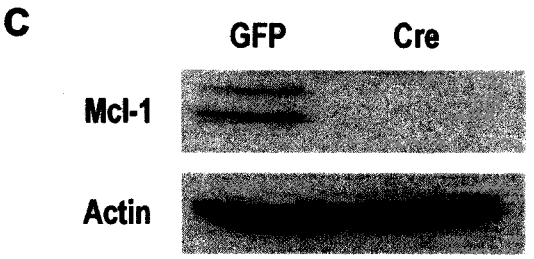
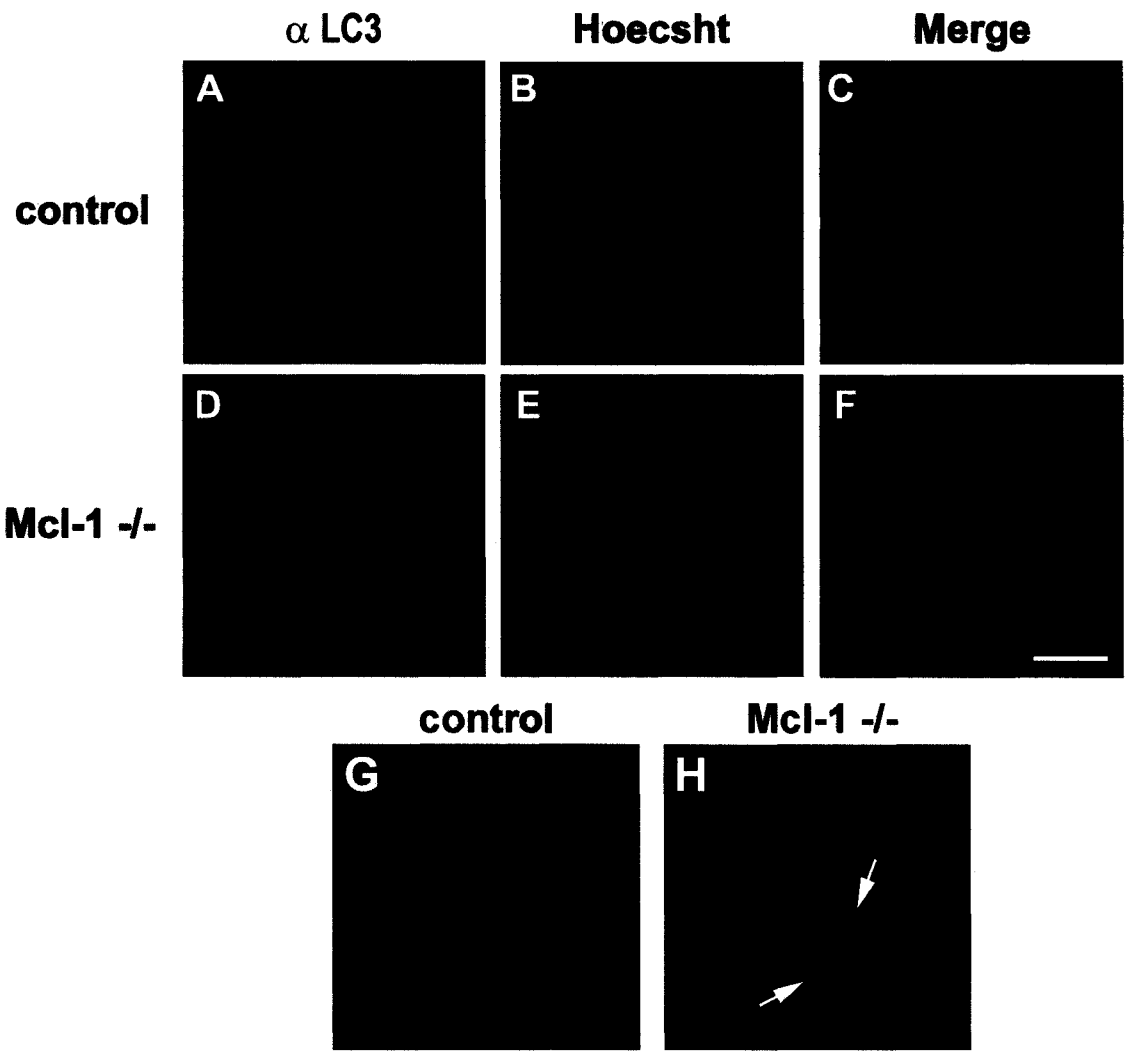


Figure 3-10. Primary cortical cultures undergo autophagy when Mcl-1 is removed. (A-I) Primary cortical cultures of E17.5 Mcl-1 f/f brains, transduced with GFP lentivirus (A-C) or Cre recombinase lentivirus (D-F) and labeled with LC3 antibody and Hoescht. (G-H) Higher magnification of control and Mcl-1 deficient neurons. Arrows point to a "positively" labeled cell for LC3 fragmentation. (I) LC3-positive cells were counted and found to be significantly higher in the Mcl-1 deficient neurons at day 10 compared to the Mcl-1 f/f and Mcl-1 f/+ neurons treated with a GFP lentivirus (P= 0.001 and 0.036, respectively) as well as the Mcl-1 f/+ neurons treated with Cre recombinase lentivirus (P= 0.002). Results represent the percentage of LC3-positive cells per total cell count. Scale bar represents 50 μm. Error bars represent standard error of the mean. n=3



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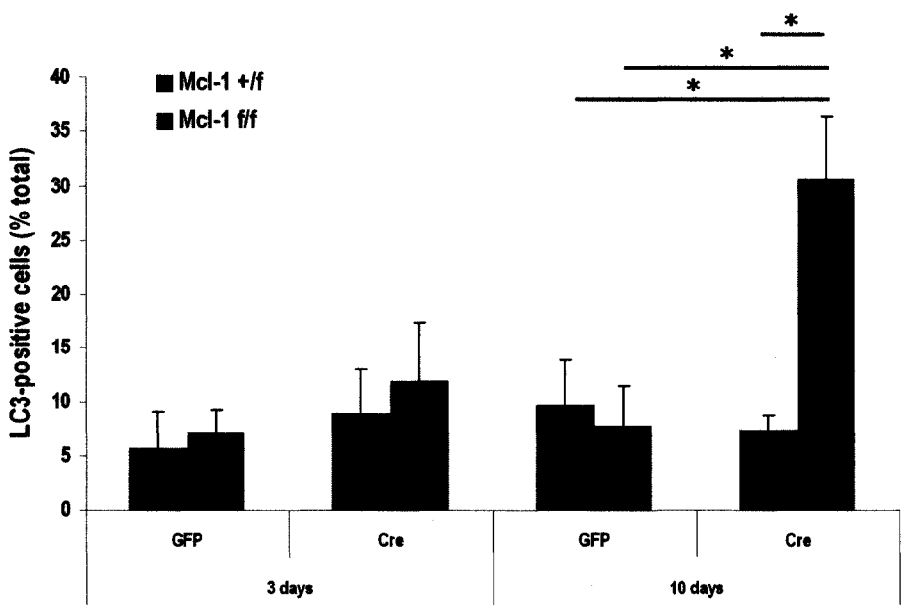

the loss of Mcl-1 in mature neurons results in an upregulation of autophagy which may be an indication of an autophagic cell death. The appearance of a deregulation of autophagy in post-mitotic cortical neurons suggests that Mcl-1's primary role in regulating neuronal survival changes as the neurons progress from progenitors and newly differentiated neurons to more mature, post-mitotic neurons.

Altogether, our findings have demonstrated that the loss of Mcl-1 in embryonic and post-mitotic neurons results in cell death of the neurons and premature death during embryonic development as well as postnatally. The two distinct forms of cell death activated, apoptotic programmed cell death in neuronal progenitors and an autophagic programmed cell death in mature, differentiated neurons, indicate that Mcl-1 functions in multiple pathways to promote neuronal survival. This is the first time that Mcl-1 has been shown to play a role in an apoptotic and non-apoptotic cell death pathway within the same tissue depending on the developmental stage of the animal. These studies also highlight Mcl-1's unique role in neuronal survival compared to other prosurvival Bcl-2 family members such as Bcl-2.

Chapter IV: Discussion

A large amount of evidence has accumulated to demonstrate the role of Mcl-1 as a key antiapoptotic regulator in many different tissue types (reviewed in Michels et al. 2005). Data implicating Mcl-1 in the regulation of neuronal apoptosis, however, has been scarce, opening only a small window into the potential role of Mcl-1 as a neuronal cell death regulator. The results presented in this thesis describe both a role for Mcl-1 in neuronal cell death *in vivo* and a requirement for this molecule in neuronal survival within both the developing and mature nervous system as well as in maintenance of a mature neuronal population *in vitro*. I hypothesized that *Mcl-1 plays an important role in programmed cell death throughout nervous system development and in maintaining mature neurons*. Through generation of neural conditional knockouts for Mcl-1, I have described a novel requirement for Mcl-1 in neuronal survival in the developing and mature central nervous system. The results presented here support the following conclusions: 1) Mcl-1 is required for proper neuronal development and the loss of this protein results in premature apoptotic death of neural precursors and newly committed neurons; 2) Mcl-1 maintains the survival of the mature neuronal populations of the postnatal brain; 3) the cell death in postnatal neurons is characterized by double membraned vesicles, suggesting a role for Mcl-1 in programmed autophagic cell death; and lastly 4) Mcl-1 is involved in the regulation of autophagy *in vitro* in cortical neurons. These findings highlight the importance of Mcl-1 as a key regulator of neuronal apoptosis in the developing telencephalon as well as in the regulation of autophagy within mature neurons.

Mcl-1 in early central nervous system development

Apoptotic programmed cell death has been long established to play a significant role in early nervous system development (Oppenheim 1991). Also well established are the roles of the Bcl-2 protein family in regulating this general process. Although there is a large amount of redundancy

among the function of the Bcl-2 family proteins, the finer points of apoptotic regulation differ depending on the tissue type, with emphasis on different Bcl-2 proteins in different tissues. With the exception of the Bcl-xL deficient mouse, previous germline knockouts of the Bcl-2 family have not displayed a developmental phenotype within the nervous system. Bcl-xL deficient mice do exhibit a vast amount of activated caspase 3 apoptotic cells within their developing nervous system (Motoyama et al. 1995, Shindler et al. 1997, Roth et al. 2000). Conditional mutant mice for Bcl-xL have revealed that some neuronal populations survive without this molecule (Savitt et al. 2005), suggesting that the requirement for Bcl-xL is not ubiquitous throughout the nervous system but rather specific to certain neuronal subtypes. Mcl-1 germline deficient mice are peri-implantation lethal, demonstrating a requirement for this molecule in proper embryonic development and implantation (Rinkenberger et al. 2000). In the past, conditional deletion of Mcl-1 has demonstrated its necessity in survival of developing B and T lymphocytes and hematopoietic stem cells (Opferman et al. 2003, Opferman et al. 2005). The results presented in this thesis demonstrate a novel requirement for Mcl-1 in the developing central nervous system and in regulation of neural precursor cell survival through the use of two separately targeted deletion models of neuronal development. Both telencephalic and neural progenitor cell specific deletion of Mcl-1 resulted in a drastic increase of apoptotic cells, suggesting that Mcl-1 is playing an obligatory role in the maintenance of these neurons as they develop by regulating the apoptotic pathway. Mcl-1 is thus the only Bcl-2 protein found to be independently required for central nervous system development.

Mcl-1 in neural progenitor cells

Programmed cell death is also an important event during early embryogenesis in which 50 to 70% of neural progenitor cells die through this process (Blaschke et al. 1996). Most commonly, neural precursor cells in the ventricular zone of the developing brain undergo a type of "proliferative

apoptosis" in order to contribute to the morphological processes involved in development such as neural tube closure and elimination of excess cells (Thomaidou et al. 1997). The significance of this process is demonstrated through mice deficient for principal proapoptotic proteins such as caspase 3, caspase 9 or Apaf-1, in which a loss of any of these molecules results in a significant reduction of apoptotic cells and an excess of neural precursor cells (Cecconi et al. 1998, Kuida et al. 1996, Kuida et al. 1998). The Bcl-2 family also plays an important role in regulating the number of neural precursor and progenitor cells throughout development. The proapoptotic Bcl-2 family proteins, Bax and Bak, have been implicated in neural precursor death during brain development. Although Bax -/- Bak -/- mice display a high rate of perinatal lethality, those animals which do survive have an accumulation of both neural precursor cells and early post-mitotic cells within the proliferative zone of the brain (Lindsten et al. 2003). Moreover, telencephalic neuronal cultures from these mice comprised largely of neural progenitor cells were found to be resistant to apoptosis caused by DNA damage (D'Sa et al. 2003). Of the antiapoptotic Bcl-2 family members previously examined, only the Bcl-x deficient mouse displayed a neuronal phenotype. The widespread apoptotic death observed in the Bcl-xL mouse, however, is largely localized to areas of the brain which contain immature post-mitotic neurons such as the intermediate and marginal zones (Motoyama et al. 1996, Roth et al. 2000). Moreover, there are neuronal subpopulations which survive in the absence of Bcl-xL (Savitt et al. 2005). The requirement for Bcl-xL in neuronal survival must, therefore, be cell specific. Our studies have revealed that Mcl-1 is a key regulator of neural precursor cell survival given that conditional deletion of Mcl-1 results in widespread apoptosis of Nestin expressing cells as well as newly committed neurons expressing β-Tubulin. Consistent with the double labeling observed in the AC3/PSA NCAM staining, other work produced in our lab, using double labeling of doublecortin (which marks newly migrating neurons) and active caspase 3, has demonstrated that over half (64.5 +/- 6.4%) of the newly migrating neurons are

undergoing apoptosis (Arbour et al. 2008). As such, Mcl-1 is the only antiapoptotic protein described to play an essential role in neural precursor cell survival to date.

Mechanism of Mcl-1 regulation in neuronal apoptosis

While we do not explore the mechanism of Mcl-1 mediated control of apoptosis here, a few scenarios are hypothesized to explain how Mcl-1 mediated regulation of apoptosis occurs on the molecular level. The proapoptotic Bcl-2 proteins bind different BH3 only proteins with varying affinity. *In vitro* binding studies have revealed that Mcl-1 associates with Bim, Puma and Bid strongly, has a weaker affinity for Noxa and Bak and does not associate with Bad at all (Certo et al. 2006, Chen et al. 2006, [2]Willis et al. 2005). Bak association has also been demonstrated in isolated mitochondria by cross-linking and immunoprecipitation (Cuconati et al. 2003, Leu et al. 2004, Nguyen et al. 2007, [2]Willis et al. 2005). As such, it has previously been proposed that Mcl-1 may function to regulate cytochrome c release and apoptosis by binding to Bak and preventing its oligomerization on the mitochondrial outer membrane and that this function is selectively inhibited by the BH3 only proteins Bim, Bid and Puma (Warr et al. 2008). Although this remains a plausible mechanism of apoptosis regulation in other tissue types, it is less likely in neurons. In neurons, the multidomain proapoptotic Bak is substituted by N-Bak, a neuronal specific splice variant of Bak, which functions as a BH3 only protein by binding predominantly Bcl-xL to induce a Bax-dependent apoptosis (Uo et al. 2005). Another candidate for Mcl-1 regulation, however, is the truncated form of Bid, tBid. Unlike other BH3 only proteins, tBid has been shown to function as a direct activator of apoptosis by translocating to the mitochondrial membrane to induce Bax and/or Bak oligomerization (Eskes et al. 2000, Wei et al. 2001). Moreover, Mcl-1 has been shown to interact with tBid and inhibit its ability to induce cytochrome c release and apoptosis (Clohessy et al. 2006). Consequently, Mcl-1 may function to regulate apoptosis in neuronal precursor cells by binding to tBid and inhibiting its ability to induce oligomerization of Bax.

Mcl-1 maintains post-mitotic neurons

Recent work done in our lab has alluded to the presence of a transitional period during differentiation of neural progenitor cells into post-mitotic cells in which the expression levels of survival signals differ and that Mcl-1 regulates this process (Arbour et al. 2008). In these studies, contrary to the apoptotic form of cell death observed in the two embryonic conditional knockouts presented in this thesis, post-mitotic neurons deficient for Mcl-1 were able to survive in culture for up to four days. This was true for both cortical neurons cultured from Foxg1: Cre Mcl-1 deficient mice and cerebellar granule neurons from animals homozygous for the floxed Mcl-1 allele which were then treated with a Cre adenovirus. A lack of any signs of apoptosis implies that these cell types undergo a change in their susceptibility to apoptosis *in vitro* when differentiating from neural progenitor cells to post-mitotic neurons. Mcl-1 is, however, expressed in post-mitotic neurons in the cortical plate at high amounts (Arbour et al. 2008). Accordingly, the results presented in this thesis have shown that deletion of Mcl-1 from post-mitotic neurons around birth results in a progressive loss of neurons. Conditional deletion of Mcl-1 from post-mitotic neurons via the CamKIIα promoter driven Cre recombinase caused progressive and severe deterioration of the cortex, identifying Mcl-1 as a necessary regulator in the maintenance of post-mitotic neurons of the cortex. The type of cell death observed in these mice, in contrast to that observed in neural progenitor cells, was reminiscent of an autophagic cell death, as suggested by the double membraned vacuoles observed with electron microscopy. The latter was subsequently confirmed by an upregulation of autophagy by punctuate LC3 staining *in vitro* in post-mitotic cortical neurons cultured from Mcl-1 floxed mice and treated with a Cre lentivirus. The use of a lentivirus allowed for observation of Mcl-1 deficient neurons *in vitro* for a much longer period of time than the adenovirus. Autophagic cell death has been described as a relatively slow process compared to apoptosis (Inbal et al. 2002). As such, extra time was necessary in order to be able to study this type

of cell death. These results indicate that the previously observed change in survival signaling regulated by Mcl-1 is likely characterized by a transition from a neural progenitor cell predominantly susceptible to an apoptotic form of cell death to a post-mitotic neuron which is predominantly sensitive to autophagy. The presence of two primary forms of cell death, apoptotic and autophagic, dependent on the developmental stage of the neuronal population suggests that Mcl-1 is a main regulator of both pathways and can play distinct roles in different neuronal populations.

Regulation of autophagy by Mcl-1

In addition to their role in apoptosis regulation, several Bcl-2 proteins have been linked to the process of autophagy. Recent studies have demonstrated the ability of an autophagic regulating protein called Beclin-1 to bind several antiapoptotic Bcl-2 proteins including Bcl-2, Bcl-x, Bcl-w and Mcl-1 (Erlich et al. 2007, Feng et al. 2007, Maiuri et al. 2007, Oberstein et al. 2007). The precise role of Bcl-2 proteins in regulating autophagy, however, has remained elusive. In one study, over-expression of either Bcl-2 or Bcl-xL resulted in an increase of autophagy, while only the loss of Bcl-2 affected the autophagic process by decreasing levels of autophagic cell death in Bax/Bak deficient mouse embryonic fibroblasts (Shimizu et al. 2004). Others have reported opposing results in which Bcl-2 expression inhibited starvation-induced autophagy in mouse cardiac muscles (Pattingre et al. 2005). Bcl-2 has been further implicated in the regulation of autophagy in that a loss of its ability to bind Beclin-1 causes an upregulation of autophagy in certain cell types (Pattingre and Levine, 2006; Pattingre et al., 2005). These data suggest that Bcl-2 family proteins regulate autophagy differently, either acting as a proautophagic or antiautophagic protein, dependent on the cell type and experimental conditions. The results presented in this thesis suggest that the antiapoptotic protein, Mcl-1 also acts as an antiautophagic protein, the loss of which causes an increase in autophagy and autophagic cell death in post-mitotic cortical neurons. It remains to be determined whether Mcl-1 also

acts as an autophagic inhibitor in other cell types or under other experimental or physiological conditions.

Interestingly, all of these proteins are multidomain antiapoptotic proteins, which may suggest that only the upstream regulators of apoptosis act promiscuously and function as regulators in other cell death pathways while the downstream "workhorse" Bcl-2 proteins retain their function at the mitochondria in regulating membrane permeability. Studies have indeed shown that cells deficient for both Bax and Bak are resistant to apoptosis but retain their ability to undergo an autophagic programmed cell death (Shimizu et al. 2004). Moreover, it has been shown that it is the pool of Bcl-2 localized to the endoplasmic reticulum which regulates autophagy and not mitochondrial targeted Bcl-2 (Pattingre et al. 2005). While Mcl-1 is largely found at the mitochondrial membrane, it is also present at the endoplasmic reticulum (Schinzel et al. 2004). Further studies are required, however, to determine if Mcl-1's role in autophagy regulation is specific to the endoplasmic associated pool. What is certain is that Mcl-1 plays an obligatory role in the regulation of autophagy within post-mitotic neurons of the cortex and the conditional mutants described here display the most pronounced autophagic phenotype with respect to the Bcl-2 protein family described to date.

Mcl-1 and mitochondrial homeostasis

Recently, there has been growing evidence to support a link between cell death mechanisms and bioenergetics (Vander Heiden and Thompson 1999, Nicholls and Ward 2000). Among these studies, one can find several which focus on the nonapoptotic functions of Bcl-2 proteins. For example, Bcl-2, Bcl-x, Bcl-w and Mcl-1 have all been shown to have antiproliferative functions, while proapoptotic Bax exhibits a proliferative effect (Schuler and Green 2005, Zinkel et al. 2006). The BH3 only protein, Bid, has been shown to participate in the DNA-damage response (Kamer et al. 2005, Zinkel et al. 2005) and Bad and Bcl-xL have been implicated in glucose and energy metabolism,

respectively (Danial et al. 2003, Vander Heiden et al. 1999). One of the more recognized functions of the Bcl-2 proteins is regulation of calcium homeostasis at the level of the endoplasmic reticulum (Hetz and Glimcher 2007, Pinton and Rizzuto 2006). Less known, however, is the role that Mcl-1 plays in calcium homeostasis at the level of the mitochondria. A recent study has shown that Mcl-1 acts to inhibit mitochondrial calcium signaling (Minagawa et al. 2005). An increase in the free mitochondrial calcium has been suggested to induce formation of the permeability transition pore, causing a release of mitochondrial calcium (Ichas et al. 1997) and a subsequent induction of apoptosis (Boehning et al. 2003). While mitochondrial calcium signaling has been demonstrated to induce apoptosis, increased cytosolic calcium levels have also been linked to the induction of autophagy (Feng et al 2005, Høyer-Hansen et al. 2007). Although further studies would be needed to ascertain such a theory, it is interesting to speculate that the dual roles of calcium signaling could be the linking factor responsible for the dual cell death mechanisms triggered by Mcl-1 deficiency described in this thesis. As such, in immature neurons and neural progenitor cells which are primed to undergo developmental apoptosis, mitochondrial calcium signaling induced by Mcl-1 deficiency triggers the apoptotic cascade. Upregulation of the apoptotic pathway was in fact seen in the Foxg1: Cre Mcl-1 fl/fl and Nestin: Cre Mcl-1 fl/fl mice through increased active caspase-3 labeling. On the other hand, the same cytosolic calcium signaling may produce an upregulation of the autophagic process in mature post-mitotic neurons, a non-dividing cell type primed to survive as long as the organism does, as was observed in the neurons of the Mcl-1 deficient CamKIIα: Cre Mcl-1 fl/fl mice as well as in the post-mitotic cortical cultures deficient for Mcl-1.

Mcl-1 and cell cycle regulation

Originally identified as an upregulated gene during differentiation of human myeloid leukemia cells (Kozopas et al. 1993), Mcl-1 has been implicated in cell cycle regulation in addition to its role in

cell death. Mcl-1 has been shown to interact with proliferating cell nuclear antigen (PCNA) in HeLa cells to cause a decrease in BrdU uptake and a slowed doubling rate, a function which has been shown to be distinct from its role in cell death regulation (Fujise et al. 2000). A short form of Mcl-1 called snMcl-1 in the nucleus in a murine myeloid progenitor cells has also been described (Jamil et al. 2005). In this study, Mcl-1's antiproliferative function was found to be linked to the ability of snMcl-1 to bind to and negatively regulate cdk1 activity. Although the proliferation data presented do not display any statistically significant differences between Mcl-1 deficient cells and wildtype, it is interesting to note that the trend suggested that the absence of Mcl-1 increased proliferation. That is, in the absence of Mcl-1, more neural progenitor cells of the ventricular zone expressed the cell cycle marker phosphohistone H3. These data are consistent with previous claims that Mcl-1 acts as an antiproliferative protein.

Implications in neurodegeneration

The Bcl-2 family has been implicated in the regulation of neuronal apoptosis in several models of neurodegeneration including hypoxic or ischemic injury (Banasiak et al. 1999, Minami et al. 2000, Shimazaki et al. 1994) seizure-induced neuronal cell death (Ananth et al. 2001, López et al. 1999, Tuunanen et al. 1999, Zhang et al. 1998), motor neuron disease such as amyotrophic lateral sclerosis (ALS) (Ekegren et al. 1999, Guégan et al. 2001, Mu et al. 1996, Shinoe et al. 2001) and axotomy-induced induced neurodegeneration (Bonfanti et al. 1996, Cenni et al. 1996, Gillardon et al. 1994, Gillardon et al. 1996, Isenmann et al. 1997). Nevertheless, our understanding of the specific role played by Mcl-1 in neuronal degeneration is limited to the increased susceptibility to excitotoxicity-induced cell death of mice heterozygous for Mcl-1 (Mori et al. 2004). The results presented in this thesis describe a requirement for Mcl-1 in neuronal development and an apoptotic phenotype in neural progenitor cells and newly committed neurons in its absence. Such a drastic phenotype in the Mcl-1

conditionally deficient mice is unlike any other antiapoptotic Bcl-2 protein examined thus far. These data suggest that Mcl-1 is a primary player in neuronal apoptosis and may have implications for many neurodegenerative models.

The contribution of apoptosis to many neurodegenerative diseases has, however, been scrutinized as of late (Graeber and Moran 2002, Olney 2003, Roth 2001) and the focus of research has shifted to include an interest in the appearance and regulation of another cell death mechanism, namely autophagic neuronal degeneration (Tolkovsky et al. 2002, Xue et al. 1999). Bcl-2 proteins have also been implicated in regulating autophagy in neurodegeneration. For example, Bcl-xL deficient neurons display an increased susceptibility to autophagic cell death *in vitro* while Bax deficiency results in a decreased susceptibility to drugs which target the lysosome and can initiate autophagy (Zaidi et al. 2001). Autophagosomal structures appear in the neurons of many neurodegenerative diseases such as Alzheimer's (Nixon et al. 2005), Parkinson's (Anglade et al. 1997), Huntington's (Kegel et al. 2000) and Creutzfeldt-Jakob disease (Sikorska et al. 2004). The autophagic phenotype in mature post-mitotic neurons described in this thesis provides evidence that the antiapoptotic protein, Mcl-1 is a key player in neuronal survival through the regulation of neuronal autophagy. As such, it is interesting to speculate that deregulation of Mcl-1 expression may play a role in one or many of these diseases, although further research would be needed to substantiate such a claim. Moreover, overexpression of Mcl-1 may protect neurons from an autophagic cell death. Mcl-1 expression is regulated at multiple levels including transcriptional regulation through E2F-1 and Hif-1 (Liu et al. 2006, Piret et al. 2005, Yang et al. 2000), as well as at the level of protein stability through proteasomal degradation and caspase cleavage (Herrant et al. 2004, Warr et al. 2005, Weng et al. 2005, [1]Willis et al. 2005), making Mcl-1 an attractive candidate for pharmacological manipulation to treat neurodegenerative diseases.

Conclusion

The studies presented in this dissertation reveal a novel role for Mcl-1 in the regulation of two different programmed cell death mechanisms in the developing central nervous system as well as in post-mitotic neurons. My results show that Mcl-1 is required *in* vivo for proper central nervous system development in which neural progenitor cells and newly differentiated neurons deficient for Mcl-1 die via an apoptotic mechanism. Our data has also shown a requirement for Mcl-1 in the maintenance of mature post-mitotic neurons *in vivo* and *in vitro*. In addition to its role as an apoptotic regulator, these results have identified a role for Mcl-1 in the regulation of autophagy in mature post-mitotic neurons.

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