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Nicole Arbour

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology
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Abstract

Involvement of Bcl-2 family proteins has been implicated in neuronal survival, injury and a number of neurodegenerative disorders. The present study was undertaken to determine the importance of Bcl-2 family members Noxa, Puma and Mcl-1 in neuronal survival and neuronal cell death following injury. Here I show that Puma, a BH3-only member of the Bcl-2 family, is required for p53-mediated neuronal cell death resulting from DNA damage injury. Further, that the transcriptional activation of p53 is required for the up-regulation of Puma and the initiation of cell death under these circumstances. I also show that Noxa, a second BH3-only member of the Bcl-2 family known to be transcriptionally up-regulated by p53, is dispensable for this form of neuronal cell death (Chapter 2). The anti-apoptotic protein Mcl-1 has been implicated on a higher level, in the regulation of the neuronal progenitor cell pool, through interactions with the Notch 1 protein. It has also shown to have a dose dependant response in that mice heterozygous for Mcl-1 display an increased sensitivity to pilocarpine induced seizure injury. The research presented here, using conditional knock out models, identifies a requirement for Mcl-1 in cortical neurogenesis. Further, I found that loss of Mcl-1 in post-mitotic neurons results in the rapid, progressive degeneration of mature cortical neurons, resulting in lethality. The mature neurons lost in these mice occur, not through an apoptotic mechanism as expected, but through an autophagic cell death pathway. This identifies Mcl-1 as a key player in both apoptotic and autophagic neuronal cell death. To confirm the importance of Mcl-1 in neurons, and identify the mechanism of its degradation in neurons, I performed *in vitro* experiments showing the degradation of Mcl-1 in neurons following DNA damage induced injury, and their further protection through maintenance of Mcl-1 protein levels within the system. I also show that loss of Mcl-1 sensitizes neurons DNA damage induced injury. Further, degradation of Mcl-1 under injury conditions occurs through caspase cleavage and proteasomal degradation (Chapter 3). These studies provide insight into the mechanisms of neuronal survival, cell death and

neurodegeneration. They also identify Puma and Mcl-1 as potential therapeutic targets for the treatment of neuronal injury and neurodegenerative diseases.

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Table of Contents

Abstract	ii
General Acknowledgements	iv
Table of Contents	vi
Table of Figures and Tables	viii
List of Abbreviations	ix
Chapter 1: General Introduction	1
1.1 Ischemic Injury and Neurodegeneration	1
1.1.1 Apoptosis in Neurodegeneration	4
1.2 Apoptosis & Cell Death	6
1.2.1 The Extrinsic Apoptotic Pathway	8
1.2.2 The Intrinsic Apoptotic Pathway	10
1.2.2.1 The Endoplasmic Reticulum and the Intrinsic Apoptotic Pathway	11
1.2.3 The Intersection of Extrinsic and Intrinsic Apoptotic Signaling	13
1.3 P53 Mediated Apoptosis	15
1.3.1 P53 Mediated Apoptosis in Neurons	17
1.4 Apoptosis and the Bcl-2 Family	20
1.4.1 Multi-Domained Bcl-2 Proteins in Apoptosis	21
1.4.1.2 Control of Mitochondrial permeability by Bcl-2 family members	22
1.4.2 BH3 Only Proteins in Apoptosis	26
1.4.3 Bcl-2 Family Proteins at the Endoplasmic Reticulum	28
1.4.4 Bcl-2 Proteins and Neuronal Apoptosis	30
1.4.4.1 Anti-apoptotic Bcl-2 Proteins in Neuronal Apoptosis	30
1.4.4.2 Pro-apoptotic Bcl-2 Proteins in Neuronal Apoptosis	32
1.5 Autophagic cell death	37
1.5.1 Autophagy in Neurodegeneration	39
1.5.2 Autophagy and the Bcl-2 family	39
1.6 Bcl-2 Family Proteins in the Control Neuronal Cell Death Paradigms	41
1.7 Research Objectives	42
Chapter 2: Cregan, Arbour <i>et al.</i> (2004) p53 Activation Domain 1 is Essential for PUMA Up-regulation and p53-mediated Neuronal Cell Death. <i>J. Neurosci.</i> 22:10003-12	45
2.1 ABSTRACT	47
2.2 Introduction	48
2.3 METHODS	50
2.3.1 Cell Culture and Recombinant Adenovirus Infection:	50
2.3.2 Mice and Genotyping	50
2.3.3 Semiquantitative RT-PCR analysis	51
2.3.4 Electrophoretic Mobility Shift Assays	51
2.3.5 Western Blot Analysis and Immunostaining	53
2.3.6 Cell Viability and Caspase Assays	53
2.4 RESULTS	55
2.4.1 Transcriptional activation is essential for p53 – mediated neuronal cell death	55
2.4.2 Reconstitution of responsiveness to DNA damage-induced apoptosis in p53 deficient neurons	59
2.4.3 Transactivation mutants of p53 activate common and distinct proapoptotic targets	63

2.4.4 Apoptotic response in neurons carrying a targeted mutation in activation domain	1
.....	63
2.4.5 PUMA is a Potent Inducer of Neuronal Cell Death	67
2.4.6 PUMA is Required for p53 Mediated Neuronal Cell Death	69
2.5 DISCUSSION	72
Chapter 3: Arbour, NA, Vanderluit, JL et al.(2007) Mcl-1 is Required for Neuronal Survival. <i>Neuron (Manuscript in preparation).</i>	76
3.1 Abstract	78
3.2 Introduction	79
3.3 Results	82
3.3.1 Mcl-1 is down-regulated following neuronal injury	82
3.3.2 Mcl-1 is Required for Cortical Neurogenesis	84
3.3.3 Mcl-1 deficient neural progenitors die by apoptosis	87
3.3.4 Loss of Mcl-1 in post-mitotic neurons (PMN) results in premature death	88
3.3.5 Loss of Mcl-1 deficient PMNs does not occur through the hallmark apoptotic pathway	93
3.3.6 PMNs lacking Mcl-1 are more sensitive to DNA damage induced neuronal cell death	95
3.3.7 Mcl-1 plays an important role in injury induced neuronal cell death	97
3.4 Discussion	102
3.5 Materials and Methods:	108
3.5.1 Mice and primary neuronal cultures	108
3.5.2 Tissue Processing and Immunohistochemistry	108
3.5.3 Electron microscopy	109
3.5.4 Cell Culture and Recombinant Adenovirus Infection	110
3.5.5 Semiquantitative RT-PCR analysis	110
3.5.6 Western Blot Analysis and Immunostaining	110
3.5.7 Camptothecin Treatment and Cell Viability Assays	111
Chapter 4: General Discussion	112
4.1 p53-mediated neuronal cell death	112
4.2 The roles of Noxa and Puma in neuronal cell death	114
4.3 The role Mcl-1 in neuronal development	115
4.4 The role of Mcl-1 in the survival of post-mitotic neurons	116
4.5 The role of Mcl-1 in autophagy: far reaching implications in the regulation of cell death	117
4.6 Mcl-1 and mitochondrial fission?	118
4.7 Mcl-1 in p53 mediated neuronal cell death	120
4.8 Significance of Research	121
Appendix 1: Curriculum Vitae	123
Appendix 2: Letters of Permission to Reprint Published Materials	128
References	129

Table of Figures and Tables

Figure 1.1 Spatial and temporal injury distribution following stroke injury.....	3
Figure 1.2 Intrinsic and extrinsic cell death pathways.	7
Figure 1.3 p53-mediated transcriptionally dependant and independent apoptotic pathways ..	16
Figure 1.4 BH3-only Bcl-2 family models of action.....	25
Table 2.1 The RT-PCR primer sets used for the detection of p53-inducible gene.....	52
Figure 2.2 P53 mutant proteins are stable, expressed at comparable levels and retain the appropriate protein conformation.	57
Figure 2.3 P53 mutant proteins localize to the nucleus.....	58
Figure 2.4 Transactivation domain 1 of p53 is essential for the induction of neuronal cell death.	60
Figure 2.5 Reconstitution of responsiveness to DNA damage-induced apoptosis in p53 deficient neurons.	62
Figure 2.6 P53 mutants cause differential upregulation of target genes in cerebellar granule neurons.....	64
Figure 2.7 Transactivation domain 1 of p53 is essential for the induction of neuronal cell death in vivo.	66
Figure 2.8 Upregulation of Noxa and PUMA mRNA in p53-mediated neuronal cell death.....	68
Figure 2.9 Forced expression of PUMA, but not Noxa is sufficient to induce neuronal apoptosis.....	70
Figure 2.10 Upregulation of PUMA is essential for the induction of p53-mediated neuronal cell death.	71
Figure 3.1. Mcl-1 is down-regulated in response to DNA damage induced cell death.....	83
Figure 3.2. Loss of Mcl-1 in developing neurons results in severe defects in cortical neurogenesis.....	86
Figure 3.3. Loss of Mcl-1 in developing neurons results in increased apoptotic activity in neuronal progenitors.....	89
Figure 3.4. Loss of Mcl-1 in post-mitotic CamKIIα:Cre Mcl-1 neurons results in rapid neuronal degeneration.	91
Figure 3.5. Mice lacking Mcl-1 in post mitotic neurons lose cortical neurons.	92
Figure 3.6. Mcl-1 mutant post-mitotic neurons undergo autophagic cell death.....	94
Figure 3.7. Mutant mice have fewer cells available for culture, but otherwise are similar to littermate controls.....	96
Figure 3.8. Loss of Mcl-1 in cortical neurons results in increased sensitivity to DNA damage induced cell death, and is due in part to Mcl-1 to degradation by caspases and the proteosome.	98
Supplemental Figure 3.1. Mcl-1 protein levels are stabilized by inhibition of caspase and proteosome activity.....	99

List of Abbreviations

Ad: Adenoviral
AD: Alzheimer's disease
ADI: Activation Domain I
ADII: Activation Domain II
ADP: adenosine diphosphate
AIF: apoptosis inducing factor
ALS: amyotrophic lateral sclerosis
ANT: adenine nucleotide translocator
ANOVA: analysis of variance
APAF-1: apoptotic peptidase activating factor 1
ATP: adenosine triphosphate
Atg: Autophagy related
Bad: Bcl-2 associated death promoter
Bak: Bcl-2 antagonist/killer
Bax: Bcl-2-associated X
Bcl-2: B-cell leukemia/lymphoma 2
Bcl-w: Bcl-2 like 2
Bcl-x: Bcl-2 like 1
BFA: brefeldin A
BH: Bcl-2 Homology domain
BID: BH3 interacting domain death agonist
BIK: BCL2-interacting killer
BMF: Bcl-2 modifying factor
BOK: Bcl-2-related ovarian killer
BSA: bovine serum albumin
CamKII α : Ca²⁺/calmodulin-dependent protein kinase II alpha
CARD: caspase recruitment domain
Caspase: cysteine aspartyl protease
cDNA: complementary DNA
CGNs: Cerebellar granule neurons
CHOP: C/EBP homologous protein
CNS: central nervous system
COX IV: cytochrome c oxidase IV
CP: cortical plate
CPT: camptothecin
Cre: Cre recombinase
CTL: control
CycD: cyclophilin D
CytC: cytochrome C
DD: death domain
DED: death effector domain
DEVD-AFC: 7-Amino-4-trifluoromethylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide
DISC: death inducing signaling complex

DM: double mutant
DNA: deoxyribonucleic acid
DP5: Neuronal death protein 5
DR5: death receptor 5
DTT: Dithiothreitol
E#: embryonic day #
E2-like: ubiquitin-conjugating enzyme like
E3-like: ubiquitin-ligase like
E2F-1: E2F transcription factor 1
EDTA: ethylenediaminetetraacetic acid
EM: transmission electron microscopy
EMSA: electrophoretic mobility shift assay
ER: endoplasmic reticulum
ES: embryonic stem
GFP: green fluorescent protein
ESR: ER stress response
FADD: Fas-associated death domain
FLIP: FLICE-inhibitory protein
fl: flox
HD: Huntington's disease
HEK 293: Human embryonic kidney cell line
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFis1: human mitochondrial fission protein 1
HRK: harakiri
IAP: inhibitor of apoptosis
InsP₃: inositol 1,4,5-triphosphate
InsP₃R: inositol 1,4,5-triphosphate receptor
IZ: intermediate zone
KCl: potassium chloride
LacZ: β-galactosidase
LC3: light chain 3
MAC: mitochondrial apoptosis induced channel
Map2: microtubule-associated protein 2
Mcl-1: myeloid cell leukemia-1
MDM2: murine double minute
MEF: mouse embryonic fibroblast
MgCl₂: magnesium chloride
MMP: mitochondrial membrane potential
MOI: multiplicity of infection
MOM: mitochondrial outer membrane
MOMP: mitochondrial outer membrane permeabilization
mRNA: messenger RNA
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mut: mutant
N-Bak: neuronal BAK
NF-κB: nuclear factor of kappa B

NGF: nerve growth factor
NMDA: N-methyl-D-aspartic acid
NPCs: neural precursor cells
OGD: oxygen glucose deprivation
P#: Post-natal day #
P300/CBP: p300/CREB binding protein
PBR: peripheral benzodiazepine receptor
PBS: phosphate buffered saline
PCD: programmed cell death
PCR: polymerase chain reaction
PD: Parkinson's disease
PERP: prolyl endopeptidase
PMN: post mitotic neurons
PMSF: phenylmethanesulphonylfluoride
PNS: peripheral nervous system
PSA-NCAM: polysialylated forms of the cell-surface glycoprotein NCAM
PTP: permeability transition pore
PUMA: p53-upregulated mediator of apoptosis
RIP: receptor interactin protein kinase
RNA: ribonucleic acid
ROS: reactive oxygen species
RT-PCR: reverse transcription PCR
tBID: truncated BID
TuJ1: Neuronal Class III β -Tubulin
SD: standard deviation
SVZ: Subventricular zone
TNF: tumor necrosis factor
TRADD: TNFR-associated death domain
TRAIL: TNF-alpha-Related Apoptosis-Inducing Ligand
TUNEL: Terminal transferase dUTP nick end labeling
VDAC: voltage dependant anion channel
VZ: ventricular zone
WT: wild-type

Chapter 1: General Introduction

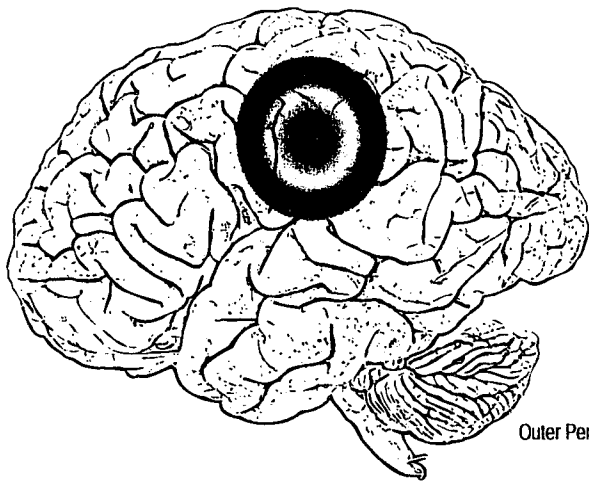
Programmed cell death in neuronal injury is a major target of research, as it provides us with viable cellular targets that if protected would result in significantly improved disease prognosis. I will begin by a general introduction giving an overview of cell death. I will describe the neuronal injuries in which these forms of cell death have been identified; the role that programmed cell death plays in neurodegeneration and briefly, current research into therapeutic approaches. The current knowledge of apoptotic pathways will then be discussed in more depth, focusing on the molecular mechanisms underlying both the intrinsic and extrinsic pathways, the various ways they intersect and the role that p53 plays in the initiation and progression of these cell death paradigms. I will also discuss the role of the endoplasmic reticulum (ER), recently identified as an important player in some apoptotic paradigms. Following this, I will present a more in-depth review of the literature directly pertaining to the studies included in this thesis specifically the roles of p53 and the Bcl-2 family in apoptosis. Finally I will discuss the more recently identified involvement of the Bcl-2 family in an additional cell death paradigm, autophagy and its role in neuronal injury. This foundation of information will provide the reader with all the necessary background knowledge and up to date information necessary to put the research presented here in context and allow the reader to evaluate it in terms of its contribution to what is currently known in the field.

1.1 Ischemic Injury and Neurodegeneration

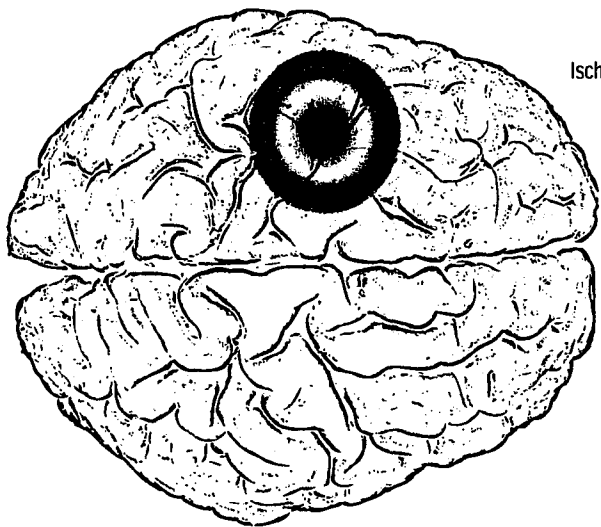
Ischemic injury, or stroke, is the third leading cause of death and long-term disability in the Western world (Lo et al., 2003; Mergenthaler et al., 2004). As such, a great deal of emphasis is being funneled into research aimed at the treatment and prevention of this debilitating disease. Ischemic injury results from an interruption of blood flow to the brain resulting in the death and dysfunction of neurons and neurological deficits reflecting the size and location of the injury. Following an ischemic injury there are two spatially and temporally different waves of cell death. First, the cells in the

immediately affected area, or the ischemic core, are irreversibly damaged due to low ATP levels, ionic disruption and metabolic failure. Cells in this affected area die within minutes by catastrophic cell death known as necrosis. The second wave of cell death occurs within a large cell population on the periphery of the ischemic core, called the penumbra (Figure 1.1). Here a severe disruption in cellular homeostasis, due to secondary effects of the death within the necrotic core, results in delayed cell death that continues for several days following the initial injury and can result in an increase of the lesion size by up to 50% (Ginsberg, 2003; Li et al., 1998b; Mergenthaler et al., 2004). This loss of the penumbral region and gradual increase in infarct size is a major contributor to the neurological damage, and resultant physiological consequences of a stroke injury (Baird et al., 1997; Dirnagl et al., 1999; Furlan et al., 1996). Cell death in the penumbral region following ischemic injury occurs through many active cell death mechanisms including excitotoxic cell death, oxidative stress, hypoxia as well as the classical apoptotic cell death pathway (for review see (Dirnagl et al., 1999; Lo et al., 2003). Ongoing research aims to identify the specific pathways that lead to the active cell death mechanisms within the penumbral region, as they provide cellular targets within the ischemic area that are still viable and may be rescued through the advent of novel neuroprotective therapies. Neuroprotection aimed at saving the injured cells of the ischemic penumbra is the focus of many ongoing therapeutic approaches. Over the past two decades research in this field has identified many mechanisms that have shown promise. Of 178 clinical trials, however, with over 100 of those being related to neuroprotection, only one therapy has currently been adopted for acute stroke therapy (Cheng et al., 2004; Kidwell et al., 2001). Although there are many therapies still in clinical trial, this lack of success in identifying effective therapeutic options only further emphasizes the need for continued innovation in neuroprotective research.

Figure 1.1 Spatial and temporal injury distribution following stroke injury. Immediately following the interruption of blood flow, cells in the ischemic core are irreversibly damaged due to lack of ATP, ionic disruption and metabolic failure. Cells in the tissue immediately surrounding the core die more slowly over the course of several days by active cell death mechanisms including excitotoxicity, oxidative stress, hypoxia and apoptosis (see text for details).



Outer Penumbra Region



Ischemic Core



Delayed Programmed Cell Death

Time (Days)

Immediate Necrosis

1.1.1 Apoptosis in Neurodegeneration

Neuronal apoptosis or type I programmed cell death (PCD) (Schweichel and Merker, 1973) is a key player in cell loss observed in many neurodegenerative diseases including Alzheimer's disease (AD) (Behl, 2000; Colurso et al., 2003; Selznick et al., 1999; Stadelmann et al., 1999), Parkinson's disease (PD) (Andersen, 2001; Hartmann et al., 2000; Hartmann et al., 2001) and Huntington's disease (HD) (Kiechle et al., 2002; Ona et al., 1999; Sanchez et al., 1999) as well as stroke (Namura et al., 1998; Tagami et al., 1999; Whittemore et al., 1994) and other traumatic brain injuries (reviewed in (Ekshyyan and Aw, 2004; Raghupathi, 2004)). Evidence of caspase activation, a hallmark of apoptosis, has been identified in neurodegeneration associated with several of these conditions (Akao et al., 1999; Cutillas et al., 1999; Galvan et al., 2006; Graham et al., 2006; Yang et al., 1998). In ischemic stroke injury, pharmacological pretreatment with broad caspase inhibitors or selective inhibitors of caspase 1 or 3 has shown some promise (Cheng et al., 1998; Endres et al., 1998; Fink et al., 1998; Hara et al., 1997). As of January 2004 however, none of the caspase inhibitors tested have made it to clinical trial, despite having shown promise in preclinical studies. This is likely due to the fact that these pharmacological agents have poor bioavailability, and are unable to cross the blood brain barrier, and are for the most part irreversible inhibitors (Cheng et al., 2004). Bcl-2 family members have also been shown to play a vital role in apoptotic neurodegeneration (for review see (Becker and Bonni, 2004)). Both pro and anti apoptotic members of this family of proteins have been implicated in stroke injury, AD, PD as well as amyotrophic lateral sclerosis (ALS) (Becker and Bonni, 2004; Mattson, 2000). Transgenic mouse models of stroke with over-expression of Bcl-2, an anti-apoptotic member of the Bcl-2 family, have shown decreased injury/infarct size following ischemic injury (De Bilbao et al., 2000; Kitagawa et al., 1998; Martinou et al., 1994; Zhao et al., 2003), whereas Bcl-2 deficient mice show increases in infarct size (Hata et al., 1999). Bax, a pro-apoptotic member of the Bcl-2 family, is up-regulated following ischemic injury (Antonawich et al., 1998; Chen et al.,

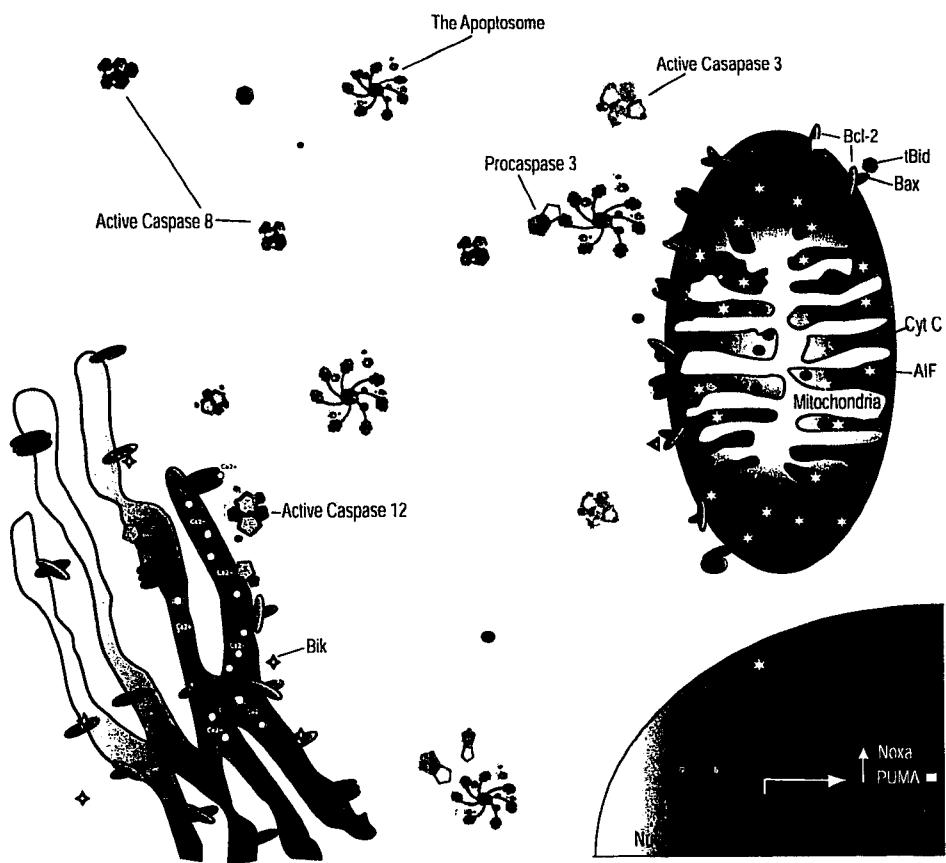
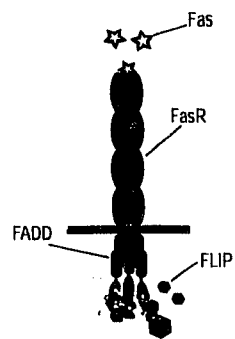
1996a; Gillardon et al., 1996; MacGibbon et al., 1997; Matsushita et al., 1998; Niwa et al., 1997), and neurons deficient in Bax are protected against growth factor deprivation, DNA damage induced cell death and axotomy (Cregan et al., 1999; Deckwerth et al., 1996; Johnson et al., 1998; Miller et al., 1997; Xiang et al., 1998). Current research implicates apoptosis as a key active cell death mechanism in many neurodegenerative paradigms. As such, recognizing the mechanisms by which this form of cell death proceeds in the nervous system is the first step to identifying novel therapeutic targets for the effective treatment and prevention of cell loss in these debilitating diseases. The following sections will discuss the mechanisms involved in the apoptotic program in greater detail.

1.2 Apoptosis & Cell Death

Apoptosis, or programmed cell death is an evolutionarily conserved active cell death mechanism that has evolved for the elimination of excess cells during tissue remodeling, but also for the removal of damaged or infected cells for the protection of the surrounding tissue (Kerr et al., 1972; Lockshin and Williams, 1965). Aberrant apoptosis however results in many diseased states that span from degenerative diseases such as AD and PD, to excessive cell proliferation resulting in cancer and autoimmune disease (Cory and Adams, 2002; Walensky, 2006). Apoptosis occurs through two main cellular pathways, both of which converge on a family of proteins, known as caspases that are the ultimate executioners of apoptosis (Figure 1.2). The first pathway known as the extrinsic pathway, originates from an extra-cellular signal transferred through a multi-protein cell surface receptor complex known as a death inducing signaling complex (DISC). Once recruited and activated, this complex sets off an intracellular caspase cascade originating with the activation of caspase 8 (Muzio et al., 1996). The second pathway is known as the intrinsic pathway. Its key players are the Bcl-2 family who provide a rheostat at the mitochondrion, and perhaps the endoplasmic reticulum, that sets the threshold of susceptibility of cells to apoptosis. When this balance is shifted towards apoptosis, it results in premeabilization of the mitochondrial membrane, loss of mitochondrial outer membrane permeabilization (MOMP), release of apoptogenic factors into the cytosol and activation of the caspase cascade (for recent reviews of the basic apoptotic pathways see (Adams, 2003; Danial and Korsmeyer, 2004; Jin and El-Deiry, 2005)). In the following section I will further delineate these pathways and their roles in apoptosis.

Caspases are a family of cysteinyl aspartate proteinases that are responsible for the propagation and execution of the apoptotic program. Healthy cells contain several caspases in their inactive precursor form, known as zymogens that are kept in this state through the activity of several regulatory molecules (Kumar, 2007). These inactive caspases are cleaved and activated through

Figure 1.2 Intrinsic and extrinsic cell death pathways. Extra cellular signals activate aggregation of death receptors (FasR), initiating the extrinsic cell death pathway. Aggregated death receptor proteins recruit intracellular adaptor proteins into the DISC formation resulting in the activation of caspase 8 and the downstream caspase cascade. The intrinsic cell death pathway occurs primarily at the mitochondria and can be activated via various intracellular signaling pathways including the extrinsic cell death pathway. Activation of the intrinsic cell death pathway is mediated by pro- and anti-apoptotic Bcl-2 family members on the mitochondrial membrane. Activation of the intrinsic pathway results in mitochondrial outer membrane permeabilization and release of apoptogenic factors including AIF, Cyt C and Smac/Diablo into the cytosol and activation of the apoptosome and downstream executioner caspases, including caspase 3. The endoplasmic reticulum can also play a role in intrinsic cell death through the control of Ca^{2+} release and activation of caspase 12/4.



various means depending on the apoptotic stimuli. Active caspases are homodimers, composed of two activated caspase monomers, each of which has two subunits, a larger one (p20) and a smaller one (p10) (Degterev et al., 2003). Activated caspase homodimers contain two active sites, one formed by each monomer on opposite sides of the activated complex, with the exception of caspase 9, which only has one active centre (Renatus et al., 2001). The caspase family can be divided into two subgroups: initiator caspases and effector caspases. Initiator caspases have long pro-domains that contain recruitment domains, either death effector domains (DED) or caspase recruitment domains (CARD), and are able to initiate proximity dependant activation. This group includes caspases 2, 8, 9 and 10. Effector caspases with shorter pro-domains are unable to self activate and rely on initiator caspases for activation (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004; Kumar, 2007). Following activation, these downstream effector caspases (predominantly caspases 3, 6 and 7) go on to cleave their various substrates within the cell (reviewed in (Timmer and Salvesen, 2007)). This proteolytic processing results in the prototypic apoptotic morphological features including cytoplasmic shrinking, membrane blebbing, intra-nucleosomal DNA fragmentation, phosphatidylserine exposure and fragmentation into membrane-enclosed apoptotic bodies (Wyllie et al., 1980). In order to tightly control the apoptotic pathway, cells are further equipped with a family of inhibitory proteins known as inhibitors of apoptosis (IAPs) that function as intrinsic regulators of the caspase cascade. These proteins are able to inhibit both initiator and executioner caspases, thus providing an additional mechanism to ensure the proper regulation of the apoptotic cascade (reviewed in (Liston et al., 2003)).

1.2.1 The Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway is activated by ligand bound cell surface death receptors including TNF, Fas and Trail family receptors, all of which contain an intracellular death domain (DD), required for the recruitment of corresponding adaptor proteins. Ligand binding to death receptors

leads to the recruitment of other death receptor proteins and the aggregation of these intracellular DDs. This is followed by the assembly of the DISC. Adaptor proteins such as FADD (Fas associated death domain), TRADD (TNFR-associated death domain), and RIP (receptor interacting protein kinase), among others are recruited to the cell surface death receptors and associate through their DD (Chinnaiyan et al., 1995; Degterev et al., 2003; Hsu et al., 1995). These adaptor proteins contains death effector domains (DED), and through these can recruit procaspase 8 (also potentially procaspase 10 and procaspase 2 although this remains somewhat controversial (Kischkel et al., 2001; Sprick et al., 2002; Wang et al., 2001a)) via association of homologous DEDs (Chinnaiyan et al., 1995). The proximity of the procaspase 8, or 10, zymogens then promotes dimerization and the autocatalysis required for activation. Active caspase 8 and/or 10 are then released from the DISC complex into the cytosol where they go on to activate effector caspases such as caspases 3, 6 and 9 (Medema et al., 1997). Caspase 8 is also known to cleave and activate the pro-apoptotic BH3-only family member BID, resulting in the activation of the intrinsic apoptotic pathway and a further amplification of the apoptotic signal (Green, 2000; Scaffidi et al., 1998). The activation of caspases at the DISC is further regulated through inhibition by proteins known as cFLIPs which have homology to Caspases 8 or 10 and are able to compete for binding at the DISC, thus inhibiting caspase activation (Griffith et al., 1998; Kim et al., 2000; Kim et al., 2002; Krueger et al., 2001; Tschopp et al., 1998). The functions of FLIPs however is not entirely understood as there is conflicting evidence suggesting that FLIPs may also have a role in sensitizing cells to death through caspase 8 activation (Chang et al., 2002).

Extrinsic cell death activation varies depending on the activated cell surface receptor. Activation of the extrinsic cell death pathway through the FAS and/or TRAIL receptors shows some cell type variability and can be further sub-divided into the requirement of the cell type for the activation of the mitochondrial pathway for the efficient mediation of cell death through the extrinsic

pathway (Jin and El-Deiry, 2005; Wang and El-Deiry, 2003). In type I cells the activation of caspase 8 is sufficient to efficiently induce the apoptotic pathway resulting in cell death. In type II cells however, efficient activation of caspases and the apoptotic pathway requires the amplification loop mediated by the caspase 8 cleavage of BID, and subsequent activation of the intrinsic pathway. This results in the release of mitochondrial pro-apoptotic factors and the formation of the apoptosome (Fulda et al., 2001; Scaffidi et al., 1998). In the latter case, caspase 9 will go on to activate caspase 3, which in turn activates caspase 8 to complete the feedback loop.

1.2.2 The Intrinsic Apoptotic Pathway

The mitochondria, and in some cases the ER (Lee et al., 1999; McCullough et al., 2001; Pinton et al., 2002; Pinton and Rizzuto, 2006; Zhu et al., 1996), sit at the central point of the intrinsic apoptotic pathway, and the pivotal event in the initiation of apoptosis via this pathway involves the MOMP. This permeabilization of the outer mitochondrial membrane is controlled by a family of pro- and anti-apoptotic proteins known as the Bcl-2 family, containing upwards of 20 members. All Bcl-2 family proteins share homology in up to four conserved regions known as Bcl-2 homology domains (BH1-4). Bcl-2 family proteins control the release of apoptotic factors (including cytochrome C (Cyt C), apoptosis inducing factor (AIF), Smac/Diablo and others) from the inter-membrane space of the mitochondria. The Bcl-2 family can be divided into two categories; the anti-apoptotic and the pro-apoptotic members. The anti-apoptotic members of this family include Bcl-2, Bcl-xl, Mcl-1 and Bcl-w these proteins typically contain Bcl-2 homology (BH) domains 1-4 and are associated with light intracellular membranes, including the mitochondrial outer membrane (MOM) and the ER (Schinzel et al., 2004). They are believed to protect the cells at the MOM where they are thought to maintain mitochondrial integrity. The pro-apoptotic Bcl-2 family members can be further subdivided into multi-domained pro-apoptotic proteins and BH3-only containing pro-apoptotic proteins. The multi-domained pro-apoptotic Bcl-2 family members include Bax, Bak and Bok, and typically possess BH domains 1-3.

They are thought of as the executioners of the Bcl-2 family, as they are required for mitochondrial mediated apoptosis (Zong et al., 2001). These pro-apoptotic Bcl-2 family proteins tend to have structures that closely resemble those of their anti-apoptotic brethren (Moldoveanu et al., 2006; Petros et al., 2004; Suzuki et al., 2000). The BH3-only proteins, including BIM, BID, Bad, BIK, Noxa, Puma, Hrk and BMF, share only one BH domain (BH3) with their multi-domained family members, but are otherwise unique. In these Bcl-2 family members lies the key to the Bcl-2 family rheostat. The BH3 only proteins function as cell death sentinels. They are situated throughout the cell and when activated by their various cellular stressors initiate the apoptotic program through interactions with their multi-domained brethren (Adams and Cory, 2007; Willis and Adams, 2005). Upon apoptotic stimuli, Bax and Bak become activated through the upstream activities of the BH3 only proteins. When activated Bax and Bak undergo a conformational change and homo-oligomerize (Walensky, 2006). These oligomers are believed to participate in the permeabilization of the MOM, however the mechanism of this permeabilization remains unclear (Green, 2005; Newmeyer and Ferguson-Miller, 2003). This MOMP results in the release of apoptogenic factors, including Cyt C, AIF, Smac/Diablo (reviewed in (Green and Kroemer, 2004)), into the cytosol. Once in the cytosol, Cyt C initiates the formation of the activator caspase protein complex known as the apoptosome. This holoenzyme complex is composed of a heptamer of Cyt C, Apaf-1 and Caspase 9 (Acehan et al., 2002; Yu et al., 2005c), and once activated, initiates the cleavage and activation of downstream executioner caspases and apoptosis (Li et al., 1997; Rodriguez and Lazebnik, 1999; Zou et al., 1997).

1.2.2.1 The Endoplasmic Reticulum and the Intrinsic Apoptotic Pathway

In addition to the role of the mitochondria in the intrinsic apoptotic pathway, there is evidence that an additional intracellular organelle, the endoplasmic reticulum (ER) also plays an important role in the life/death decision making process (for reviews see (Boyce and Yuan, 2006; Breckenridge et al., 2003a; Szegezdi et al., 2006). The ER has been shown to sensitize cells to intrinsic and extrinsic cell

death cues, as well as initiate specific death responses through the certain ER stress response (ESR) pathways. The ESR primarily functions to slow down normal ER function in order to alleviate accumulations of unfolded proteins, lipid imbalances or changes in ER lumen redox or ionic conditions (for reviews see (Ma and Hendershot, 2001; Schroder and Kaufman, 2005a, b)). Persistence of an ER stressor however, can lead to the initiation of the apoptotic program resulting from ER dysfunction. In the event of ER dysfunction, some of the core components of the ESR pathway participate in the induction of ER-induced apoptosis. ESR related transcription factors are, in some cases, able to directly activate and inhibit apoptotic pathways in response to persistent ER stress. One of these transcription factors is CHOP, which has been shown to promote ER-mediated apoptosis (Zinszner et al., 1998), likely through the down-regulate of anti-apoptotic Bcl-2 (McCullough et al., 2001), as well as the up-regulate the death receptor protein DR5 (Yamaguchi and Wang, 2004). ER stress can also activate both Bcl-2 family proteins, as well as caspases localized to the ER. Inhibition of ER stress induced apoptosis by anti apoptotic Bcl-2 has been well documented (Annis et al., 2001; Thomenius et al., 2003), and loss of both pro-apoptotic Bax and Bak is also protective against ER-stress (Oakes et al., 2005; Scorrano et al., 2003; Zong et al., 2003). There is also evidence suggesting that Bax and Bak are required for the mediation of signal integration between the mitochondria and the ER, as mouse embryonic fibroblasts (MEFs) treated with the ER-to-Golgi vesicle transport inhibitor brefeldin A (BFA) require the presence of Bax at both the ER and the mitochondria to go through the normal apoptotic process (Scorrano et al., 2003). The caspase cascade is also activated in response to ER stress. Mouse caspase 12, and one of it's human homologues caspase 4, are both localized to the ER and activated by ER, but not non-ER stressors (Hitomi et al., 2004; Nakagawa et al., 2000). Caspase 7 has also been shown to translocate to the ER in response to certain apoptotic stressors and may also play a role in the initiation and

amplification of the caspase cascade originating at the ER (Boyce and Yuan, 2006; Chandler et al., 1998; Rao et al., 2001).

1.2.3 The Intersection of Extrinsic and Intrinsic Apoptotic Signaling

Although it was previously stated that the intrinsic and extrinsic pathways converge at the point of caspase activation, there are several other points at which these pathways meet, it frequently occurs in the process of amplifying the apoptotic cascade. Following Caspase 8 and/or 10 activation, the active caspases are released from the DISC complex into the cytosol and proceed to activate the effector caspases 3, 6 and 9. Caspase 8 can also cleave and activate BID, a BH3 only member of the Bcl-2 family (Gross et al., 1999b; Li et al., 1998a; Luo et al., 1998; Yin et al., 1999). Once activated, truncated BID (tBID) will proceed to the mitochondria where it is known to interact with multi-domained Bcl-2 family members, initiating the intrinsic pathway through MOMP and formation of the apoptosome. Bcl-xl has also been shown to be able to disrupt the formation of the DISC complex in the plasma membrane, and thus to inhibit the extrinsic apoptotic pathway in mouse lung endothelial cells exposed to hypoxia/reoxygenation conditions (Wang et al., 2004). In addition to this, Bcl-xl, but not Bcl-2 is also able to inhibit TRAIL-induced apoptosis in type II cells, providing a specific linker between the intrinsic and extrinsic pathways (Keogh et al., 2000). Caspase 8 can also be further activated through a positive feedback amplification of caspases following activation of the intrinsic apoptotic pathway. Both caspases 3 and 6 are able to cleave and activate caspase 8 to amplify the intracellular apoptotic signal (Cowling and Downward, 2002; Jin and El-Deiry, 2005; Tang et al., 2000).

P53 is another protein that links the intrinsic and extrinsic apoptotic pathways together. The p53 tumor suppressor protein is a key player in the response to cellular stressors, leading to either cell cycle arrest or apoptosis (for reviews see (Michalak et al., 2005; Vousden, 2000; Vousden and Lu, 2002). Although predominantly referenced as a key initiator of the intrinsic apoptotic pathway,

p53 also plays a role in extrinsic apoptosis through the up-regulation of death receptors DR5/Killer and DR4 (Guan et al., 2001; Wu et al., 1997), and has also been implicated in the regulation of FAS (Bennett et al., 1998; Owen-Schaub et al., 1995). Further, p53 has also been shown to up-regulate BID and SIVA, two pro-apoptotic protein involved in intracellular propagation of the apoptotic signal through the extrinsic pathway (Fortin et al., 2004; Prasad et al., 1997; Sax et al., 2002).

In summary, apoptosis occurs through both intrinsic and extrinsic pathways (Figure 1.2). The extrinsic pathway is activated by extracellular stimuli and may or may not require the participation of the intrinsic pathway for efficient propagation of the cell death signal. The intrinsic pathway is regulated predominantly at the mitochondria, but may also involve activation of apoptotic signals through the ER. Although these pathways intersect at various points, their final point of convergence involves the activation of the caspase cascade.

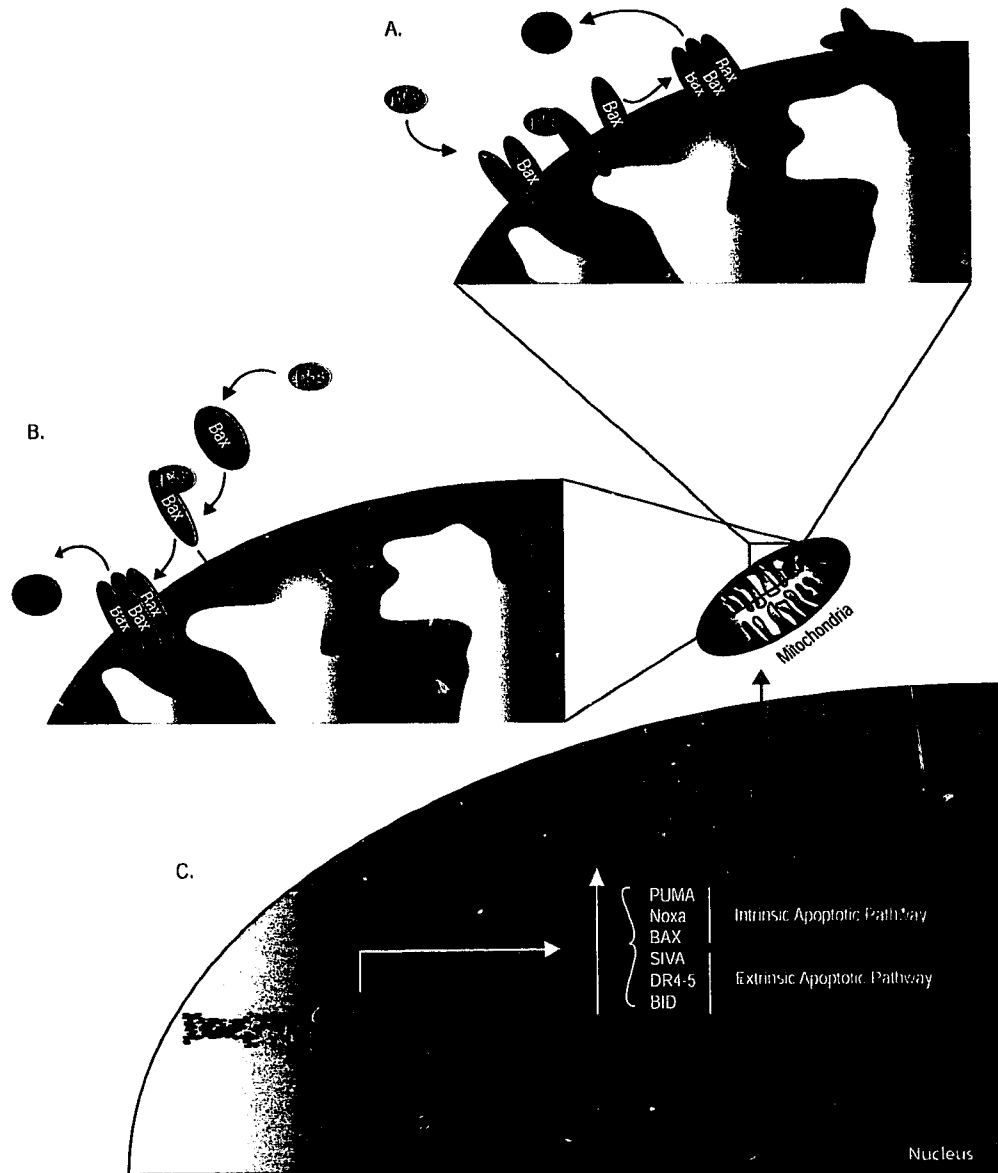
1.3 P53 Mediated Apoptosis

The P53 tumor suppressor gene encodes a transcription factor that is a key regulator of many cellular processes. The p53 protein acts as a cellular stress sensor and is activated in response to DNA damage, hypoxia, aberrant growth signals, oncogene expression and nucleotide depletion (reviewed in (Giaccia and Kastan, 1998; Levine, 1997)). In response to such stimuli, p53 can respond through the regulation of various intracellular programs including cell cycle arrest, senescence, DNA repair, survival or apoptosis and most recently autophagy (Bensaad et al., 2006; Crighton et al., 2006; Green and Chipuk, 2006; Michalak et al., 2005). P53 has been implicated in the progression of many diseased states and loss of p53 is observed in greater than 50% of human cancers (Levine, 1997). Although it has been shown to be required for the regulation of several cellular processes, it is through the regulation of apoptosis that p53 is believed to exert its tumor suppressor functions and influences on disease.

The p53 tumor suppressor protein mediates apoptosis predominantly through the intrinsic pathway. As a transcription factor, its role in apoptosis has been believed to occur mostly at the level of transcription, through the transcriptional up-regulation of intrinsic pro-apoptotic proteins including Bax (Miyashita and Reed, 1995), APAF-1 (Fortin et al., 2004; Moroni et al., 2001; Robles et al., 2001), PUMA (Nakano and Vousden, 2001; Yu et al., 2001), and Noxa (Oda et al., 2000). P53 can also however influence the extrinsic apoptotic pathway through the transcriptional up-regulation of death receptor proteins and pro-apoptotic BH3-only family member BID (Figure 1.3C) (Guan et al., 2001; Sax et al., 2002; Wu et al., 1997). There is also evidence suggesting that p53 induced transcriptional repression is also an important factor for mediating hypoxia induced apoptosis (Koumenis et al., 2001). Further, p53 has been shown to transcriptionally repress the activity of NF- κ B (Aleyasin et al., 2004; Ikeda et al., 2000; Webster and Perkins, 1999), which supports survival

Figure 1.3 p53-mediated transcriptionally dependant and independent apoptotic pathways A)

At the mitochondria, p53 is able to bind to and neutralize anti-apoptotic Bcl-2 family members, allowing pro-apoptotic Bax and Bak to oligomerize and cause MOMP. B) Within the cytosolic compartment of the cell, p53 is also believed to be able to bind to Bax, resulting in its activation and translocation to the mitochondria and the unleashing of the apoptotic cell death pathway. C) The canonical role of p53 in the transcriptional activation of pro-apoptotic genes at the nucleus remains a pivotal role in the apoptotic pathway.



signaling in neurons through up-regulation of anti-apoptotic proteins including Bcl-2 and IAPs (Mattson et al., 2000). Recently p53 has also been shown to up-regulate TIGAR, a protein that inhibits glycolysis resulting in an overall decrease in reactive oxygen species (ROS) (Bensaad et al., 2006; Green and Chipuk, 2006). This suggests an additional mechanism by which p53 is able to protect cells apoptosis caused by ROS. Recent work had been focused on the development of small molecules to modulate the function of p53, including modulation of its transcriptional activity (Mujtaba et al., 2006).

In addition to its role in transcriptional regulation, recent studies have shown p53 to play a more direct role in the initiation of the intrinsic apoptotic pathway through direct protein-protein interactions (for a review on this see (Moll et al., 2005)). Within the past 5 years there has been emerging evidence showing that p53 directly interacts with Bcl-2 family both at the level of the mitochondria (Dumont et al., 2003; Erster et al., 2004; Leu et al., 2004; Mihara et al., 2003) as well as within the cytoplasmic compartment of the cell (Chipuk et al., 2004; Schuler and Green, 2005; Speidel et al., 2006). P53 has been shown to interact directly with both Bcl-2 and Bcl-xl, to antagonize their abilities to stabilize the MOM both *in vitro* and *in vivo* (Figure 1.3A) (Bonafe et al., 2004; Chipuk et al., 2004; Marchenko et al., 2000; Mihara et al., 2003; Sansome et al., 2001). Further, within the cytosol, p53 has been shown to interact with and activate cytosolic Bax (Figure 1.3B) (Chipuk et al., 2004; Schuler and Green, 2005). The importance of p53 in the propagation of apoptotic signals has been unequivocally demonstrated by several researchers. A great deal of work however, remains to be done to discern the involvement of p53-mediated cell death in various disease scenarios.

1.3.1 P53 Mediated Apoptosis in Neurons

The p53 tumor suppressor gene has been identified as a key apoptotic factor in neuronal injury. P53 involvement has been implicated in the progression of several neurological disorders

including AD, PD and HD (for review see (Culmsee and Mattson, 2005; Morrison et al., 2003)). Increases in p53 expression have also been observed following various forms of neuronal injury including ischemia (Cheng et al., 2003; Chopp et al., 1992; Crumrine et al., 1994; Culmsee et al., 2001b; Li et al., 1994), hypoxia (Banasiak and Haddad, 1998; Halterman and Federoff, 1999) and excitotoxicity (Cregan et al., 1999; Xiang et al., 1998). *In vitro* studies have further shown that the over-expression of the p53 protein is, in itself, sufficient to trigger apoptosis in post-mitotic cerebellar granule neurons (CGNs) (Slack et al., 1996). Mice carrying a germline deletion of p53 incur significantly less brain damage following excitotoxic or stroke injuries, consistent with the belief that p53 is a key player in these neuronal injury paradigms (Crumrine et al., 1994; Morrison et al., 1996; Xiang et al., 1996). Extensive research over the past several years has shown that the absence of p53 is protective in a wide variety of neuronal injury models including MPTP-induced neurotoxicity (Trimmer et al., 1996), traumatic brain injury (Martin et al., 2001), DNA damaging agents (Morris et al., 2001; Xiang et al., 1998) and glutamate (Chen and Chuang, 1999; Uberti et al., 1998) among others. Further, recent work has shown p53 blocking peptides to be neuroprotective following acute brain injury, and suggests that small molecule inhibitors may serve as potential neuroprotective agents (Culmsee et al., 2003; Culmsee et al., 2001b). These studies emphasize the importance of p53 as a key apoptotic factor following neuronal injury and underscore the necessity of uncovering the mechanisms by which p53 induces the death of post-mitotic neurons.

To summarize, p53 plays a role in many cellular processes and is the determining factor between cell death and survival following certain types of apoptotic stimuli. P53 has been shown to be involved in apoptosis not only through its role as a transcription factor, but also directly at the level of the mitochondria through protein-protein interactions with Bcl-2 family proteins. P53 has been implicated in many neuronal cell death paradigms and recent research targeting p53 with pharmacological inhibitors has shown great promise in the inhibition of neuronal apoptosis.

1.4 Apoptosis and the Bcl-2 Family

The Bcl-2 family of proteins are important regulators of mitochondrial-mediated (intrinsic) apoptosis (Gross et al., 1999a). Although Bcl-2 family members are generally agreed upon as being the regulators intrinsic apoptosis, how exactly this occurs remains controversial. The apoptotic rheostat is controlled through the balance of pro- and anti-apoptotic Bcl-2 family members. The ultimate control of this balance lies in the interaction of the pro-apoptotic BH3-only members of the Bcl-2 family.

BH3-only Bcl-2 family members exert their apoptotic activities upstream of Bax and Bak. Work done in MEFs deficient in both Bax and Bak has shown that these cells cannot be induced to die by up-regulation of pro-apoptotic BH3-only proteins (Cheng et al., 2001; Zong et al., 2001). The method by which the BH3-only proteins exert their pro-apoptotic effect is still remains in contention (Figure 1.4). One model of BH3-only interactions suggests that BH3-only proteins are activated via various apoptotic stimuli, and upon activation they engage anti-apoptotic Bcl-2 family members through insertion of their BH3 domain into the hydrophobic groove formed by BH1, BH2 and BH3 domains of their binding partners (Sattler et al., 1997; Walensky, 2006). This interaction frees up the sequestered multi-domained pro-apoptotic family members Bax and Bak to permeabilize the outer mitochondrial membrane and release the pro-apoptotic contents of the intermembrane space into the cytosol, resulting in activation of the caspase cascade (Figure 1.4A). In the second model of Bcl-2 family activity the BH3-only proteins are further subdivided into sensitizers and activators, where certain BH3-only family members, or activators (BIM, BID and Puma) can directly bind to and activate Bax and Bak, while the other BH3-only proteins, or sensitizers more traditionally bind to and inhibit the activities of anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-xl (Figure 1.4B) (Cartron et al., 2004; Certo et al., 2006; Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002; Oh et al., 2006; Walensky et al., 2006). This model is still very controversial, as some groups contend that

there is no binding between these BH3 only family members and their pro-apoptotic relatives except under detergent conditions known to alter the Bax conformation (Hsu and Youle, 1998; Tan and Ting, 2000; Willis et al., 2007).

1.4.1 Multi-Domained Bcl-2 Proteins in Apoptosis

Multi-domained Bcl-2 family proteins include both pro- (including Bax and Bak) and anti-apoptotic Bcl-2 family members (including Bcl-2, Bcl-xl, and Bcl-w). These proteins are considered to be the gatekeepers of cell death. Associated with light intracellular membranes, particularly the MOM, the ER, and occasionally the nucleus, anti-apoptotic multi-domained Bcl-2 family members are the front line defense against MOMP and apoptosis via the intrinsic pathway (Schinzel et al., 2004). Interactions between pro- and anti-apoptotic Bcl-2 family members have been well documented. In healthy cells both Bcl-xl and Mcl-1 are required to sequester Bak, which is localized in complexes on intracellular membranes (Cuconati et al., 2003; Schinzel et al., 2004; Willis and Adams, 2005; Willis et al., 2005). For Bak to be activated, it must be released not only from one, but both of these anti-apoptotic Bcl-2 family members through their inhibition by one or more BH3-only proteins (Chen et al., 2005; Nijhawan et al., 2003; Willis et al., 2005). Bax however is localized primarily to the cytosol and loosely associated to the mitochondrial outer membrane in an inactive conformation prior to apoptotic stimuli (Nechushtan et al., 1999; Suzuki et al., 2000; Wolter et al., 1997). Many proteins have been implicated in its retention in the cytosol, including 14-3-3, Ku70, and Humanin, however release from these cytosolic chaperones is not sufficient in itself for activation of the Bax protein and initiation of the intrinsic apoptotic pathway (Nomura et al., 2003; Sawada et al., 2003a; Sawada et al., 2003b; Yuan et al., 2003b). Upon activation by apoptotic stimuli, Bax changes conformation and inserts itself into the mitochondrial membrane resulting in MOMP and the release of apoptogenic factors including AIF, Cyt C and Smac/DIABLO into the cytosol, activating the caspase cascade (Kroemer et al., 2007; Wolter et al., 1997).

Studies of mice deficient in individual multi-domained Bcl-2 family proteins has revealed unique requirements for each individual family member, and suggests distinct roles in various systems. The first of these germline knockout mice, deficient in Bcl-2, are viable, however they exhibit dramatic apoptosis in the lymphoid compartment, and polycystic kidney disease (Veis et al., 1993). Bcl-xl deficient mice are embryonic lethal, at E13, showing increased apoptotic activity hematopoietic compartment as well as the nervous systems (Motoyama et al., 1995). Loss of anti-apoptotic Bcl-w results in male infertility and testicular degeneration, as does the loss of pro-apoptotic Bax (Knudson et al., 1995; Print et al., 1998; Ross et al., 1998). Loss of Bak failed to reveal any phenotype on its own, however cumulative loss of both Bax and Bak result in several severe developmental defects, including accumulations of excess cells in the nervous and hematopoietic systems. These animals exhibit significant embryonic lethality however, 10% of double deficient animals do survive to adulthood (Lindsten et al., 2000). These observations lead to the theory that Bax and Bak are fairly interchangeable and can compensate for one another in most tissues. Unique among Bcl-2 family members, Mcl-1 germ line mutant mice are peri-implantation lethal, suggesting a requirement for Mcl-1 early in development (Rinkenberger et al., 2000). The various phenotypes resulting from the loss of individual multi-domained Bcl-2 family members suggests that although they may have similar expression patterns under certain circumstances, they all play individual roles in the control of apoptosis.

1.4.1.2 Control of Mitochondrial permeability by Bcl-2 family members

Anti-apoptotic Bcl-2 family members inhibit apoptosis in large part through interaction with their pro-apoptotic brethren (Chen et al., 2005; Cuconati et al., 2003; Schinzel et al., 2004; Willis and Adams, 2005; Willis et al., 2005). They have also however been shown to be involved in the inhibition of mitochondrial swelling and loss of mitochondrial membrane potential (MMP) (Marchetti et al., 1996; Vander Heiden et al., 1997), the regulation of intracellular Ca²⁺ levels (Baffy et al., 1993;

Pinton et al., 2000; Pinton et al., 2002; Pinton and Rizzuto, 2006), in blocking the generation of reactive oxygen species (ROS) (Hockenbery et al., 1993; Kane et al., 1993; Zamzami et al., 1995), and modulating the exchange of ATP/ADP between the mitochondrial matrix and the cytosol following cell death stimuli (Vander Heiden et al., 1999). The inhibition of mitochondrial swelling and loss of mitochondrial membrane potential, coupled with the ability of anti-apoptotic Bcl-2 family proteins to modulate ATP/ADP levels has been attributed to Bcl-2 proteins playing a direct role in the regulation of the mitochondrial outer membrane permeability (MOMP) (Marzo et al., 1998; Shimizu et al., 1998; Shimizu et al., 2000).

Structurally, multi-domained Bcl-2 pro-and anti-apoptotic family members resemble the membrane insertion domains of diphtheria toxin, which lead to the hypothesis that Bcl-2 family members may also form pores in membranes (Muchmore et al., 1996; Petros et al., 2004). While there is *in vitro* data suggesting the formation of these pores (Jonas et al., 2005; Schendel et al., 1998; Shimizu et al., 2000; Shimizu et al., 1999), and that at physiological pH Bcl-2 is able to inhibit the ability of Bax to form channels (Antonsson et al., 1997), this work has all been done in synthetic membranes and may not be physiologically relevant. It was previously suggested that Bcl-2 family members are involved in the regulation of the mitochondrial permeability transition pore (PTP) which is putatively composed of components of both the inner and outer mitochondrial membranes including the voltage dependant anion channel (VDAC), adenine nucleotide translocator (ANT), the peripheral benzodiazepine receptor (PBR), and cyclophilin D (Cyc D) (Marzo et al., 1998; Shimizu et al., 2000; Verma et al., 2006). Recent evidence using Cyc D deficient mice however, suggests that the PTP regulates necrotic and some forms of ischemia-reperfusion cell death, but not apoptotic cell death pathways (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005).

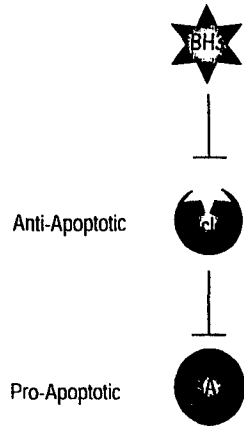
In spite of their exclusion from the formation of the PTP, multi-domained Bcl-2 family members Bcl-2, Bcl-xl, Bax and Bak have all been shown to interact with VDAC (Ferri et al., 2000;

Shimizu and Tsujimoto, 2000; Vander Heiden et al., 2000; Vander Heiden et al., 2001). There is evidence that Bcl-xl is able to maintain VDAC in an open conformation, allowing ATP/ADP exchange, and inhibiting mitochondrial swelling in isolated mouse liver mitochondrion. In mitochondria isolated from cells that had undergone growth factor withdrawal, recombinant Bcl-xl was able to keep VDAC open, allowing the passage of creatine phosphate, but not Cyt C (Vander Heiden et al., 2001). Further, pro-apoptotic BH3-only Bcl-2 family member BID is able to cause the closure of VDAC, in artificial membranes, suggesting regulation of VDAC by some but not all Bcl-2 family proteins (Rostovtseva et al., 2004). As VDAC is a major constituent of the mitochondrion and is required for the movement of organic ions, including respiratory substrates, ATP and ADP, it is conceivable that this interaction and regulation of VDAC by Bcl-2 family members occurs as part of the apoptotic process (Chipuk et al., 2006; Lemasters and Holmuhamedov, 2006; Rostovtseva et al., 2002).

Recently published data implicates the multi-domained Bcl-2 family members in the formation of a newly described channel known as the mitochondrial apoptosis induced channel (MAC), of which Bax and/or Bak are hypothesized to be major structural components (Dejean et al., 2005; Dejean et al., 2006; Guo et al., 2004; Pavlov et al., 2001). This channel is believed to be the channel through which Cyt C is released, based on pore size and electrophysiological characteristics. Further, the onset of MAC activity, as demonstrated by patch clamp experiments, coincides with Cyt C release (Dejean et al., 2005; Gross et al., 1998; Guo et al., 2004; Martinez-Caballero et al., 2004; Pavlov et al., 2001). Pores formed by oligomeric Bax in synthetic membranes have been shown to exhibit strikingly similar conductance levels to those of MAC, and form channels that would easily

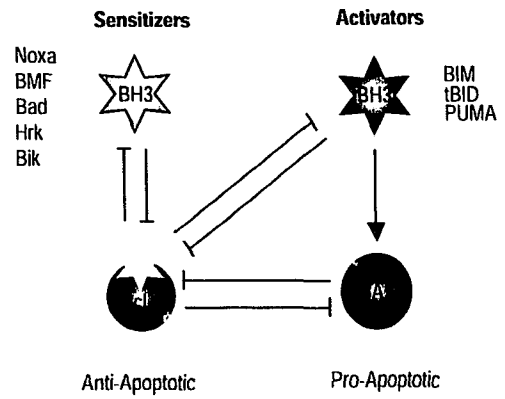
Figure 1.4 BH3-only Bcl-2 family models of action. There are several models of BH3-only Bcl-2 family activities. A) One model of BH3-only activity suggests that BH3-only proteins act as pro-apoptotic “de-repressors” by binding to and inhibiting their anti-apoptotic multi-domain Bcl-2 family counterparts (Bcl-2, Bcl-xl, Mcl-1). B) A second model of BH3-only activity suggests that the BH3-only family can be further subdivided into activator proteins and sensitizer proteins. Activators are able to directly bind to and activate pro-apoptotic multi-domain proteins such as Bax or Bak. Sensitizers however act similarly to the “de-repressors” of the first model, and are only able to bind to and inhibit their anti-apoptotic counterparts, thus liberating the pro-apoptotic proteins to cause MOMP and initiate the caspase cascade. C) BH3-only Bcl-2 proteins can also be divided by their apoptotic potency through their ability to interact with multiple members of the multi-domain anti-apoptotics. Some, including PUMA, and BIM are able to interact with all the anti-apoptotic Bcl-2 family members and as such elicit a more potent cell death response. Others including BMF, Bad and Noxa can only bind to a smaller subset of Anti-apoptotics and elicit a weaker apoptotic effect.

A.



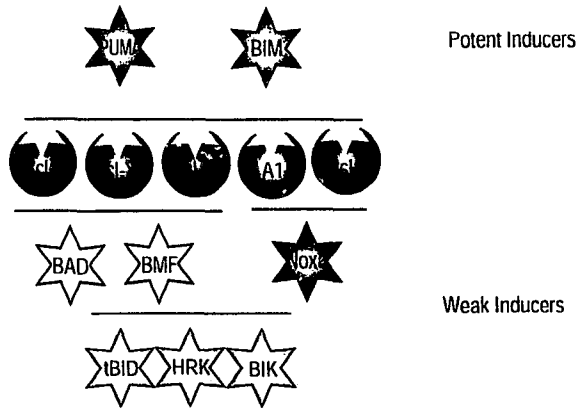
Model I: Indirect Activators

B.



Model II: Sensitizers/Activators

C.



allow for the passage of Cyt C (Dejean et al., 2006; Roucou et al., 2002). MAC activity is also absent in cells lacking both Bax and Bak, or staurosporine treated Hela cells immunoprecipitated of Bax, suggesting that Bax and Bak may be structural components of the MAC channel (Dejean et al., 2005; Dejean et al., 2006). Further, over-expression of Bcl-2 is sufficient to inhibit MAC activity (Pavlov et al., 2001). Although this data is based primarily on patch-clamp conductance experiments done in mitochondria, and the biochemistry supporting the results is purely correlative, the results do none-the-less support the existence of an apoptotic pore formed of and controlled by the Bcl-2 family of proteins.

1.4.2 BH3 Only Proteins in Apoptosis

The BH3-only members of the Bcl-2 family of proteins share only a common 9 amino acid BH3 domain with the other family members, but are otherwise unique (Huang and Strasser, 2000; Puthalakath and Strasser, 2002). This BH3 domain has been shown to be required for the interaction with other Bcl-2 family members in the induction of apoptosis (Cheng et al., 2001; Wang et al., 1996; Zong et al., 2001). With greater than ten BH3-only proteins presently described, it is easy to understand how they can be involved in very different cell death pathways. The hetero-dimerization of BH3-only proteins to other members of the Bcl-2 family is mediated by the insertion of the BH3 domain into a hydrophobic cleft formed between BH1, BH2 and BH3 domains on the surface of target Bcl-2 family members (Sattler et al., 1997). BH3-only proteins can be activated through transcriptional up-regulation (Han et al., 2001; Harris and Johnson, 2001; Imaizumi et al., 1997; Inohara et al., 1997; Nakano and Vousden, 2001; Oda et al., 2000; Whitfield et al., 2001; Yu et al., 2001), or post-translational modification (Li et al., 1998a; Luo et al., 1998; O'Connor et al., 1998; O'Reilly et al., 2000; Puthalakath et al., 1999; Puthalakath et al., 2001), and are activated in response to differing stress stimuli (Adams and Cory, 2007; Bouillet and Strasser, 2002; Germain et al., 2005; Labi et al., 2006; Puthalakath and Strasser, 2002). As mentioned above, there are conflicting

theories as to the exact mechanism of action of BH3-only activation of the apoptotic pathway. In addition to this however, BH3-only proteins have also been found to possess varying apoptotic strength depending on their ability to antagonize specific subsets of anti-apoptotic Bcl-2 family members (Figure 1.4C). Certain BH3-only family members exhibit an indiscriminate ability to bind anti-apoptotic Bcl-2 family proteins, and are therefore very potent initiators of apoptosis (ie PUMA, BIM). Others BH3-only members however, are able to inhibit only a select subset of anti-apoptotic Bcl-2 proteins, resulting in weaker apoptotic effects (ie Noxa, BAD, BMF, etc) (Chen et al., 2005). There is also evidence of co-operativity among BH3-only family members with more than one of the weaker pro-apoptotic BH3-only proteins working together to elicit a potent killing response (Chen et al., 2005; Germain et al., 2005).

In addition to induction potency, knock-out studies of BH3-only proteins also demonstrate stimulus and tissue specificity (Bouillet et al., 1999; Coultas et al., 2004; Imaizumi et al., 2004; Ranger et al., 2003; Sax et al., 2002; Villunger et al., 2003a; Villunger et al., 2003b; Yin et al., 1999). BIM has been found to be important for haematopoietic cell homeostasis, as mice deficient in this gene accumulate 2-5 fold increased numbers of lymphoid and myeloid cells (Bouillet et al., 1999; Bouillet and Strasser, 2002; Puthalakath and Strasser, 2002). BID deficient mice however, appear normal, but are resistant to cell death induced by anti-Fas, and loss of pro-apoptotic BID may enhance cell death response to chemosensitivity in a p53-dependant manner (Bouillet and Strasser, 2002; Puthalakath and Strasser, 2002; Sax et al., 2002; Yin et al., 1999). Bad deficient mice are viable and show no overt phenotype. With aging however, the mice develop diffuse large B cell lymphoma of germinal centre origin. Bad deficient cells did however show cell type and stimulus related sensitivities, for example, Bad deficient MEFs showed no increased sensitivity to growth factor withdrawal, but had increased sensitivity when treated in combination with Fas ligand (Ranger et al., 2003). Studies have shown Noxa and PUMA to be up-regulated under conditions of p53-

mediated cell death in mouse embryonic fibroblasts (MEFs) (Oda et al., 2000) and colon cancer cell lines (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2003; Yu et al., 2001). Studies with mice carrying a null mutation for PUMA have shown that it is a key player in the apoptotic pathway. PUMA deficiency confers similar protection against apoptotic stimulus as p53-deficiency (Jeffers et al., 2003; Villunger et al., 2003). Work done in cell lines has also shown that PUMA may be regulated by factors other than p53, such as E2F-1 (Hershko and Ginsberg, 2003) and p73 (Melino et al., 2003), emphasizing its importance in the apoptotic pathway. The primary function of the Noxa protein seems to be at the mitochondrial level (Seo et al., 2003 115). Recent work with Noxa-deficient mice suggests that it may play a minor role in the apoptotic pathway in fibroblasts, but that it is dispensable in other tissues (Villunger et al., 2003). Mice lacking Blk, the murine homologue of BIK, are developmentally normal, suggesting that it may play a redundant role on apoptosis (Coultas et al., 2004). Its human homologue, BIK however, has been shown to play an important role in ER-mediated apoptosis, and mitochondrial cristae remodeling during apoptosis, and is able to co-operate with BH3-only protein Noxa in the activation of Bax dependant apoptosis (Germain et al., 2005; Germain et al., 2002; Mathai et al., 2005). Although BMF initially implicated in cell death involving anoikis, mice lacking BMF are developmentally normal (Labi et al., 2006). There is preliminary evidence however that BMF may play a role in excitotoxic cell death (Slack lab unpublished data). BH3-only protein DP5/HRK deficient mice were also developmentally normal, closer examination however revealed that loss of DP5/HRK rescued neurons from NGF withdrawal and axotomy induced cell death (Imaizumi et al., 2004). Taken together, although loss of individual BH3-only proteins does not seem to result in any gross phenotype, these results strongly suggest different stimulus and tissue specificities among BH3-only Bcl-2 family members.

1.4.3 Bcl-2 Family Proteins at the Endoplasmic Reticulum

Although Bcl-2 family proteins have been long known to be mediators of the mitochondrial-mediated intrinsic apoptotic pathway, their localization on the ER, and its significance to apoptosis was largely overlooked (Krajewski et al., 1993; Lithgow et al., 1994; Schinzel et al., 2004; Zong et al., 2003). The Bcl-2 protein itself however, has been known to play an important role in the regulation of intracellular calcium within the ER (Baffy et al., 1993; Lam et al., 1994; Magnelli et al., 1994). Recently evidence has emerged demonstrating the importance of Bcl-2 family members in mediating apoptosis resulting from ER stress (Distelhorst and McCormick, 1996; Scorrano et al., 2003; Wei et al., 2001). Both Bcl-2 pro and anti-apoptotic family members have been shown to contribute to control of ER-mediated apoptosis. Pro-apoptotic Bax and Bak have been shown to localize to an initiate apoptosis at the ER as well as the mitochondria (Zong et al., 2003). Further, loss of both Bax and Bak result in cells that are resistant to ER-mediated cell death stimuli (Oakes et al., 2005; Scorrano et al., 2003). Recently Bak, but not Bax, has also been shown to induce swelling and restructuring of the ER in a Bcl-xl dependant manner, implicating a unique role for Bak at the ER (Klee and Pimentel-Muinos, 2005). A number of pro-apoptotic BH3-only Bcl-3 family members including PUMA (Li et al., 2006; Luo et al., 2005; Nickson et al., 2007; Reimertz et al., 2003), BIK (Germain et al., 2005; Germain et al., 2002; Mathai et al., 2005), Noxa (Germain et al., 2005; Li et al., 2006), BIM (Morishima et al., 2004), BID (White et al., 2005) have also been implicated in ER-mediated apoptosis. Over-expression of anti-apoptotic ER-targeted Bcl-2 has been shown to protect cells from ER-, but not mitochondrial-mediated apoptotic stimuli (Annis et al., 2001; Hacki et al., 2000; Rudner et al., 2001; Thomenius et al., 2003; Wang et al., 2001b). Exactly how Bcl-2 family members regulate ER-stress mediated apoptosis however remains a somewhat contentious issue (Distelhorst and Shore, 2004). Anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-xl have been shown to inhibit thapsigargin induced ER-stress induced apoptosis through functional interactions with inositol 1,4,5-triphosphate receptor (InsP₃R) (Chen et al., 2004; White et al., 2005). In these studies, both anti-apoptotic proteins were

shown to endogenously interact with InsP₃R, and Bcl-xl was shown to inhibit ER calcium release by increasing the sensitivity of the channels to very low levels of ligand (InsP₃) (Chen et al., 2004; White et al., 2005). Further, interactions between Bcl-xl and the InsP₃R can be inhibited through sequestration of the Bcl-xl proteins with Bax or tBID (White et al., 2005). In a separate study using Bax/Bak double deficient MEFs, an interaction of Bcl-2 with InsP₃R type 1 was shown to result in increased phosphorylation of the receptor resulting in an increased Ca²⁺ leak, conflicting with the previously mentioned reports (Oakes et al., 2005). Although the exact mechanism of Bcl-2 family Ca²⁺ regulation at the ER remains to be fully understood, their involvement in ER-stress mediate apoptosis is indisputable.

1.4.4 Bcl-2 Proteins and Neuronal Apoptosis

Many Bcl-2 family proteins are involved in the apoptotic response in the nervous system. During development, the expression of Bcl-2, Bcl-xl, Bax, and BID in CNS have been studied by immunohistochemistry. Bcl-2, the founding member of this family, is expressed at both the mRNA and protein levels in the developing nervous system (Krajewska et al., 2002; Merry et al., 1994). Bcl-2 and Bax expression peaked at E11 and remained high until E14-15 and decrease to relatively low level at birth. In contrast, Bcl-xl remained high once it is induced and only declined about 1 week postnatally. BID expression was found at a high level during embryonic as well as adult life. A Comparative immunohistochemical study of Bcl-2 and Mcl-1 done in human tissues showed differential regulation of these two multi-domained anti-apoptotic proteins, although very little Mcl-1 was observed in neurons under non-injury conditions (Krajewski et al., 1995).

1.4.4.1 Anti-apoptotic Bcl-2 Proteins in Neuronal Apoptosis

Genetic manipulations of anti-apoptotic Bcl-2 family members within the mouse have helped to determine the various roles these proteins play within the nervous system. Bcl-2 targeted deficiency studies suggest that Bcl-2 is expendable for CNS development. Loss of motor, sensory

and sympathetic neuron populations within the peripheral nervous system (PNS) during early postnatal life however, suggests that there may be a requirement for Bcl-2 in the maintenance and survival of these post-mitotic populations.(Michaelidis et al., 1996). Bcl-xl germline knock out mice show profound neuronal abnormality which leads to massive apoptosis in the CNS and lethality at E13.5 (Motoyama et al., 1995; Roth et al., 2000). The cell death can be seen as early as E11.5 in post-mitotic differentiating neurons in the brain and spinal cord. Germline knockout mice doubly deficient in both Bax and Bcl-xl, or Caspase 3 and Bcl-xl still exhibit embryonic lethality, but the massive apoptosis in the CNS is rescued (Roth et al., 2000; Shindler et al., 1997). Primary neurons cultured from Bax and Bcl-xl deficient mice exhibited rates of spontaneous apoptosis indistinguishable from those of wildtype cultures, suggesting that some of this observed apoptosis is likely due to secondary effects resulting from the effects of germline deletion on other systems. Moreover, neuron specific conditional knock out mice, where loss of Bcl-xl is limited to catecholaminergic neurons, have shown Bcl-xl to be largely dispensable for the development and survival of neurons, further supporting the notion that the massive apoptosis observed in the CNS of the germline deficient mice may be a secondary to systematic problems (Savitt et al., 2005). Targeted deficiency of Mcl-1 results in growth arrest at embryonic day 3, therefore any direct effect its absence has on the nervous system remains to be determined (Rinkenberger et al., 2000). Recently, a study has been conducted to determine whether Mcl-1 heterozygous mice are more resistant to seizure-induced neuronal loss (Mori et al., 2004). A comparison of the sensitivity of mouse strains to pilocarpine induced seizure revealed that elevated endogenous Mcl-1 expression in hippocampal neurons correlated with increased resistance to cell death (Mori et al., 2004). Further, studies have shown Mcl-1 expression to be associated with neural progenitor survival, involvement in Notch1-mediated expansion of the neural precursor pool (Oishi et al., 2004), as well as the development of cerebellar granule neurons (Zhang and D'Ercole, 2004).

1.4.4.2 Pro-apoptotic Bcl-2 Proteins in Neuronal Apoptosis

Germline knockouts of Bax, Bak or both of these pro-apoptotic multi-domained Bcl-2 family members also fail to exhibit an extreme phenotype that one might expect if Bax, Bak or both were independently necessary for neuronal cell death (Deckwerth et al., 1996; Lindsten et al., 2000; White et al., 1998). Since single targeted germline deletion of Bax or Bak have less severe effects on neuronal development than the loss of other pro-apoptotic proteins such as caspase 3, APAF-1 or caspase 9, the induction of caspases during developmental is either independent of multi-domained pro-apoptotic Bcl-2 family members, or occurs through a pathway other than the intrinsic cell death pathway (Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Yoshida et al., 1998). Bax and Bak deficient neural progenitor cells however, are more resistant to growth factor deprivation and DNA damage when compared to either mutation alone (Lindsten et al., 2003). In contrast to this, the deletion of Bax alone is sufficient for the protection of neurons in differentiated, post-mitotic neurons, as well as a variety of cellular insults including trophic factor withdrawal and DNA damage, whereas, deletion of Bak provided no protection at all, and deletion of both Bax and Bak was no more protective than Bax deletion alone (Cregan et al., 1999; Deckwerth et al., 1996; Putcha et al., 2002; Xiang et al., 1998). This is in contrast to other cell types, in which the deletion of both Bax and Bak is required to prevent cell death, and suggest that Bak is not a redundancy mechanism in post-mitotic neurons (Lindsten et al., 2003; Lindsten et al., 2000; Wei et al., 2001).

Recently it has been shown that several neuronal subtypes, including cortical, cerebellar granular and sympathetic neurons do not express the full length multi-domain Bak, but express a splice variant of Bak (N-Bak) containing only a BH3 domain, leaving Bax as the key multi-domained pro-apoptotic protein in these cells (Sun et al., 2001; Uo et al., 2005). Over-expression of studies in Bax deficient neuronal cell cultures have demonstrated that N-Bak induced neuronal cell death is Bax dependant. In a manner similar to other BH3-only proteins, N-Bak has been found to bind to and

inhibit anti-apoptotic Bcl-xl, which in turn induces Bax translocation to the mitochondria and the apoptotic cascade (Uo et al., 2005). In sympathetic neurons deprived of nerve growth factor (NGF) however, N-Bak has been found to exhibit an anti-apoptotic behavior that is conformation dependant, but that still exhibited pro-apoptotic activity in non-neuronal cell types (Sun et al., 2003; Sun et al., 2001). These studies suggest that N-Bak may be a differentially regulated to be pro- or anti-apoptotic depending on cellular subtype. Full length Bak has also been reported to exhibit both pro- and anti-apoptotic behaviors through murine CNS development (Fannjiang et al., 2003). In these studies Bak was found to play an anti-apoptotic role in response to kainic acid induced excitotoxic injury, which is in contrast to studies suggesting that Bax or Bax/Bak deficient neurons are no more sensitivte to excitotoxic cell death induced by NMDA than wildtype neurons (Miller et al., 1997). Although there is evidence of involvement of Bak in the nervous system, its exact role in neuronal injury remains to be determined.

Several BH3-only Bcl-2 family proteins have been shown to play important roles in neuronal cell death. PUMA, a pro-apoptotic BH3-only protein that is directly induced by p53, can mediate p53 dependent DNA damage induced neuronal cell death in cerebellar granule neurons (Cregan et al., 2004; Nakano and Vousden, 2001; Wyttenbach and Tolkovsky, 2006; Yu et al., 2001). It has also been shown to be induced following tunicamycin treatment, an inducer of ER-stress induced apoptosis in neuroblastoma cells, as well as following forebrain ischemia in rat hippocampal neurons (Reimertz et al., 2003). PUMA knockout mice are viable and exhibit no developmental defects (Villunger et al., 2003). Up-regulation of PUMA alone is sufficient to induce cell death in CGNs and sympathetic neurons, whereas other p53 inducible BH3-only proteins Noxa and BIM cannot (Cregan et al., 2004; Wyttenbach and Tolkovsky, 2006). Further, although Noxa and PUMA are required for DNA-damage induced capsase-3 activation in neural precursor cells (NPCs) *in vitro*; only PUMA deficiency was able to protect them *in vivo* (Akhtar et al., 2006a; Oda et al., 2000). Noxa however,

was found to be a critical mediator in axotomy induced motor neuron cell death, as Noxa deficiency results in increases in surviving motor-neurons, following injury (Kiryu-Seo et al., 2005). These results demonstrate that PUMA and Noxa are likely to play different roles in different, but equally important roles in neuronal injury.

NGF withdrawal in sympathetic neurons has been shown to involve both BH3-only proteins BIM and DP5/Hrk (Imaizumi et al., 2004; Putcha et al., 2001; Whitfield et al., 2001). Both BIM and DP5/Hrk are highly expressed in the nervous system (Imaizumi et al., 1997; Kuida et al., 1996; O'Reilly et al., 2000). There are at least three BIM isoforms identified: BIM_{EL}, BIM_L, and BIM_S, which are generated by alternative splicing of BIM transcripts (Marani et al., 2002; O'Connor et al., 1998). BIM_{EL} and BIM_L are sequestered by the dynein motor complex, and associated with the microtubules until activated. Upon activation, BIM dissociates from the microtubules and translocates to the mitochondria where it is involved in the induction of MOMP. (O'Connor et al., 1998). BIM_{EL} is the major form of BIM in sympathetic neurons, cerebellar granule neurons, and dorsal root ganglion neurons, and has been implicated in neuronal apoptosis in AD and ischemic injury (Biswas et al., 2007; Inta et al., 2006; O'Reilly et al., 2000; Yao et al., 2007). BIM deficient mice show a transient reduction in developmentally regulated apoptosis of the dorsal ganglion neurons, but have no CNS abnormalities at birth (Bouillet et al., 1999; Putcha et al., 2001). Neurons isolated from BIM deficient mice are, however, partially protected against NGF withdrawal (Whitfield et al., 2001). In addition to its role in NGF withdrawal, BIM involvement has also been implicated in axotomy of retinal ganglion cells, thrombin-induced apoptosis in cortical neurons, and beta-amyloid peptide induced neuronal death (Biswas et al., 2007; Rao et al., 2007; Wakabayashi et al., 2005; Yao et al., 2007). DP5 is the murine homologue of human Harakiri (HRK) which has a BH3 domain and a hydrophobic membrane insertion sequence. It was the first BH3 only protein identified to have a function in NGF withdrawal induced death in sympathetic neurons (Imaizumi et al., 2004). Similar to BIM, DP5/Hrk has been

found to be involved in NGF withdrawal in sympathetic neurons, cortical neurons treated with amyloid beta peptide and axotomized motor-neurons, (Imaizumi et al., 2004; Imaizumi et al., 1999; Imaizumi et al., 1997).

Although less is known about the role of the remaining BH3 only pro-apoptotic family members in neuronal cell death, there is some promising research supporting their involvement in neuronal apoptosis. BID is a BH3-only protein involved in the extrinsic or death receptors pathway that mediates cross talk between intrinsic and extrinsic pathways. Cleavage of BID to tBID by caspase 8 results in Bax activation, and initiation of the intrinsic apoptotic pathway (Luo et al., 1998). BID deficient mice exhibit normal neuronal development, and although BID was not originally found to play a role in naturally occurring or genotoxin-induced neuronal cell death (Leonard et al., 2001; Yin et al., 1999), loss of BID has recently been shown to be protective against traumatic brain injury and to reduce infarct size following ischemic injury (Bermppohl et al., 2006; Plesnila et al., 2001; Yin et al., 2002). Further, BID deficient cortical neurons have also been found to be more resistant to cell death induced by oxygen-glucose deprivation, than their wildtype counterparts (Culmsee et al., 2005). Recent work has been aimed at developing small molecule inhibitors of BID to be used in neuroprotective therapies, with some of the recent candidates showing promise in in vitro systems against glutamate, OGD and beta-amyloid induced neuronal cell death (Culmsee and Plesnila, 2006; Culmsee et al., 2005). Bad another BH3 only protein is sequestered in its phosphorylated form in the cytosol in resting cells by the chaperone protein 14-3-3. Upon activation, Bad is dephosphorylated and released from 14-3-3 to interact with Bcl-xl at the mitochondrial membrane and initiate the intrinsic cell death pathway (Zha et al., 1996). Bad knockout mice also show no abnormalities in the nervous system (Ranger et al., 2003), however CGNs isolated from transgenic mice expressing a phosphorylation resistant Bad show increased sensitivity to cytotoxic insults (Datta et al., 2002). Bad up-regulation has also been recently reported following transient focal ischemia in rats, and motor

neurons following spinal cord injury suggesting that Bad signaling may be playing a more important role in neuronal cell death than has been previously thought (Kamada et al., 2007; Yu et al., 2005a). Although current knowledge of the involvement of BH3-only proteins in neuronal cell death implicates a number of these proteins in various injury paradigms, the evidence suggests that their roles are cell type and stimulus specific. More research is required to further elucidate the exact roles of individual BH3-only proteins in neuronal apoptosis.

The control of the intrinsic cell death pathway is intimately linked to the ability of the pro-apoptotic multi- domain Bcl-2 family proteins to oligomerize causing MOMP. This results in the release of apoptogenic factors, such as AIF and Cyt C, into the cytosol and downstream caspase activation. The remaining Bcl-2 family proteins, both pro- and anti-apoptotic, act upstream of this event and mediate MOMP. Although there is evidence of tissue and stimulus specific requirements for the activities of various Bcl-2 family members, no individual Bcl-2 family members have been shown to be responsible for the control of cell death in neurons. There is however evidence that many Bcl-2 family proteins do play some role in the regulation of neuronal cell death. Further research into the roles of Bcl-2 family proteins is required to elucidate the specific requirements for Bcl-2 family proteins in the regulation and control of neuronal cell death pathways.

1.5 Autophagic cell death

Autophagy or self-eating, is an evolutionarily conserved cellular stress response mechanism responsible for bulk degradation and recycling of cellular components (Klionsky and Emr, 2000). Autophagy can be divided into three distinct categories: microautophagy involving the invagination of preexisting membranes to engulf portions of cytosol, or organelles (Muller et al., 2000); chaperone-mediated autophagy which involves the delivery of proteins with the targeting sequence KFERQ, through designated lysosomal transporters, to lysosomes for degradation (Majeski and Dice, 2004); and macroautophagy which involves the formation of *de novo* sequestering vacuoles for the degradation and recycling of intracellular organelles (Shintani and Klionsky, 2004). Macroautophagy (referred to as autophagy from here on in) is the main pathway responsible for the turnover of organelles and long-term proteins within the cell and will be the basis of this discussion. In mammalian cells autophagy is believed to be involved in many physiological processes including starvation response, cell growth control, anti-aging, and innate immunity (reviewed in (Levine and Klionsky, 2004). Deregulation of autophagy had also been implicated in a number of diseased states including cancer, cardiomyopathy (Yan et al., 2006), as well as muscular and neurodegenerative diseases (Boland and Nixon, 2006; Chu, 2006; Yuan et al., 2003a). Autophagy can be induced by serum deprivation, high cell density, pharmacological agents such as rapamycin and tamoxifen, or tumor suppressors including p53, and PTEN (Arico et al., 2001; Crighton et al., 2006; Feng et al., 2005; Gunn et al., 1977; Kisen et al., 1993; Knecht et al., 1984; Kopitz et al., 1990; Noda and Ohsumi, 1998; Otsuka and Moskowitz, 1978; Petiot et al., 2000).

Autophagic mechanisms are best studied in yeast, however are conserved through to higher eukaryotic organisms (Levine and Klionsky, 2004). During the autophagic process a pre-autophagic cup structure forms in the cytosol, and is elongated engulfing cytosolic components, including organelles into a double membraned vesicle known as an autophagosome. The autophagosome

subsequently fuses with a lysosome to form an autolysosome, degrading the contents of the autophagosome as well as its inner membrane (Komatsu et al., 2007; Mizushima et al., 2002a). Induction of autophagy and vesicle nucleation in mammalian systems as well as the origins of the membrane used for the *de novo* synthesis of the preautophagic vacuole are, as of yet, not well understood (Levine and Klionsky, 2004). Beclin-1, the mammalian homologue of yeast ATG 6, is believed to play an important role in the initiation of mammalian autophagosome formation, by mediating the localization of other autophagy proteins to the preautosomal membrane (Kihara et al., 2001; Liang et al., 1998; Mizushima et al., 2002a; Pattingre and Levine, 2006). Once initiated, the membrane elongation and the subsequent formation of the double membraned autophagosome occurs through a ubiquitylation-like protein conjugation system involving autophagy related proteins Atg 3, 5, 7, 10 and 12 as well as the microtubule-associated protein 1 light chain 3 (LC3) (Tanida et al., 2004). The two proteins targeted for modification are Atg12 and the LC3 protein, where Atg12 modification is required for the formation of preautophagosomes and LC3 modification is required for the formation of autophagosomes (Kabeya et al., 2000; Mizushima et al., 2001). Both Atg12 and LC3-I are activated by the same E¹-like enzyme, Atg7. Following activation Atg12 is transferred to an E2-like enzyme, Atg10, and LC3-I to a second, Atg3. Atg12 is then conjugated to Atg5, forming the autophagosomal precursor, whereas LC3-I is modified to a membrane bound form known as LC3-II (Mizushima et al., 1998; Mizushima et al., 2001; Mizushima et al., 2002b; Nemoto et al., 2003; Tanida et al., 2001). Atg12-Atg5 is lost following completion of the double membraned autophagosome, LC3-II however remains present in preautophagosomes through to the fusion of autophagosomes with lysosomes, where it gets degraded by hydrolytic enzymes (Kabeya et al., 2000). The autophagic process is required for maintenance of homeostasis and survival in mammals. Mice missing one copy of Beclin-1 show an increased incidence of spontaneous tumourgenesis, whereas complete germline knockouts are embryonic lethal E7.5 and ES cells derived from these

mutant mice show deficits in autophagy (Qu et al., 2003; Yue et al., 2003). Further mice lacking the Atg 5 or 7 genes are unable to survive neonatal starvation and die shortly after birth (Komatsu et al., 2005; Kuma et al., 2004).

1.5.1 Autophagy in Neurodegeneration

Autophagy has been frequently described in neuronal development and neurodegenerative diseases (for review see (Boland and Nixon, 2006; Yuan et al., 2003a)). Although the CNS is relatively protected from nutrient deprivation induced autophagy (Mizushima, 2004), autophagy can be stimulated in the brain by neurotrophin deprivation (Xue et al., 1999) and abnormal protein aggregation, as is seen in PD and AD (Fortun et al., 2003; Yamamoto et al., 2006). Autophagy is also activated in response to hypoxia (Zhu et al., 2005) and excitotoxicity (Borsello et al., 2003), both factors known to play an important role in neuronal cell death following ischemic injury (Dirnagl et al., 1999; Lo et al., 2003). Overactive autophagy or failure of autophagy has been implicated in several neurodegenerative diseases including AD (Cataldo et al., 2004; Nixon et al., 2005; Yu et al., 2005b), HD (Kegel et al., 2000; Ravikumar et al., 2004), PD (Anglade et al., 1997; Zhu et al., 2003), prions diseases (Sikorska et al., 2004), cerebral ischemia (Adhami et al., 2006; Adhami et al., 2007) and juvenile neuronal ceroid lipofuscinosis (Cao et al., 2006). Autophagic cell death is key to survival, as demonstrated by mice with germline deficiencies in either Atg5, Atg7 or Beclin-1, none of whom survive long after birth (Komatsu et al., 2005; Kuma et al., 2004; Yue et al., 2003). Further, autophagy has been shown to be required for neuronal survival, as suppression of autophagy through loss of either Atg5 or Atg7 in the CNS results in neurodegeneration in mice (Hara et al., 2006; Komatsu et al., 2006). The link between autophagy and neurodegenerative diseases suggests an increase in the complexity of programmed cell death in neurodegeneration.

1.5.2 Autophagy and the Bcl-2 family

Recent evidence suggests that Bcl-2 family proteins may be involved in the regulation not only of apoptosis, but also of autophagic cell death. Beclin-1 was initially identified as a Bcl-2 interacting protein and a tumor suppressor gene, but was later recognized as the mammalian homologue of yeast protein Atg6 (Liang et al., 1999; Liang et al., 1998; Qu et al., 2003). Recent work has shown that Bcl-2 binds to beclin-1 and in so doing inhibits autophagic cell death in mammalian HEK 293 cells, and *in vivo* in mouse cardiac tissue. Only Bcl-2 localized to the ER is able to inhibit autophagy however, and not that localized to the mitochondria (Pattingre et al., 2005). Bcl-xl has also been shown to bind Beclin-1 through a putative BH3-only domain, which has lead to the suggestion that Beclin-1 may also act upstream of Bcl-2 family proteins and could potentially function in the apoptotic pathway (Furuya et al., 2005; Oberstein et al., 2007). Results showing an augmentation of the apoptotic signal by over-expression of Beclin-1 in human gastric cancer cells treated with cis-diamminedichloroplatinum lend some support to this theory (Furuya et al., 2005). Other downstream participants in the autophagic pathway have been shown to be required for p53-mediated apoptotic activity however, and it remains unclear as to whether Beclin-1 is directly involved in this apoptotic potentiation or whether the activation of the autophagic pathway in concert with the apoptotic pathway result in a potentiation of cell death through downstream autophagic effectors (Crighton et al., 2006). Further complicating matters, in MEFs lacking both Bax and Bak, over-expression of Bcl-2 and Bcl-xl were found to potentiate, not inhibit, autophagic cell death (Shimizu et al., 2004). MEFs lacking both Bax and Bak, although resistant to apoptosis, are still able to undergo autophagic cell death when treated with etoposide, or when undergoing growth factor withdrawal (Lum et al., 2005; Shimizu et al., 2004). Although the exact regulation of autophagy by Bcl-2 family proteins remains unclear, there is ample evidence suggesting the involvement of Bcl-2 family members in the regulation of autophagic cell death.

1.6 Bcl-2 Family Proteins in the Control Neuronal Cell Death Paradigms

To establish the role of Bcl-2 family members in p53-mediated neuronal cell death, I performed a series of *in vitro* assays to determine the regulation of Bcl-2 family genes following initiation of the p53-mediated cell death pathway. Previous research done in our lab has established an important role for Bax in p53-mediated neuronal cell death, so our efforts were focused on identifying BH3-only proteins and anti-apoptotic Bcl-2 family proteins that showed changes in regulation following DNA-damage induced neuronal cell death (Cregan et al., 1999). Based on levels of messenger RNA following DNA damage injury induced by the topoisomerase I inhibitor camptothecin, a known activator of p53-mediated apoptotic cell death (Morris et al., 2001; Xiang et al., 1998), BH3-only proteins Noxa and Puma were identified as ideal candidates for our initial studies. Both PUMA and Noxa are known to be up-regulated by the p53 transcription factor (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Using DNA damage injury, I will show that PUMA, but not Noxa plays a key role in the regulation of p53-mediated neuronal cell death.

Mcl-1, an anti-apoptotic member of the Bcl-2 family was also identified as a potential candidate for study, as it was found to be rapidly down-regulated following DNA-damage induced injury. Although well known as an oncogene, the role of Mcl-1 in neuronal injury remains unclear. Mcl-1 haplo-insufficiency has been shown to sensitize neurons to pilocarpine induced seizures (Mori et al., 2004), the pre-implantation lethality of the germline Mcl-1 knockouts however has inhibited further study of this model (Rinkenberger et al., 2000). The recent advent of the conditional Mcl-1 mutant has provided the perfect model in which to study the role of Mcl-1 in neuronal injury (Opferman et al., 2005; Opferman et al., 2003). Using this model, I will demonstrate an absolute requirement for Mcl-1 in neuronal survival. Further, I will argue that Mcl-1 plays a regulatory role not only in apoptosis, but also in autophagic cell death.

1.7 Research Objectives

Given the obvious link between p53 and neuronal injury and neurodegenerative diseases, p53-mediated neuronal cell death was studied. My objective was to examine the role of Bcl-2 family members in the regulation of p53-mediated neuronal injury. To do this I assessed the requirement for BH3-only proteins Noxa and PUMA in the propagation of DNA damage induced injury. I also determined a role for Mcl-1 in neuronal injury and went on to identify a novel role for Mcl-1 in the development and survival of neurons *in vivo*.

The overall objective of my doctoral thesis is to identify Bcl-2 family members that are key regulators in p53-mediated neuronal injury. Therefore, in the present studies, I aim to: 1) Determine the roles of BH3-only proteins Noxa and PUMA in p53-mediated neuronal cell death. 2) Determine the role of Mcl-1 in the development and survival of neurons.

1. To determine the roles of BH3-only proteins Noxa and PUMA in p53-mediated neuronal cell death (Chapter 2).

Given the evidence demonstrating the involvement of p53 in neuronal injury and neurodegenerative diseases, and the fact that both PUMA and Noxa were recently identified pro-apoptotic p53 target genes, I sought to determine the importance of these genes in DNA-damage induced neuronal cell death.

I hypothesized that BH3-only Bcl-2 family member PUMA was required for p53-mediated neuronal cell death.

To test this I introduced PUMA or Noxa, using adenoviral constructs, into primary cultured cerebellar granule neurons and assessed their ability to independently induce neuronal cell death. Using *in vitro* studies I established a requirement for BH3-only protein PUMA, but not Noxa in the mediation of DNA damage induced neuronal cell death. This study determined the importance of BH3-only protein PUMA in transcriptionally dependant p53-mediated neuronal cell death.

2. To determine the role of Mcl-1 in the development and survival of neurons (Chapter 3).

We identified Mcl-1 as being the only multi-domained Bcl-2 family member to be differentially regulated following DNA-damage induced neuronal injury. Further, we determined that by maintaining levels of Mcl-1 in these damaged cells we were able to show protection against neuronal injury. Decreased Mcl-1 levels in neuronal was also shown to increase sensitivity to pilocarpine induced seizures (Mori et al., 2004). Given this information we set out to determine the role of Mcl-1 in neuronal development and survival *in vivo*.

I hypothesized that Mcl-1 is required for neuronal survival.

To test this I set out to determine the impact of a Mcl-1 null mutation on the development and survival of neurons in the CNS, using conditional deletions of Mcl-1 under the control of the FoxG1 and nestin promoters (Berube et al., 2005; Hebert and McConnell, 2000). I also used a conditional deletion of Mcl-1 under the control of the Cank11 α promoter to determine the impact of postnatal Mcl-1 loss on post-mitotic neurons (Casanova et al., 2001). Further cells were isolated from these mutant mice in order to determine the role of Mcl-1 in p53-mediated neuronal cell death *in vitro*. This study

identified the importance of Mcl-1 in neuronal development and survival. Further, it established a previously unknown role for Mcl-1 in autophagic cell death.

These studies are described in the following chapters with the specific objectives of each defined at the beginning of each section. The results of these studies will then be examined in the context of the breadth of our current knowledge on the role of Bcl-2 family proteins in the regulation of neuronal injury in the general discussion as the culminating chapter this of dissertation.

Chapter 2: Cregan, Arbour *et al.* (2004) p53 Activation Domain 1 is Essential for PUMA Up-regulation and p53-mediated Neuronal Cell Death. *J. Neurosci.* 22:10003-12

This first manuscript represents the results of my studies of the roles of Noxa and PUMA in p53-mediated neuronal injury.

The experiments in this manuscript were carried out by S. P. Cregan and N. A. Arbour. N. A. Arbour contributed 50% of the experimental work, including cell culture and all of the Noxa and Puma data, with the exception of the final Puma deficiency data. The manuscript was written by N. A. Arbour with guidance and editorial assistance from the co-authors.

2.1 ABSTRACT

The p53 tumor suppressor gene has been implicated in the regulation of apoptosis in a number of different neuronal death paradigms. Due to the importance of p53 in neuronal injury, we questioned the mechanism underlying p53-mediated apoptosis in neurons. Using adenoviral mediated gene delivery, reconstitution experiments and mice carrying a knock-in mutation in the endogenous p53 gene, we show that the transactivation function of p53 is essential to induce neuronal cell death. While p53 possesses two transactivation domains that can activate p53 targets independently, we demonstrate that the first activation domain (AD1) is required to drive apoptosis following neuronal injury. Furthermore, the BH3 only proteins, Noxa and PUMA, exhibit differential regulation by the two transactivation domains. Here, we show that Noxa can be induced by either activation domain whereas PUMA induction requires both activation domains to be intact. Unlike Noxa the upregulation of PUMA alone is sufficient to induce neuronal cell death. We demonstrate therefore that the first transactivation domain of p53 is indispensable for the induction of neuronal cell death.

2.2 Introduction

The p53 tumor suppressor gene is up-regulated in multiple neuronal death paradigms including ischemia (Cheng et al., 2003; Chopp et al., 1992), hypoxia (Banasiak and Haddad, 1998; Halterman and Federoff, 1999) and excitotoxicity (Cregan et al., 1999; Xiang et al., 1998). Over-expression of p53 alone is sufficient to trigger apoptosis in post-mitotic neurons (Slack et al., 1996); consistent with this, p53 null mice exhibit reduced brain damage following excitotoxicity and stroke (Crumrine et al., 1994; Morrison et al., 1996). These studies emphasize the importance of p53 as a key apoptotic factor following neuronal injury.

It has become clear that the apoptotic mechanisms induced by p53 depends on the tissue type and insult (for review see (Ryan et al., 2001)). P53 can function as a transcription factor; however, its requirement for the induction of apoptosis remains controversial. P53 contains two distinct activation domains (ADI and ADII). Studies involving ectopic expression of p53 proteins containing inactivating deletions or mutations within ADI have generated conflicting results. It has been shown that the transcriptional function of ADI is essential for the induction of apoptosis in certain cellular systems (Attardi et al., 1996; Jimenez et al., 2000), but not in others (Chen et al., 1996b; Ding et al., 2000; Haupt et al., 1995). Further, it has been shown that mutations in ADII can diminish apoptotic activity and arrests cells in the G1 phase of the cell cycle (Zhu et al., 2000). In some systems, therefore, p53-mediated apoptosis requires ADI to be intact.

In addition to its role in transactivation, there is increasing evidence that p53 is capable of transcriptional repression, further suggesting the importance of p53 in regulating gene expression during apoptotic cell death (Koumenis et al., 2001; Murphy et al., 1996; Nakade et al., 2004; Seto et al., 1992; Venot et al., 1998). P53 has also been shown to induce cell death in a transcriptionally independent manner (Bennett et al., 1998; Caelles et al., 1994; Haupt et al., 1995). Studies have shown that p53 can function at the mitochondrial level (Chipuk et al., 2004; Marchenko et al., 2000;

Mihara et al., 2003); (Bonini et al., 2004). Differences in cell type and apoptotic stimulus may account for the variability in the required mechanism by which p53 induces apoptosis. In summary, there are several p53-dependent apoptotic pathways; one requiring transactivation, a second that functions through transcriptional repression, and a third that functions exclusively at the mitochondrial level.

Despite the importance of p53 in the regulation of neuronal apoptosis, little is known regarding the mechanisms by which p53 mediates apoptosis in post-mitotic neurons. In view of the diverse mechanisms by which p53 functions, in this study we questioned the mechanism underlying p53-mediated apoptosis in neurons. Our results demonstrate that the transactivational function of p53 is essential to induce neuronal cell death. While two distinct domains have been identified, AD1 is critical for the induction of the BH3-only Bcl-2 family member, PUMA, which is required to drive neuronal cell death.

2.3 METHODS

2.3.1 Cell Culture and Recombinant Adenovirus Infection:

Cortical and cerebellar granule neurons were cultured as described previously (Cregan et al., 1999; Fortin et al., 2001). The cDNA for WTP53, p53^{fl} (Kubbutat et al., 1997), p53 Δ V, p53 Δ Pro and p53 Δ 22/23 were a kind gift from Dr. Karen Vousden. The p53 double transactivation mutant, p53 Δ DM, was generously provided by Dr. Xinbin Chen. To generate the mutation for the second activation domain (ADII) alone (p53 Δ 53/54), WTP53 cDNA was digested with BsrDI and the resulting 5' fragment was ligated to the corresponding 3' cDNA fragment from BsrDI digested p53 Δ DM. The cDNA for Noxa and cDNA for HA-PUMA were kind gifts from Dr. Eri Oda (Oda et al., 2000) and Dr. Bert Vogelstein (Yu et al., 2001) respectively. N-terminal 3xFlag tag was added by subcloning full-length Noxa cDNA into p3XFLAG-myc-CMV-24 (Sigma). Recombinant adenoviral vectors carrying expression cassettes for Noxa, PUMA, wildtype or mutant human p53 proteins were constructed, purified, and titered as previously described (Cregan et al., 2000). Adenoviral vectors were added to cell suspensions immediately before plating.

2.3.2 Mice and Genotyping

Mice carrying a targeted null mutation for P53 were obtained from Jackson Laboratories (Bar Harbor), and p53 genotyping was done by PCR as previously described (Fortin et al., 2001). P53QS mice expressing p53 containing point mutations at codons 25 and 26 (L25Q,W26S) were a generous gift from Dr. Geoffrey Wahl. Genotyping of these mice was performed as previously described

(Jimenez et al., 2000). Mice carrying a targeted null mutation for PUMA were a generous gift from Dr. Andreas Strasser and genotyping of these mice was performed as previously described (Villunger et al., 2003).

2.3.3 Semiquantitative RT-PCR analysis

Total RNA was isolated from cells using Trizol isolation reagent according to the manufacturer's instructions (Invitrogen). Pilot experiments were performed to determine the linear range of amplification with respect to quantity of starting template and PCR cycles. The primers used for the detection of p53-inducible genes are shown in Table 2.1. 25-100 ng of total RNA was used for cDNA synthesis and targeted gene amplification using the SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis was carried out at 48°C for 45 min followed by a 2 min initial denaturation step at 94°C. This was followed by 35 cycles (Noxa, BTG2, Dda3), 30 cycles (APAF-1, PUMA, STINP, SCOTIN, PERP) or 25 cycles (S12) at 94°C for 30 s, 55-62°C for 30 s, and 72°C for 1 min. The resulting products were sequenced to confirm identity.

2.3.4 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed on total protein extracts as described (Fortin et al., 2001), with the following modifications. Cells were harvested, centrifuged, and extracted in lysis buffer (100 mM HEPES, pH 7.4, 5 mM MgCl₂, 2.5 mM EDTA, 20% glycerol, 0.5 M KCl, 0.5 mM PMSF, 0.1% NP-40, 5 µg/mL aprotinin, 2 µg/mL leupeptin, and 20 µM sodium orthovanadate) and assayed for protein concentration by the method of Bradford (Bio-Rad Laboratories protein assay reagent). Protein extract (10–20 µg) was incubated with an excess of indicated ³²P-labeled double-stranded DNA probes (60,000 cpm/0.2 ng of DNA). Oligonucleotides used included 5'-

Table 2.1 The RT-PCR primer sets used for the detection of p53-inducible genes

Gene	Sequence	
	5' - Sense - 3'	5' - Anti-Sense - 3'
NOXA	CAACGCGGGCAGAGCTACCTGA	TGGGCTTGGGCTCCTCATCCTGCTC
APAF-1	GACATCAAAACATCCTACATCA	CTTCGGCAGCTAACACAG
DDA3	CTGCTGCCCACCGTGAGTTCTTG	GCGCTTGGATGCCCTTTCTGAGTT
PUMA	CCTCAGCCCTCCTGTCACCAG	CCGCCGCTCGTACTGCCGCTTG
STINP	AAATGTTTGTAGGCGAAGTCAC	GTAAAACATGGGGGAGGAGT
SCOTIN	CACCTGCTCCTGCTGCTGTCTGTA	GGGAATCCATGTAGGTCGGGTTGT
BTG2	AGCCACGGGAAGGGAACC	GCTGGGGCTGGCTGAGTC
PERP	ATCCTGCCCTGCTGCTGCTCA	GGCGCCCCCAAAAGGTCATC
S12	GGAAGGCATAGCTGCTGG	CCTCGATGACATCCTTGG

ATGGAGGCACGTCCCCAGCGACAGCAGGCTC-3' (APAF1) and 5'-CCTGCCTTGCCTGGACTTGC-3' (P21) corresponding to the p53 consensus binding sequences within the Apaf-1 (Fortin et al., 2001) and p21 (Macleod et al., 1996) promoters, respectively. The binding reaction (25 μ l) was carried out at room temperature for 20 min in binding buffer (50% glycerol, 250 mM KCl, 100 mM Hepes, pH 7.4, 5 mM DTT, 5 mg/mL BSA, and 0.5% Triton X-100) with 0.1 μ g sonicated herring sperm DNA, and 1 μ L of p53 Pab421 monoclonal antibody was added to the binding buffer (Ab-1; Oncogene Research Products). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide was added to the binding reaction and incubated for 20 min before the addition of labeled probe. Complexes were resolved on a 5% polyacrylamide, 1x tris-glycine gel, dried, and visualized by autoradiography.

2.3.5 Western Blot Analysis and Immunostaining

Cells were fixed and permeabilized in ice-cold methanol for 20 minutes, rehydrated in 3 changes of PBS, and incubated for 2 hours with primary antibody to p53 (CM1, Novacastra Laboratories Ltd.) or Cox IV (Molecular Probes). Cells were washed with PBS and then incubated for 1 hour with Alexa 488- and/or Alexa 546-conjugated secondary antibodies (Molecular Probes). Cells were again washed in PBS and counterstained with Hoechst. Western blot analysis was performed as described previously (Cregan et al., 1999) with antibodies against p53 (CM1, Novacastra Laboratories Ltd.), Flag (Sigma), HA (Santa Cruz Biotech) and actin (SC-1616, Santa Cruz Biotechnologies) as a loading control.

2.3.6 Cell Viability and Caspase Assays

Cell survival was measured by three methods: LIVE/DEAD, TUNEL, and MTT assays. At the times indicated, neuronal viability was determined using the LIVE/DEAD viability/cytotoxicity kit

(Molecular Probes) following manufacturer's instructions. TUNEL labeling was used to visualize cells with fragmented DNA. At the indicated times following treatment, cells were fixed in 4% paraformaldehyde for 20 min, washed in three changes of PBS, and then incubated for 1 h at 37°C with 75 µl of a cocktail (Roche Diagnostics Inc.) consisting of 0.5 µl terminal transferase, 0.95 µl biotin-16-dUTP, 6.0 µl CoCl₂, 15.0 µl 5x TdT buffer, and 52.55 µl distilled water. The reaction was stopped by incubation in 4x SSC buffer followed by three washes in PBS. Cells were then labeled with a streptavidin Cy2 secondary antibody (Jackson ImmunoResearch Laboratories) for 45 min at room temperature and counterstained with Hoechst 33258 (1 µg/µl) for 5 min. Images were captured using a ZEISS Axiovert 100 equipped with a Sony HAD 3CCD power color video camera, and analyzed using Northern Eclipse software. The fraction of TUNEL-positive cells as a percentage of total cell number was determined. For both the Live/Dead and TUNEL assays a minimum of 500 cells was scored for each treatment and the data represents the mean and SD from a minimum of three independent experiments. In certain experiments viability was assessed using the colorimetric MTT survival assay (Cell Titer Kit, Promega, Madison, WI) that measures the mitochondrial conversion of the tetrazolium salt to a blue formazan salt as described previously (Slack et al., 1996).

For the caspase activity assay, cells were harvested and extracted in caspase lysis buffer (consisting of 1 mM KCl, 10 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 5 µg/ml leupeptin, 2 µg/ml aprotinin, and 10% glycerol) for 15 minutes on ice, and 10 µg of protein was used to determine caspase activity as previously described (Cregan et al., 1999). Caspase activity is reported as the ratio of fluorescence output in treated samples relative to corresponding untreated controls.

2.4 RESULTS

2.4.1 Transcriptional activation is essential for p53 – mediated neuronal cell death.

P53 is a multifunctional protein whose mechanism of action largely depends on the cell type or the death stimulus. To understand the mechanism by which p53 participates in injury-induced neuronal death, we determined which functional domains were required for the induction of apoptosis. We constructed a panel of adenoviral vectors carrying wildtype and specific deletion/point mutants of p53 (Fig. 2.1). To facilitate a functional comparison between these constructs, we first verified that all p53 mutants: (a) were expressed at comparable levels by Western analysis and immunohistochemistry; (b) retained the correct conformation by performing electrophoretic mobility shift assays (EMSA); and (c) were localized to the appropriate cellular compartment by immunofluorescence and confocal microscopy.

Western analysis revealed that all p53 mutant proteins were expressed at levels comparable to wildtype p53 (Fig. 2.2A), while immunohistochemistry demonstrated that all the adenoviral constructs demonstrate comparable infectivity (Fig. 2.2B). To ensure that the protein conformation was not disrupted by specific deletions or site-directed mutagenesis, P53 sequence specific DNA binding activity was analyzed by EMSA. All p53 mutants, except the DNA binding mutant Ad-p53 Δ V, could effectively bind DNA exhibiting complex formation comparable to that seen with wildtype p53 (Fig. 2.2C). Finally, since none of these mutants disrupted the nuclear localization domain, they localized appropriately to the nucleus (Fig. 2.3A). These experiments demonstrate that the p53 mutants used in this study are equally stable and maintain an appropriate protein conformation.

Recently, studies have revealed that p53 could translocate to the mitochondria and was capable of inducing apoptosis exclusively at this site (Bonini et al., 2004; Marchenko et al., 2000; Mihara et al., 2003; Sansome et al., 2001). To determine whether wildtype p53 or any of the mutant p53 proteins

were targeted to the mitochondria, neurons were labeled with antibodies directed against p53 and a mitochondrial marker, α -CoxIV. Analysis of p53 immunostaining by confocal microscopy revealed p53 localization exclusively within the nucleus. No colocalization with the mitochondria was found with overexpressed wildtype p53, even when nuclear condensation was evident, nor was there colocalization with the double transactivation mutant Ad-p53 Δ DM (Fig. 2.3B). These studies show that, in neuronal cells, p53 does not localize to the mitochondria and functions primarily in the nucleus. The following results demonstrating the requirement of an intact transactivation domain further supports this conclusion.

To determine which functional domains of p53 are required for the induction of neuronal cell death, cerebellar granule neurons (CGNs) were transduced with adenoviral vectors expressing wildtype or mutant p53 proteins and assessed for apoptosis. Following direct expression of p53, cells were assayed for caspase activation, by DEVD-AFC cleavage, and for survival, using two independent assays: Live-Dead staining and TUNEL labeling (Fig. 2.4 A,B,D). Caspase activity closely paralleled cell death assays (Fig. 2.4C). The most efficient constructs for inducing caspase activation and apoptosis were Ad-p53wt and Ad-p53 Δ I. This was expected since Ad-p53 Δ I lacks the MDM2 binding domain which should enhance protein stability and thereby promote apoptotic activity. Loss of the proline rich motif impaired apoptotic activity, revealing only partial caspase activation and TUNEL staining. Since p53 has two transactivation domains, we examined the importance of these domains in the induction of apoptosis. Inactivation of either domain resulted in a significant decrease in p53-induced apoptosis. However, inhibition of AD1 appeared to have a more profound effect on the ability of p53 to induce cell death (Fig. 2.4A). Cells expressing p53 Δ 22/23 exhibited only 23%

Figure 2.1 Schematic representation of p53 constructs. Various p53 constructs were used to determine the importance of each functional domain for neuronal apoptosis. The p53 Δ I construct contains a deletion of the conserved box 1 sequence, which abolishes MDM2 binding without affecting DNA binding activity. The p53 Δ 22/23 construct contains two inactivating point mutations within the first transactivation domain (ADI); residues 22 and 23 were mutated from Leu and Trp to Glu and Ser, respectively. P53 Δ 53/54 has mutations at residues 53 and 54 from Trp and Glu to Phe and Ser, respectively, which inactivates the second transactivation domain (ADII). The p53DM contains both transactivation domain mutations (p53- Δ 22/23 and p53- Δ 53/54). P53 Δ Pro is a deletion of the proline rich region of the p53 protein, P53- Δ 173L mutant has a point mutation at residue 173 to Leu. and p53 Δ V is a deletion of the conserved box V sequence of p53, resulting in inactivation of DNA binding.

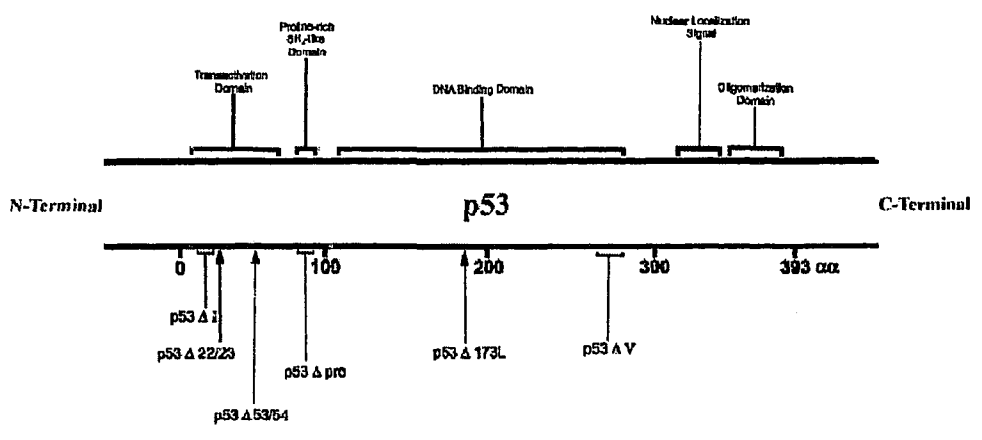


Figure 2.2 P53 mutant proteins are stable, expressed at comparable levels and retain the appropriate protein conformation. (A) Western Blot showing comparable expression levels of p53 mutant proteins. CGNs were infected at 25 MOI with the indicated Ad-p53 construct, and protein lysates were collected at 36 hours post infection (30 μ g protein). (B) Neurons were infected with the indicated Ad-p53 construct at 25 MOI. After 36 hours, neurons were fixed and immunostained for p53 and counterstained with Hoechst. Data represents the mean and standard deviation of three independent experiments (C) EMSA: Protein was extracted from neurons 36 hours after infection with the indicated Ad-p53 constructs. P53 binding activity to the APAF1 and p21 p53 response elements were assayed by electrophoretic mobility shift assay. Binding reactions were carried out with neuronal extracts (10-20 μ g protein) and the indicated oligonucleotides in the presence of p53 antibody (Ab1). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide was added to the binding reaction and incubated for 20 min before the addition of labeled probe. All p53 constructs tested efficiently bound DNA with the exception of ΔV , which inactivates DNA binding. (n=3).

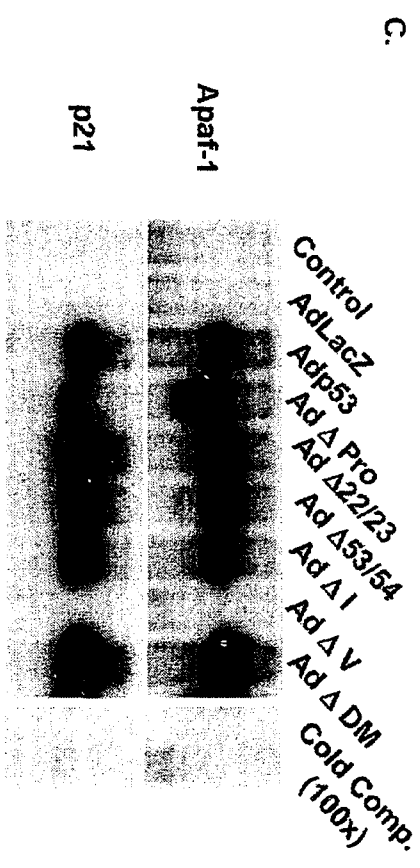
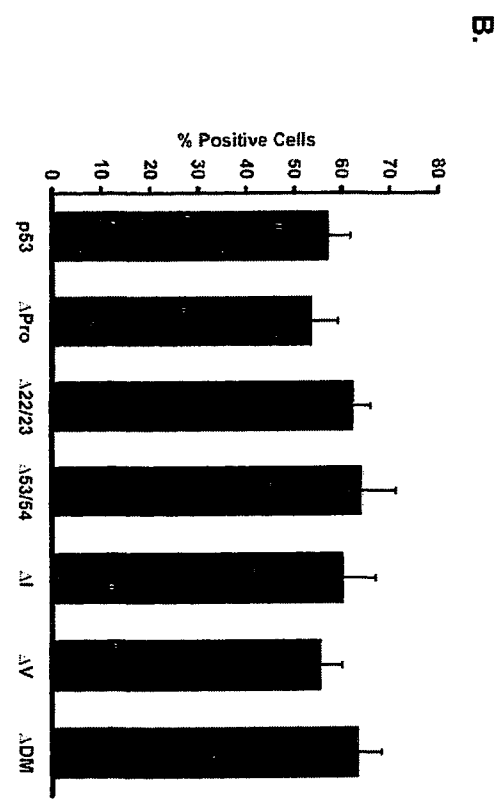
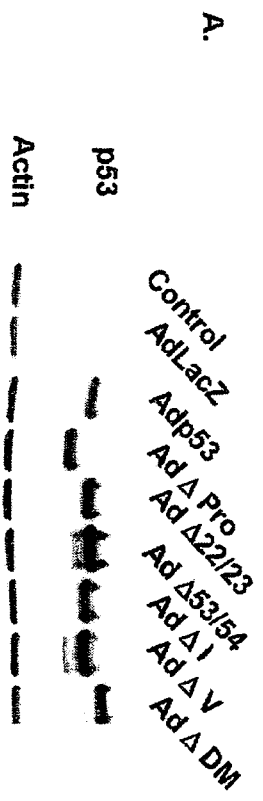
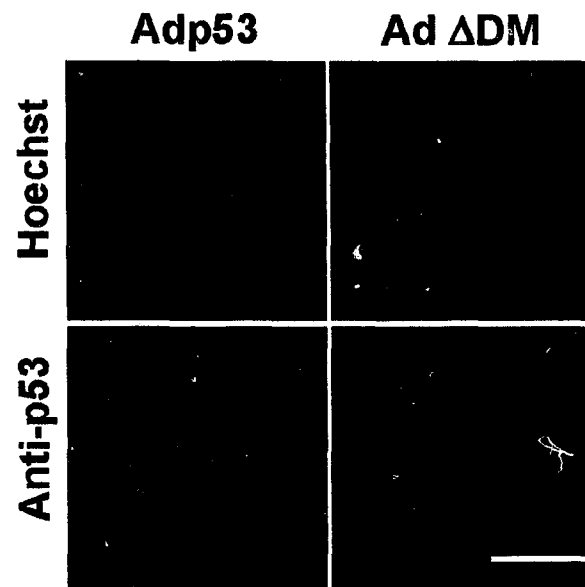
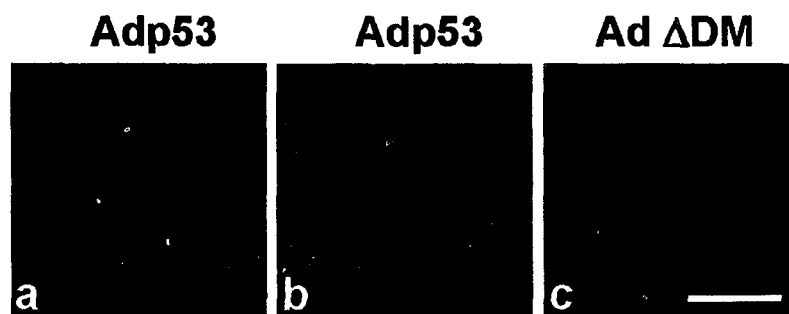


Figure 2.3 P53 mutant proteins localize to the nucleus. Neurons were infected with Ad-p53WT or Ad-p53DM at 25 MOI. After 48 hours, neurons were fixed and immunostained for p53 (A&B) and/or mitochondrial-specific CoxIV (B) or counterstained with Hoechst (A). P53 remained localized to the nucleus even under conditions of cell death, as denoted by condensed nuclei (b).

A.



B.

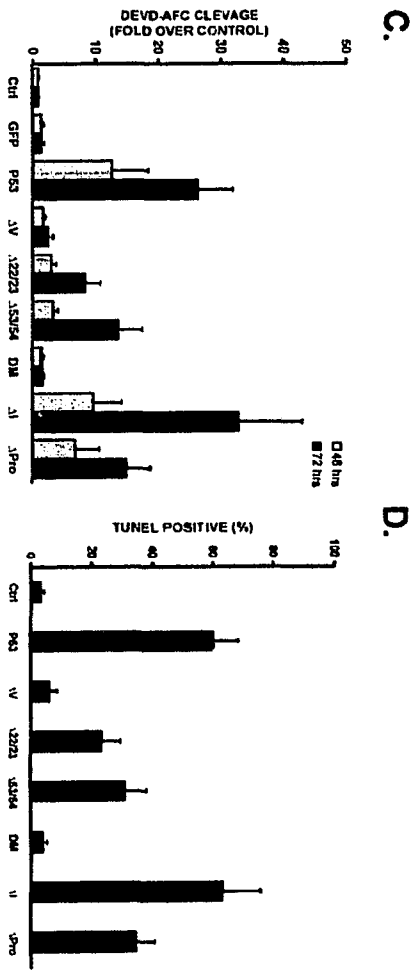
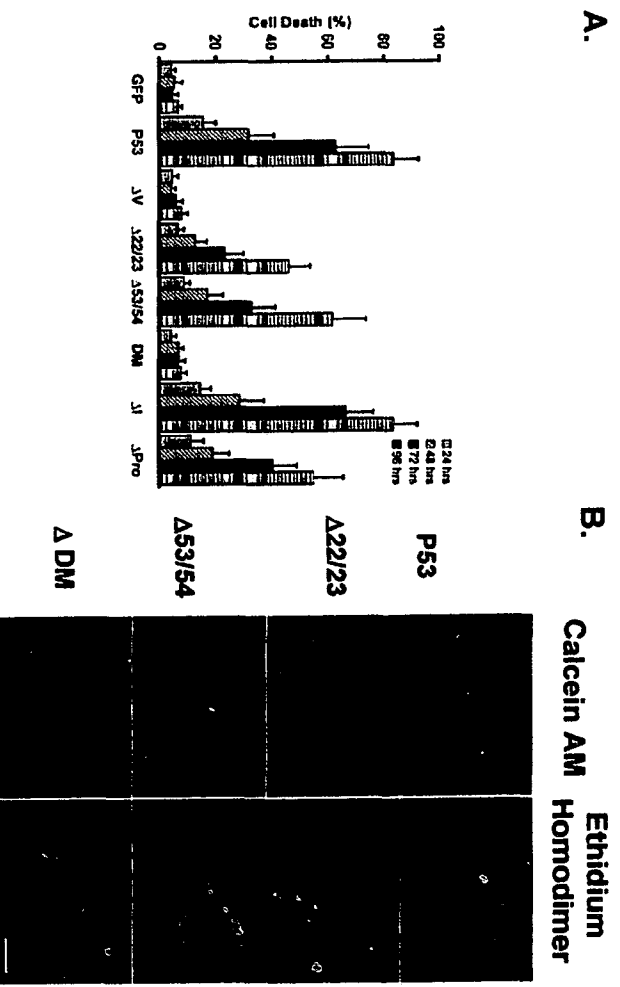


apoptosis at 72 hours relative to wildtype p53 which led to more than 63% cell death by 72 hours. In contrast, mutation of the second transactivation domain, Ad-p53 Δ 53/54, resulted in a more modest reduction in apoptotic activity with approximately 34% of cells dying by 72 hours. When both transactivation domains were inactivated (Ad-p53 Δ DM) there was no detectable increase in caspase activity or apoptotic death relative to control cells expressing Ad-GFP. Similarly, the DNA binding domain mutant, p53 Δ V, was apoptotically inert. In fact, the p53 Δ DM and p53 Δ V mutants were unable to induce caspase activation or cell death even when the time course was extended to 96 hours. In conclusion, the results of these studies demonstrate that neuronal apoptosis induced by direct expression of p53 requires DNA binding activity and an intact transcriptional activation domain. Furthermore, the results suggest that AD1 may play a more prominent role in the transcriptional induction of proapoptotic genes.

2.4.2 Reconstitution of responsiveness to DNA damage-induced apoptosis in p53 deficient neurons

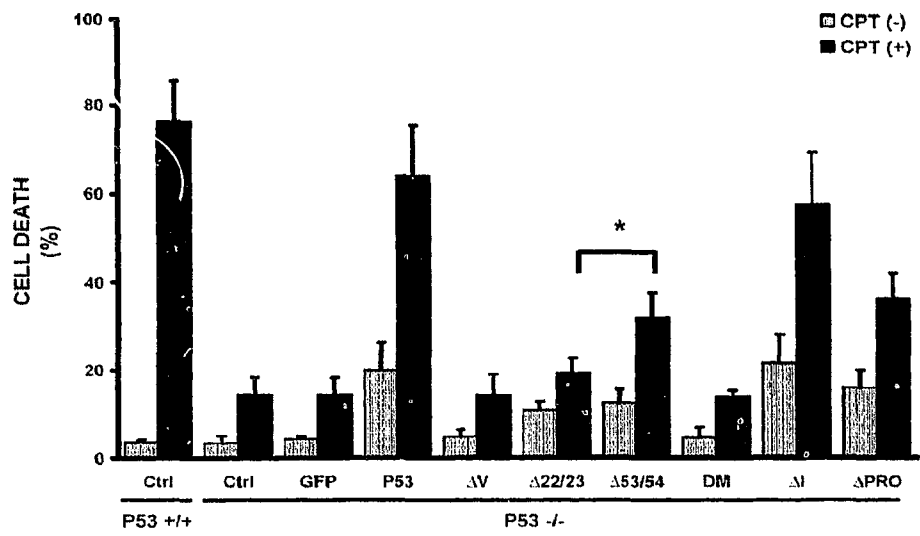
It is well known that p53 function can be modulated by post-translational modifications or the coactivation of other death signals (Morris et al., 2001; Xu, 2003). We therefore examined the function of wildtype and mutant p53 proteins in response to camptothecin, a DNA damaging agent which is known to induce cell death through a p53-dependent mechanism (Morris et al., 2001; Xiang et al., 1998). Wildtype and mutant p53 proteins were expressed in p53-deficient neurons to determine whether they could reestablish sensitivity to DNA damage induced apoptosis. CGNs were transduced with adenoviral vectors expressing wildtype or mutant p53 proteins and then challenged with the DNA damaging agent camptothecin. Cell death was monitored within 24 hours of drug treatment to minimize the contribution of p53 expression alone to the death response. Consistent with previous

Figure 2.4 Transactivation domain 1 of p53 is essential for the induction of neuronal cell death. CGNs were infected with wild type Ad-p53 or p53 mutant constructs at 25MOI. (A) LIVE/DEAD viability/cytotoxicity assay (Molecular Probes) was performed at 24, 48, 72 and 96 hours post infection. (B) Photomicrographs of Live/Dead cell assay. Bar, 100 μ m (C) Caspase-3 activity was measured at 48 and 72 h by DEVD-AFC cleavage. (D) The fraction of TUNEL-positive cells was measured 72 h after infection. Data represents the mean and standard deviation of five independent experiments (n=5).



studies, 76% of wildtype neurons treated with camptothecin underwent apoptosis after 24 hours (Fig. 2.5). Uninfected or Ad-GFP infected p53-deficient cells exhibited only 10 – 15% cell death (Fig. 2.5). Cells reconstituted with wildtype p53 (Ad-p53wt) or a p53 mutant lacking the MDM2 binding domain (Ad-p53 Δ I) were rescued in their apoptotic response revealing 64% and 57% cell death respectively. Cells expressing p53 with a mutation in the proline rich motif (Ad-p53 Δ Pro) revealed an intermediate apoptotic response of 37% cell death. Interestingly, mutants lacking either one of the transactivation domains (Ad-p53 Δ 22/23, Ad-p53 Δ 53/54) also exhibited intermediate levels of apoptosis (Fig. 2.5). Consistent with our results obtained by direct over expression, mutation of the first transactivation domain, p53 Δ 22/23, severely impaired the ability to rescue apoptosis, exhibiting only a slightly higher kill than p53-deficient neurons (19% cell death). In contrast, cells expressing the mutation in the second transactivation domain, p53 Δ 53-54, resulted in a partial rescue of the death response such that 32% of neurons had undergone cell death (Fig. 2.5). This corresponded to approximately half the activity obtained following reconstitution with wildtype p53. In contrast, expression of p53 proteins either lacking function of both transactivation domains (p53DM), or DNA binding activity (p53 Δ V) could not restore the apoptotic response to camptothecin. Consistent with results obtained by direct expression of the p53 mutants in neurons, reconstitution experiments revealed that the transcriptional activity of p53 was essential for the induction of neuronal cell death after DNA damage. While p53 contains two distinct transactivation domains, there appears to be a difference in the ability of these domains to induce apoptosis. The finding that the ADI mutant exhibited a significantly greater defect than ADII in the rescue of the apoptotic response, suggests that ADI may play a more prominent role in the induction of proapoptotic genes. We therefore asked whether these p53 mutants could induce known p53 transcriptional players effectively.

Figure 2.5 Reconstitution of responsiveness to DNA damage-induced apoptosis in p53 deficient neurons. Cortical neurons obtained from p53-deficient mice or wild-type littermates were infected with Ad-p53 or Ad-p53 mutant constructs and 16 hours later were challenged with 10 μ M camptothecin. The reconstitution of apoptotic cell death was determined by LIVE/DEAD assay 24 hours after treatment. * $P < 0.05$ by two-way analysis of variance compared to wildtype followed by t-test. Data represents the mean and standard deviation of three independent experiments ($n=3$).



2.4.3 Transactivation mutants of p53 activate common and distinct proapoptotic targets

The results presented above suggest that the transactivation function is essential for p53-mediated neuronal cell death; however, the two transactivation domains exhibit slightly differential apoptotic effects. We therefore asked whether there might be a difference in the target genes activated by these two domains. The induction of a series of proapoptotic p53 target genes by each of the p53 mutants was examined and compared using semiquantitative RT-PCR. The proapoptotic genes examined were Noxa (Oda et al., 2000), Apaf-1 (Fortin et al., 2001), PUMA (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001), and Perp (Attardi et al., 2000). Consistent with caspase activation and survival assays, maximal activation of proapoptotic genes was achieved with wildtype p53, and the MDM2 binding mutant (Ad-p53 Δ I) (Fig. 2.6). In contrast, no induction over GFP controls was found with the DNA binding mutant (Ad-p53 Δ V) or Ad-p53 Δ DM lacking both transactivation domains. Cells expressing p53 lacking the proline rich motif, Ad-p53 Δ Pro, exhibited target specific differences in gene induction relative to GFP controls. Some of the transcriptional targets for p53 could be equally induced by either one of the transactivation domains. For example, the targets Noxa, Apaf-1, and Perp could be efficiently induced by either one of the two transactivation domains. In contrast, there was a significant loss of activity in the induction of PUMA when either one of the transactivation domains were lost. Indeed PUMA mRNA levels were close to control levels, in cells expressing either the ADI or the ADII mutant.

2.4.4 Apoptotic response in neurons carrying a targeted mutation in activation domain 1

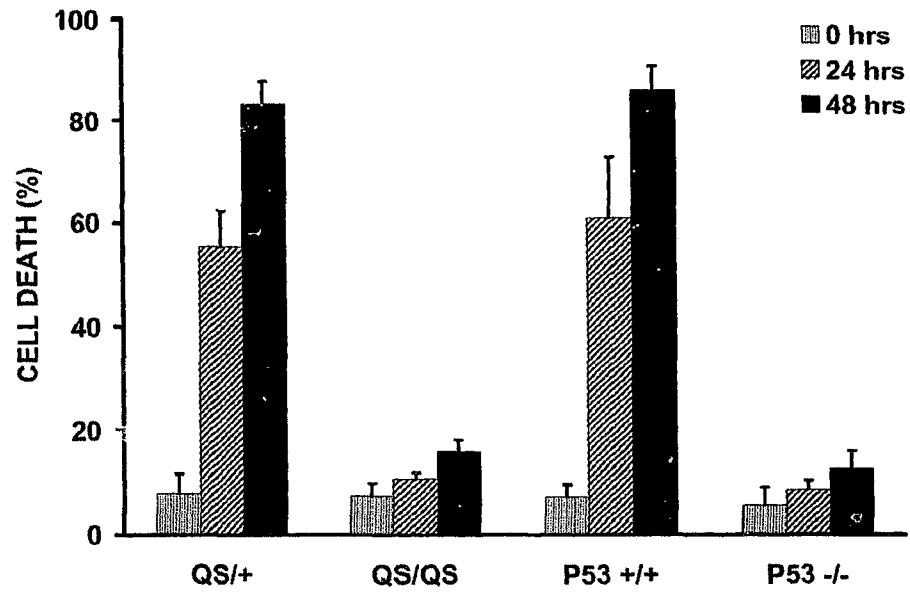
Figure 2.6 P53 mutants cause differential upregulation of target genes in cerebellar granule neurons. Total RNA was extracted from cerebellar granule neurons 40 hours after infection with Ad-p53 or Ad-p53 mutant constructs as indicated and analyzed for Noxa, Apaf-1, PUMA, and Perp or S12 expression using semiquantitative RT-PCR.

Based on caspase assays, apoptotic activity, and reconstitution assays, it appears that for Puma-mediated induction *in vitro*, both activation domains are important for the initiation of apoptosis in neuronal cells. We next asked whether a mutation in one of the existing transactivation domains in the endogenous p53 gene is sufficient to abrogate the p53-mediated apoptotic response as seen in the overexpression and reconstitution experiments above. To answer this question, we used p53QS knock-in mice which express a p53 protein containing a double point mutation of amino acid residues 25 (Leu-Gln) and 26 (Trp-Ser) analogous to the 22/23 mutation in the human p53 protein (Jimenez et al., 2000). Primary cortical neurons were cultured from p53QS mice along with their heterozygous littermates. As a basis for comparison, cells were also cultured from p53 knockout and wildtype mice of the same genetic background. Cell survival was assessed at 24 or 48 hours after camptothecin treatment to determine whether p53QS neurons were affected in their response to DNA damage (Fig. 2.7A). While 86% of wildtype cells had undergone apoptosis by 48 hrs, only 13% of p53 null cells were apoptotic. Neurons homozygous for the QS mutation exhibited only 16% cell death, a response similar to p53-deficient neurons. Mice heterozygous for the QS mutation exhibited 83% cell death similar to that observed in wildtype cells. This suggests that AD1 is essential for p53 induction of apoptosis in post-mitotic neurons.

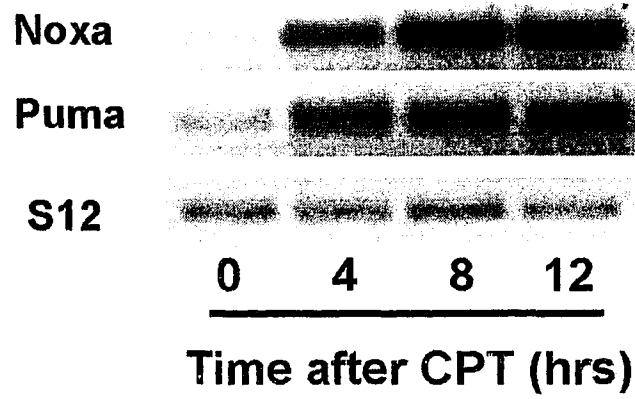
We then examined the ability of the p53QS protein to induce expression of p53 target genes following camptothecin treatment. We specifically examined the two p53 target genes, PUMA and Noxa, due to their differential induction profiles in response to adenoviral mediated expression of p53. In wildtype mice, both Noxa and PUMA are upregulated in response to camptothecin treatment (Fig. 2.7B). While camptothecin induced expression of Noxa mRNA was essentially unaffected in p53QS neurons relative to wildtype neurons, upregulation of PUMA mRNA was completely abrogated (Fig. 2.7C). These results suggest that similar to the response seen with ectopically expressed p53

Figure 2.7 Transactivation domain 1 of p53 is essential for the induction of neuronal cell death in vivo. (A) Cortical neurons obtained from QS mice, p53-deficient mice or wildtype littermates were treated with 10 μ M camptothecin and cell survival was determined by LIVE/DEAD assay at the indicated times. Cell death is reported as a percentage of corresponding untreated control cultures. Data represents the mean, and standard deviation from three independent experiments (n=3). (B) Total RNA from cortical neurons from CD1 mice treated with camptothecin was collected and analyzed at indicated times for Noxa, PUMA and S12 expression using semiquantitative RT-PCR. (C) Cortical neurons from QS mice, p53 deficient mice or wildtype littermates were treated with 10 μ M camptothecin and after 9 hours total RNA was collected and analyzed for Noxa, PUMA and S12 expression using semiquantitative RT-PCR.

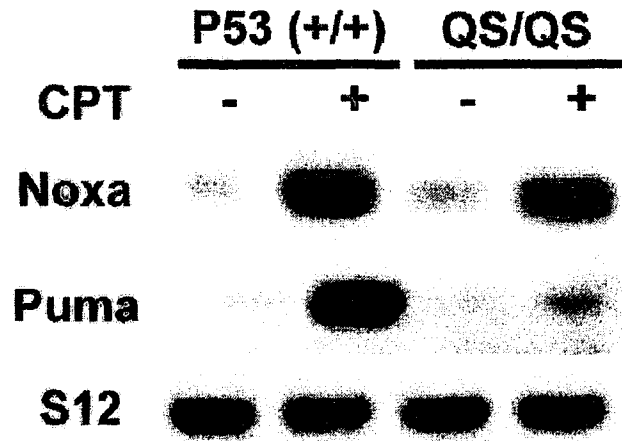
A.



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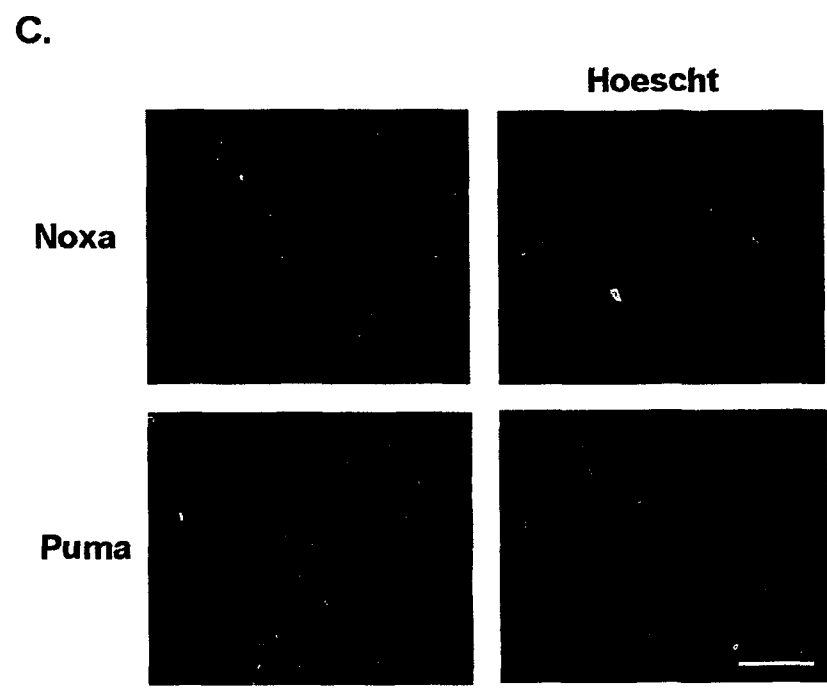
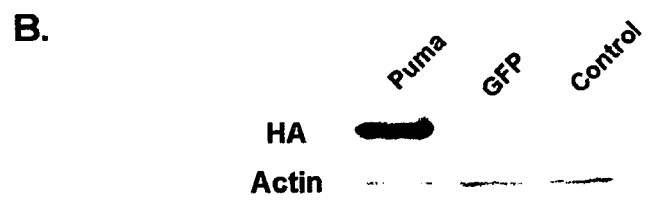
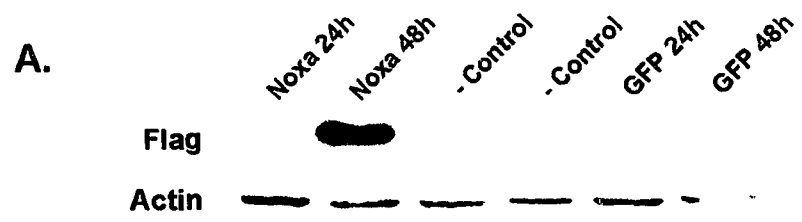
mutants, the endogenous mutant, p53QS is unable to activate expression of PUMA. AD1 therefore is essential for the transcriptional induction of PUMA and neuronal apoptosis in response to DNA damage-induced injury.

2.4.5 PUMA is a Potent Inducer of Neuronal Cell Death

Since PUMA upregulation correlated closely with the ability to induce apoptosis, while Noxa induction did not, we directly tested the apoptotic activity of each of these BH3-only proteins in post-mitotic neurons. To test this, adenoviral vectors were constructed carrying Noxa and PUMA expression cassettes. Western blots were performed to confirm the efficient expression of these proteins (Fig. 2.8A&B). Cerebellar granule neurons expressed Noxa as early as 24 hours after infection, with high expression levels present at 48 hours. In contrast, PUMA expression was not readily detectable in neurons (data not shown) likely due to its ability to rapidly induce cell death (Fig. 2.9C). Consistent with this, PUMA could be efficiently expressed in HEK 293 cells, which are deficient in BAX function due to the presence of E1B (Graham et al., 1977) (Fig. 2.8B). Due to the strong pro-apoptotic affect of Puma immunohistochemistry of both Noxa and Puma was performed in Bax deficient neurons, to verify that both BH3-only proteins were equally well expressed. Immunohistochemistry revealed efficient transduction levels of 65-70% for both adenoviral constructs (Fig. 2.8C). To examine the ability of these BH3-only proteins to induce neuronal apoptosis, primary neurons were infected with Noxa or PUMA vectors at varying doses to generate a dose response curve (Fig. 2.9A and B). Our survival assays clearly showed that PUMA induced a rapid apoptotic response, such that at 12.5 MOI, greater than 50% cells were dead at 24 hours. In contrast, Noxa failed to induce significant apoptosis at 24 hours even at 200 MOI. To determine whether Noxa induced a delayed apoptotic response, a time course survival assay was performed with cerebellar granule neurons infected at 50 MOI with either Noxa, PUMA or a GFP control vector (Fig. 2.9C). Again, our results demonstrated

Figure 2.8 Upregulation of Noxa and PUMA mRNA in p53-mediated neuronal cell death.

Western blots showing efficient transduction of Ad-Noxa3XFlag and AD-PumaHA. (A) Cerebellar granule neurons were infected with Ad-Noxa3XFlag or Ad-GFP at 50 MOI. Protein lysates were collected 48 hours post-infection and were blotted for Noxa expression with an antibody against Flag or for actin as a loading control. (B) HEK 293 cells were infected with Ad-PumaHA or Ad-GFP at 25 MOI. Protein lysates were collected 12 hours post-infection and were blotted for PUMA expression with an antibody against HA or for actin as a loading control. (C) Neurons were infected with Ad-Noxa-Flag or Ad-PumaHA at 50 MOI. After 24 hours, neurons were fixed and immunostained for Flag or HA and counterstained with Hoechst. Bar, 25 μ m. Figures are a representative of three independent experiments (n=3).



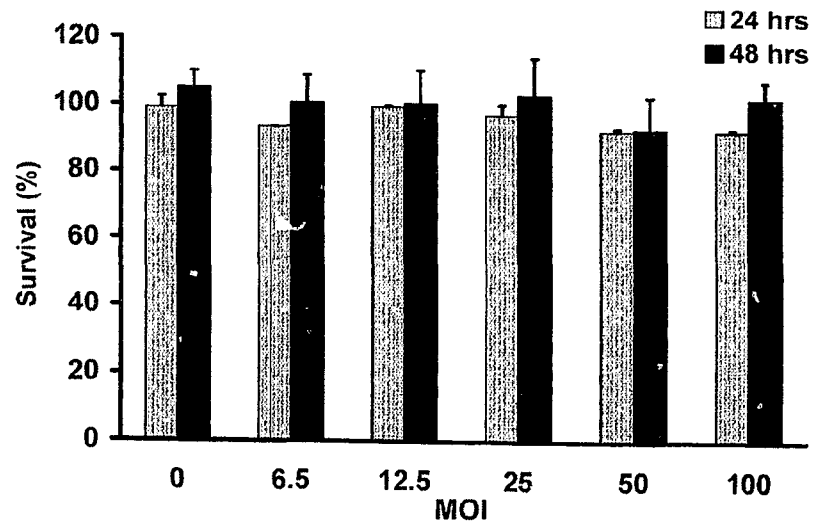
that cultures expressing PUMA exhibited a rapid apoptotic response such that greater than 80% of cells were dead by 48 hours. Cells expressing Noxa, however, exhibited minimal cell death even at 96 hours after infection. In conclusion, these results demonstrate that the p53 target gene PUMA is a potent inducer of apoptosis, whereas Noxa on its own is inefficient at triggering cell death. In summary, we have shown that AD1 of p53, within residues 1-42(Chang et al., 1995; Lin et al., 1994; Unger et al., 1993), is essential to induce neuronal cell death is due to its efficacy at inducing required proapoptotic target genes such as PUMA.

2.4.6 PUMA is Required for p53 Mediated Neuronal Cell Death

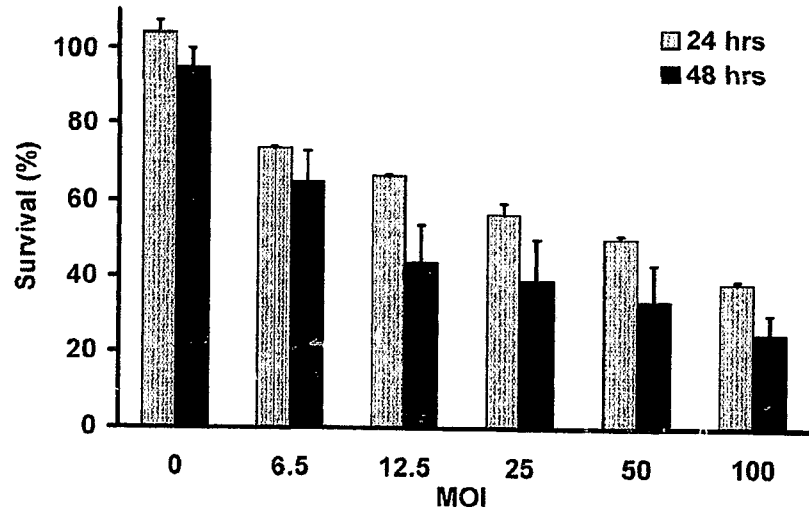
We have shown that the upregulation of PUMA by p53 requires the presence of both transactivation domains and that enforced expression of PUMA is sufficient to induce neuronal cell death. To determine whether PUMA is essential for p53 mediated apoptosis, we infected CGNs derived from PUMA-deficient mice or wildtype littermates with adenovirus expressing either p53, p53 Δ DM, or GFP. In figure 10 we demonstrate that PUMA-deficient neurons are significantly more resistant to p53 induced cell death at 48, 72, and 96 hours, relative to littermate controls. These results demonstrate that the upregulation of PUMA is required for p53 induced neuronal apoptosis.

Figure 2.9 Forced expression of PUMA, but not Noxa is sufficient to induce neuronal apoptosis. Cerebellar granule neurons were infected with Ad-Noxa3XFlag (A), Ad-PumaHA (B) or Ad-GFP control at the indicated MOI. Neuronal survival was determined at the indicated times by MTT assay. Survival is measured as a percentage of Ad-GFP treated control cells. (C) Cerebellar granule neurons were infected with Ad-Noxa3XFlag or Ad-PumaHA at 50 MOI. Neuronal survival was determined at the indicated times by MTT assay. Survival was measured as a percentage of Ad-GFP treated control cells. Data represents the mean and standard deviation from three independent experiments (n=3).

A.



B.



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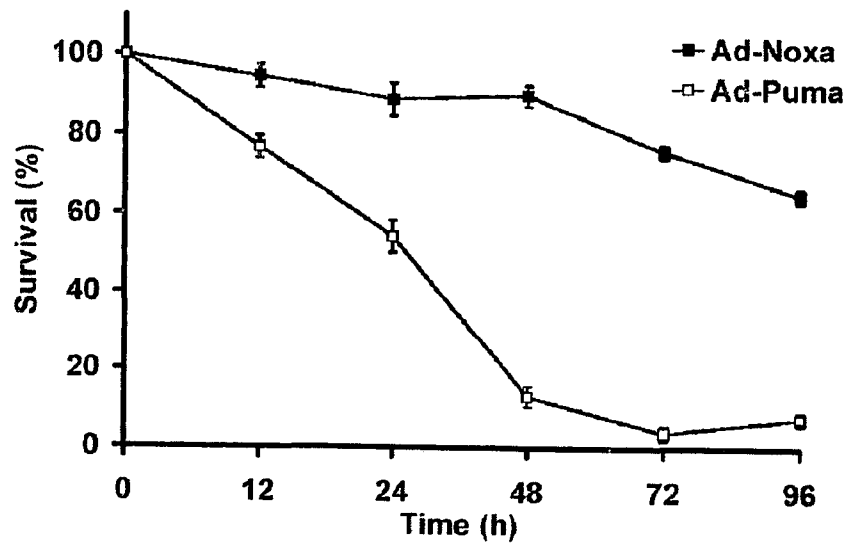
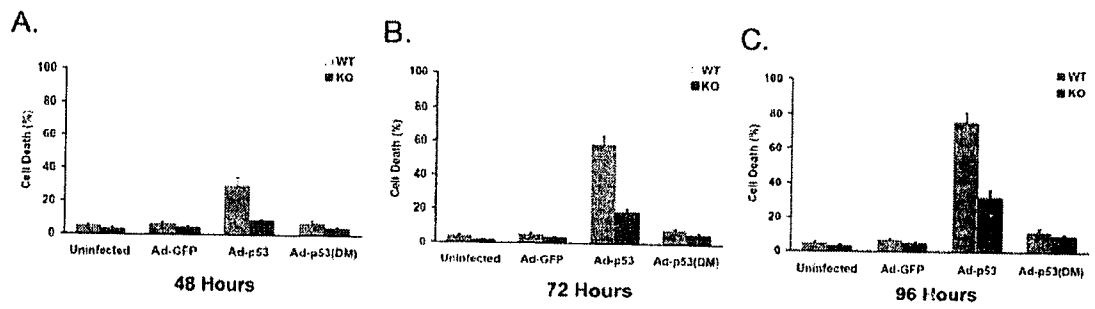


Figure 2.10 Upregulation of PUMA is essential for the induction of p53-mediated neuronal cell death. CGNs obtained from PUMA-deficient mice or control littermates were infected with wild-type Ad-p53, p53 Δ DM, or GFP constructs at 15 MOI. LIVE/DEAD viability/cytotoxicity assay (Molecular Probes) was performed at 48 hr (A), 72 hr (B), and 96 hr (C) after infection. Data represent the mean and SD of three independent experiments (n = 3).



2.5 DISCUSSION

The involvement of the p53 tumor suppressor has been demonstrated in neuronal cell death induced by acute injury and neurodegenerative diseases (Banasiak and Haddad, 1998; Cheng et al., 2003; Chopp et al., 1992; de la Monte et al., 1998; de la Monte et al., 1997; Halterman and Federoff, 1999; McGahan et al., 1998; Watanabe et al., 1999). Understanding the underlying apoptotic pathways is essential for the development of effective neuroprotective therapies. Despite the importance of p53 in neuronal apoptosis, little is known regarding the mechanisms by which p53 induces cell death. The results of our studies support a number of conclusions. First, we showed that p53-mediated transactivation is essential for the induction of neuronal cell death. Second, we demonstrated that AD1 of p53 is essential for the induction of target genes involved in neuronal apoptosis. Third, we showed that the induction of the proapoptotic BH3-only protein, PUMA, which requires the presence of both activation domains to trigger neuronal apoptosis. Fourth, that PUMA is required for p53-mediated neuronal cell death. These studies suggest that PUMA may provide a therapeutic target for the treatment of acute neuronal injury.

Recent studies have revealed that p53 is a multifunctional protein and can induce apoptosis by diverse mechanisms. Most notably, p53 has recently been shown to induce apoptosis exclusively at the mitochondria (Bonini et al., 2004; Mihara et al., 2003). In these studies, p53 mutants were constructed that failed to translocate to the nucleus and to activate the transcription of known p53 target genes. These p53 mutants could, however, translocate to the mitochondria where they were shown to sequester antiapoptotic BCL-family protein and induce apoptosis (Mihara et al., 2003). Also, when glial cells were treated with pifithrin- α , a compound that has been reported to protect neurons from ischemic or excitotoxic insult by specifically inhibiting p53 DNA binding ability (Culmsee et al., 2001a; Culmsee et al., 2001b), p53 was still able to induce apoptosis through direct activity at the

mitochondria (Bonini et al., 2004). Although the mitochondrial-mediated mechanism appears to be important in certain cell types and perhaps in response to specific stimuli, our studies demonstrate that this is not essential for the induction of neuronal apoptosis. Examination of double stained neuronal cells by confocal microscopy did not reveal any colocalization of p53 with the mitochondria, even when undergoing cell death, as determined by condensed nuclei. Consistent with the absence of direct p53-mitochondrial apoptotic activity, inactivation of ADI resulted in a loss of apoptotic activity when the mutant p53 Δ 22/23 was over-expressed. Furthermore, mutants lacking the first activation domain failed to reconstitute p53 responsiveness in p53-deficient cells exposed to camptothecin. Mutation of ADI in the endogenous gene, p53^{QS}, leads to a complete loss of apoptotic activity in response to DNA damage. It should be noted however, that the experiments presented here are limited to apoptosis induced by enforced p53 expression or DNA-damage but cannot rule out the possibility that different types of p53-mediated death stimuli may recruit other transcription-independent mechanisms. These results highlight the fact that p53 – induced apoptotic mechanisms are cell type specific and that p53 transactivation function is critical for neuronal apoptosis.

P53 contains two distinct transcription activation domains and the importance of these domains in the induction of apoptosis appears to be dependent on the cell type examined. While ADI has been shown to be important for cell death in primary thymocytes (Chao et al., 2000; Jimenez et al., 2000), studies using human lung carcinoma cells revealed that ADI was dispensable for apoptosis. In this instance, AD2 was shown to be required (Zhu et al., 1998).

Recently, studies have shown that p53-mediated transcriptional activity correlated directly with the amount of p53 interaction with p300/CREB-binding protein (p300/CBP) (Liu et al., 2003). Further, both activation domains, and to a lesser extent the proline rich domain of p53, are necessary for interaction with p300/CBP and for histone acetylation of the proximal p21 promoter required for

p21 transactivation (Liu et al., 2003). The loss of p300/CBP binding activity could account for the decreased levels of apoptotic activity observed in the ADI and Δ Pro mutants. Although the proline rich region has been previously shown to be necessary for apoptosis (Baptiste et al., 2002; Zhu et al., 1999), other data suggest that its deletion may influence function by altering local protein structure (Edwards et al., 2003). This alteration of protein structure leads to differential promoter interactions, and therefore may actually be dispensable for apoptotic activity (Edwards et al., 2003). The importance of ADI and ADII may depend on the subset of proapoptotic genes induced and the requirement of p53 target genes may be cell type specific. Using site directed p53 mutants, we have shown that each transactivation domain has independent effects on the induction of proapoptotic p53 target genes. Most notably, Noxa is readily induced by mutants carrying a mutation in ADI or ADII whereas PUMA can only be induced when both transactivation domains are present. Based on these findings we examined the proapoptotic activity of BH3-only proteins Noxa and PUMA in postmitotic neurons.

The BH3-only members of the Bcl-2 family of proteins are a unique group that only share a common nine amino acid BH3 domain with the other family members (Huang and Strasser, 2000; Puthalakath and Strasser, 2002). This BH3 domain has been shown to be required for the interaction with other Bcl-2 family members in the induction of apoptosis (Cheng et al., 2001; Wang et al., 1996; Zong et al., 2001). Previous studies have shown that Noxa and PUMA, two members of the BH3-only family, are upregulated under conditions of p53-mediated cell death in mouse embryonic fibroblasts (MEFs) (Oda et al., 2000) and colon cancer cell lines (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2003; Yu et al., 2001). Studies with mice carrying a null mutation for PUMA have shown that it is a key player in the apoptotic pathway. PUMA deficiency confers similar protection against apoptotic stimulus as seen with p53-deficiency (Jeffers et al., 2003; Villunger et al., 2003). Work

done in cell lines has also shown that PUMA may be regulated by factors other than p53, such as E2F-1 (Hershko and Ginsberg, 2003) and p73 (Melino et al., 2003), emphasizing its importance in the apoptotic pathway. The primary function of the Noxa protein seems to be at the mitochondrial level (Seo et al., 2003 115). Recent work with Noxa-deficient mice suggests that it may play a minor role in the apoptotic pathway in fibroblasts, but that it is dispensable in other tissues (Villunger et al., 2003). Several results in the present study suggest that Noxa is not likely to play an essential role in p53-mediated neuronal cell death. First, Noxa was efficiently induced by ectopic expression of p53 mutants that are defective in the induction of cell death. Secondly, Noxa was still induced following camptothecin treatment in p53^{QS} neurons yet these cells did not undergo cell death. Finally, enforced expression of Noxa was inefficient at triggering neuronal apoptosis. In contrast, p53-mediated induction of PUMA strongly correlated with apoptosis and ectopic expression of PUMA itself was sufficient to induce apoptosis. Further, PUMA-deficient cerebellar granule neurons were found to be substantially more resistant to cell death induced by p53 overexpression than littermate controls even at time points as late as 96 hours after infection. Thus, our studies demonstrate that PUMA, unlike Noxa, is a potent inducer of neuronal apoptosis, and plays a key role in neuronal injury-induced cell death.

The results demonstrate that p53 requires a functional transcriptional activation domain, specifically AD1, to induce the death of post-mitotic neurons. While multiple p53 target genes are induced in neurons, the proapoptotic BH3-only protein, PUMA, is a potent inducer of apoptosis in neuronal injury. Our results show that upregulation of PUMA is a key determinant in p53-mediated neuronal cell death and suggest that PUMA may serve as a key therapeutic target for the treatment of acute brain injury.

**Chapter 3: Arbour, NA, Vanderluit, JL et al.(2007) Mcl-1 is Required for Neuronal Survival.
*Neuron (Manuscript in preparation).***

This manuscript represents the results of my studies of the roles of Mcl-1 in neuronal survival.

The experiments in this manuscript were carried out by N. A. Arbour, J.L. Vanderluit, and J.N.Legrand. N. A. Arbour contributed 50% of the experimental work, including all cell culture data, and 90% of the animal breeding for the *in vivo* data. J.N.Legrand did most of the *in vivo* immunohistochemistry under the guidance of J.L.Vanderluit. N.A. Arbour and J.L. Vanderluit did the data interpretation with guidance from R.S. Slack. The manuscript was written by N. A. Arbour with guidance and editorial assistance from the co-authors.

3.1 Abstract

Anti-apoptotic Bcl-2 family member Mcl-1 is believed to play a unique and critical role in response to swiftly changing environmental cues or acute cellular stress in many systems during apoptosis; in part because of rapid transcription and post translational modification. The study of Mcl-1 in the nervous system however, has been very limited. Here we show the importance Mcl-1 in the neuronal survival. Mcl-1 flox mice were crossed into three lines of mice expressing Cre during neurogenesis or in post mitotic neurons. Mice with Mcl-1 deleted in the developing cortex are embryonic lethal and exhibit significant developmental defects in cortical neurogenesis, and excessive apoptosis. In mice where Mcl-1 is removed in more mature, post-mitotic neurons there is a severe loss of neurons observed, which occurs through a deregulated autophagic process. Lethality in these mutant mice occurs around 2 months of age. These results demonstrate that Mcl-1 is required for embryonic neuronal development, as well as maintaining the survival of post-mitotic neurons. Further, that Mcl-1 acts not only through the regulation of apoptotic pathways but through the regulation of autophagic pathways as well. Mcl-1 deficient neurons are also more susceptible to DNA damage, and Mcl-1 is down-regulated at both the transcriptional and post-translational levels in wild type cells. Further, sustained expression of Mcl-1, in this system, can protect against DNA damage induced neuronal cell death. This suggests that the control of Mcl-1 level/activity is paramount for neuronal survival following DNA damage injury. Our results indicate that Mcl-1 is necessary for proper neuronal development and survival, and that it achieves this through the control of multiple active cell death pathways including apoptosis and autophagic neuronal cell death.

3.2 Introduction

Bcl-2 family members are believed to be the gatekeepers of cell death in neurons, as in other tissues, however to date no single Bcl-2 family member has been shown to be absolutely required in the nervous system (reviewed in (Akhtar et al., 2004)). Myeloid cell leukemia 1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 family of proteins. It was initially identified as a gene activated in the human monocytic cell line ML-1, and found to have sequence similarity to Bcl-2 (Kozopas et al., 1993). Germline knockouts of Mcl-1 are peri-implantation lethal at E3.5 (Rinkenberger et al., 2000), and are the only germline knockouts of a Bcl-2 family member to date to exhibit such a severe phenotype (Bouillet et al., 1999; Coultas et al., 2004; Hockenbery et al., 1993; Knudson et al., 1995; Lindsten et al., 2000; Motoyama et al., 1995; Print et al., 1998; Ranger et al., 2003; Ross et al., 1998; Villunger et al., 2003; Yin et al., 1999). Furthermore Mcl-1 has been shown to be essential in the development and maintenance of B and T lymphocytes and the survival of hematopoietic stem cells (Opferman et al., 2005; Opferman et al., 2003). Mcl-1 is understood to inhibit cell death primarily through interactions with pro-apoptotic Bcl-2 family members, including multi-domained (Willis et al., 2005) and BH3-only Bcl-2 family members (for review see (Cory and Adams, 2002)). The affinity of interaction between Bcl-2 family members varies significantly, with Mcl-1 showing increased binding affinity for Noxa, PUMA, BIM_{EL} and BMF (Chen et al., 2005; Kuwana et al., 2005).

Mcl-1 is rapidly regulated via transcription (Croxtton et al., 2002; Piret et al., 2005; Yang et al., 1996), and post translational modification (Domina et al., 2000; Herrant et al., 2004; Inoshita et al., 2002; Maurer et al., 2006; Michels et al., 2004) and is believed to play a critical role in response to swiftly changing environmental cues and acute cellular stress (reviewed in (Craig, 2002)). Mcl-1 is further regulated by rapid turnover due to caspase cleavage (Michels et al., 2004) as well as through proteasome degradation through targeting by the BH3-only E3 ubiquitin ligase Mule (Zhong et al., 2005). This rapid and tightly regulated system makes Mcl-1 unique among anti-apoptotic Bcl-2 family

members. The unique regulation of Mcl-1, coupled with the specificity of binding to Bcl-2 family proteins make it particularly well suited to respond to acute neuronal injury.

Despite the importance of Mcl-1 in early apoptosis, little is known regarding its role in neuronal survival and injury-induced neuronal cell death. Initial immunohistochemical analysis comparing the protein regulation of Bcl-2 and Mcl-1 in human tissues showed differential regulation of these two multi-domained anti-apoptotic proteins, with very little Mcl-1 was observed in neurons under non-injury conditions (Krajewski et al., 1995). Recent studies, however, have shown Mcl-1 to play a role in development and cell death within the nervous system. Up-regulation of Mcl-1 has been shown to play a role in Notch-1 regulated survival of neural precursor cells (NPCs) (Oishi et al., 2004), as well as in maintaining the survival of granule cells during migration and differentiation (Zhang and D'Ercole, 2004). Studies have also demonstrated that heterozygous Mcl-1 germline deletion results in increased susceptibility of neurons to cell death caused by pilocarpine induced seizure injury (Mori et al., 2004). These studies suggest a role for Mcl-1 in maintenance of the nervous system, however, many questions remain as to its importance within the CNS, and its activity and regulation therein.

Unique among Bcl-2 family members, Mcl-1 germ line mutant mice are peri-implantation lethal, suggesting a requirement for Mcl-1 in development (Rinkenberger et al., 2000). Bcl-xl deficient mice are also embryonic lethal, at E13, showing increased apoptotic activity in the brain (Motoyama et al., 1995), conditional knock out studies however have shown Bcl-xl to be largely dispensable for neuronal survival (Savitt et al., 2005). Mice with a Bcl-2 targeted deletion show normal neuronal development, but exhibit significant loss of sympathetic neurons, motor neurons and sensory neurons during early postnatal life (Michaelidis et al., 1996). Although these studies demonstrate the importance of Bcl-2 family proteins in the maintenance of certain cell populations within the CNS, none have been shown to be essential for neuronal survival.

The sensitivity of heterozygous Mcl-1 germline deletion mutants to seizure induced injury suggests an important role for Mcl-1 in the mediation of injury induced neuronal cell death; however the lethality of this germline knock out has prevented further study of Mcl-1 in this system. In the present study, we take advantage of conditional models to identify the role of Mcl-1 in the nervous system. Using three neuron-specific conditional null mutations, we demonstrate for the first time that Mcl-1 is required for cortical development, neuronal survival, and the regulation of autophagic cell death. Loss of Mcl-1 in neuronal progenitors results in embryonic lethality and a significant reduction of cortical neurogenesis. Widespread apoptosis of neuroblasts immuno-positive for Nestin and Tuj1 demonstrates a requirement for Mcl-1 for the survival of newborn neurons. In contrast, deletion of Mcl-1 in post-mitotic neurons results in widespread autophagic death of cortical neurons. These results demonstrate that Mcl-1 is required for the survival of neurons at all stages of development. We also show a rapid, injury induced regulation of Mcl-1, in primary cortical neurons that is important for the induction of neuronal cell death. Further, mitochondria in cortical neurons lacking Mcl-1 are shorter and have a peri-nuclear localization. Our results demonstrate different roles for Mcl-1 in the regulation of multiple mechanisms of cell death in neuronal development and survival.

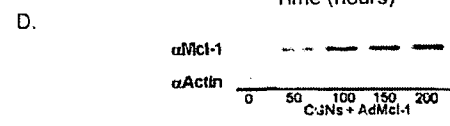
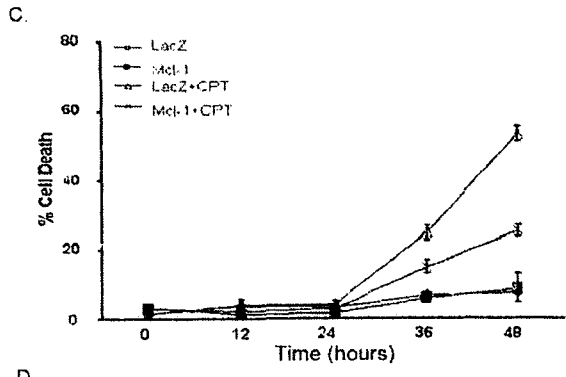
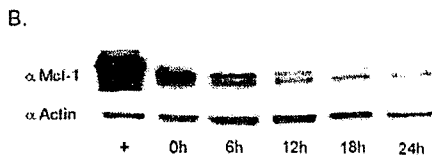
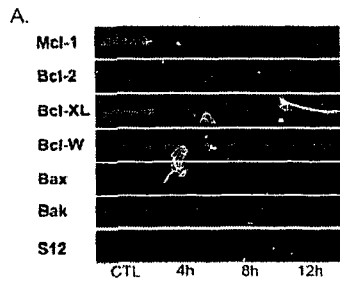
3.3 Results

3.3.1 Mcl-1 is down-regulated following neuronal injury

Bcl-2 family proteins are important regulators of mitochondrial-mediated apoptosis (Gross et al., 1999a) and are believed to hold a delicate balance between life and death of the cell (Gross et al., 1998; Gross et al., 1999a; Puthalakath and Strasser, 2002; Yang et al., 1997). Since the multi-domain Bcl-2 family members play a critical role in cell death, we asked which if any of these Bcl-2 family members were transcriptionally regulated following DNA damage induced neuronal cell death. Primary cortical neurons were cultured and treated with 10 μ M camptothecin after 2 days *in vitro* (DIV). Although we did not observe changes in the mRNA levels of most of the multi-domain Bcl-2 family members, we did observe a rapid down-regulation of Mcl-1 transcript (Figure 3.1A). This change in mRNA levels is followed by a more gradual down-regulation of Mcl-1 protein levels (Figure 3.1B). The rapid downregulation of Mcl-1 prior to the onset of neuronal cell death suggests that Mcl-1 may have an important role in the regulation of neuronal survival.

To determine if maintaining levels of Mcl-1 would protect against neuronal injury, we constructed an adenoviral vector containing the full length, FLAG-tagged Mcl-1 expression cassette (Ad-Mcl-1-3XFLAG). Efficient protein expression of the Mcl-1 vector in neurons was confirmed by western blot (Figure 3.1D). Primary cerebellar granule neurons (CGNs) were infected at the time of plating, with increasing multiplicities of infection (MOIs) of Ad-Mcl-1-3XFLAG, and protein expression was examined after 2 days *in vitro* (DIV). To assess the protective role of Mcl-1 in neurons, CGNs were infected with Ad-Mcl-1-3XFLAG at time of plating and treated with 10 μ M Camptothecin after 2 DIV. Cells maintaining high expression of Mcl-1 showed a nearly 2-fold greater protection

Figure 3.1. Mcl-1 is down-regulated in response to DNA damage induced cell death. (A) Total RNA from cortical neurons from CD1 mice treated with 10 μ M camptothecin was collected and analyzed at the indicated times for Mcl-1, Bcl-2, Bcl-xl, Bcl-w, Bax, Bak and S12 expression using semi-quantitative RT-PCR. (B) Cortical neurons were treated with 10 μ M camptothecin. Protein lysates were collected at indicated times following treatment and initially were blotted for Mcl-1 expression and then for actin as a loading control. (C) CGNs were infected with Ad-Mcl-1-3XFlag or Ad-GFP control at 75 MOI and treated with 10 μ M camptothecin, 48 hours post-infection. LIVE/DEAD viability/cytotoxicity assay was performed at the indicated times. (n=3) (D) Western blot showing efficient transduction of Ad-Mcl-13XFlag. CGNs were infected with the indicated MOI of Ad-Mcl-1-3XFLAG. Protein lysates were collected 48 hours post-infection and were blotted for Mcl-1 expression then for actin as a loading control. * P <0.05



(AdLZ $24.7 \pm 2.3\%$ cell death and AdMcl-1 at $14.8 \pm 2.0\%$ cell death) against DNA damage induced cell death relative to cultures treated with LacZ (LZ) control at 36 hours after treatment, which is maintained at 48 hours (AdLZ $53.3 \pm 2.3\%$, AdMcl-1 $25.5 \pm 1.9\%$) as demonstrated by LIVE/DEAD assay (Fig. 3.1C). These results show that Mcl-1 plays an important role in maintaining neuronal viability in response to cell death stimuli.

3.3.2 Mcl-1 is Required for Cortical Neurogenesis

As Mcl-1 is the only antiapoptotic multidomain Bcl-2 family protein downregulated following acute neuronal injury, we set out to determine if Mcl-1 plays important role in maintaining neuronal survival. Since Mcl-1 germline deletion results in pre-implantation lethality (Rinkenberger et al., 2000), we used conditional mutants to assess the role of Mcl-1 in neuronal development. Animals in which cre-recombinase was inserted at the FoxG1 locus (Hebert and McConnell, 2000) were interbred with mice carrying a floxed Mcl-1 allele (Opferman et al., 2003). Cre knocked in to the FoxG1 locus is expressed in neural progenitors throughout the telencephalon at E8 and reaches maximal levels at E17 (Shimamura and Rubenstein, 1997; Tao and Lai, 1992). FoxG1Cre have been previously characterized (Hebert and McConnell, 2000) and our previous studies have revealed efficient cre-mediated recombination of floxed alleles specifically in the developing telencephalon (Ferguson et al., 2002). This model allows us to selectively assess the role of Mcl-1 in neural precursor cells during telencephalic development.

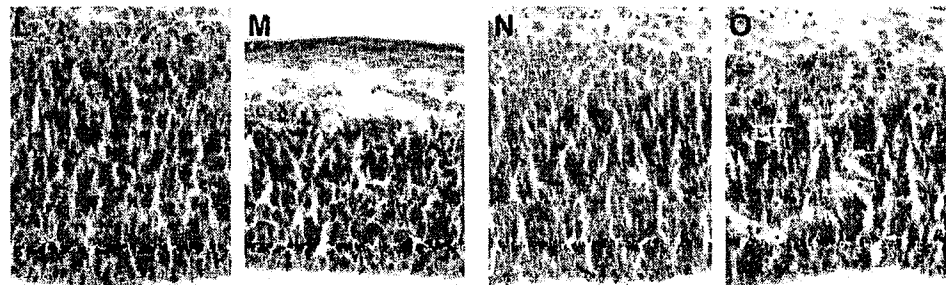
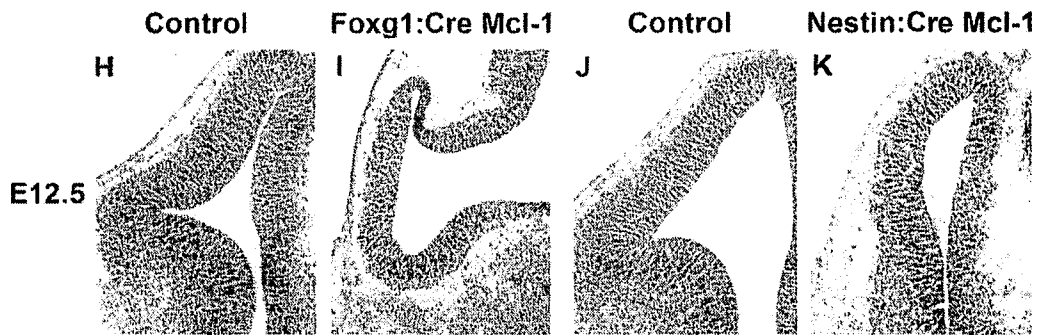
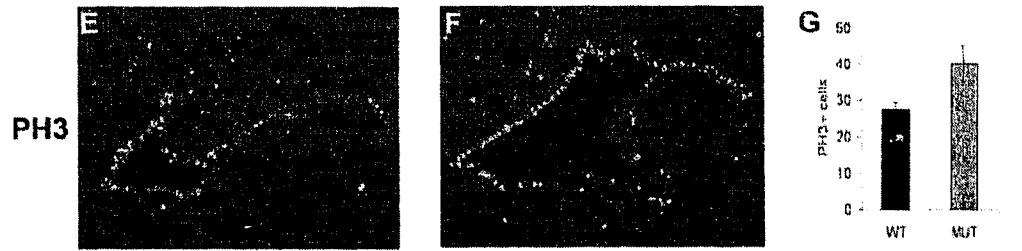
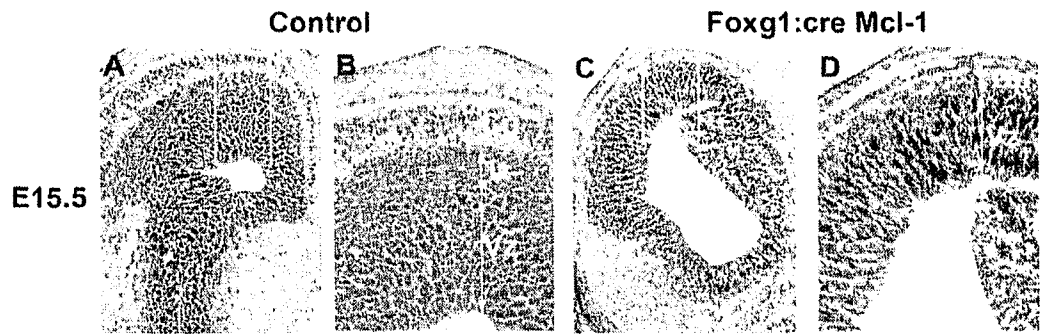
Conditional deletion of Mcl-1 specifically within the forebrain resulted in embryonic lethality at E16-17, however embryos collected at earlier time points occurred at expected Mendelian ratios. To assess the role of Mcl-1 in neuronal development, embryos were collected at E15.5 corresponding to mid-neurogenesis. This stage is characterized by a large population of neuronal progenitors (Nestin+

cells) within the ventricular and subventricular zones (VZ/SVZ), early committed neuroblasts (doublecortin+) within the (SVZ/IZ) and a growing population of postmitotic neurons (Tuj1+) in the developing cortical plate (CP). Animals lacking Mcl-1 in the developing telencephalon exhibit a dramatic neural phenotype with the overall size of the Mcl-1 mutant telencephalon being significantly smaller (2.9 fold) relative to littermate controls (Figure 3.2 A,C). Morphological assessment of coronal sections through the telencephalon revealed a striking reduction in the size of the cortical plate. In contrast to littermate controls in which layering of the VZ/SVZ, IZ and CP are clearly delineated, Mcl-1 mutant mice lacked a clear distinction between the IZ and CP (Figure 3.2 B,D). Furthermore, measurements at 3 distinct levels through the rostral-caudal extent of the telencephalon revealed a complete loss of cortical plate formation at all levels in the embryonic forebrain of Mcl-1 mutant mice.

The dramatic reduction of cortical plate formation lead us to question whether this defect occurred before or after cortical plate formation. A defect in the initiation of the cortical plate could be associated with Mcl-1 having a role in neural progenitor proliferation or commitment; whereas a defect following formation of the cortical plate would be attributed to Mcl-1 playing a role in the survival of post-mitotic neurons. To determine if Mcl-1 affects neural progenitor proliferation, we compared the size of the progenitor pools in Mcl-1 mutants and littermate controls. Measurement of the overall number of proliferating cells using the M-phase cell cycle marker, phospho-histone H3 (PH3) revealed similar numbers of proliferating cells (Figure 3.2 F-H). These results demonstrate that the lack of cortical neurons does not result from defective proliferation.

If Mcl-1 regulates survival of postmitotic neurons however, the formation of the cortical plate should be initiated and cells would die during the maturation process either enroute or after reaching the cortical plate. To determine if formation of the cortical plate was initiated at an earlier time point, embryos were collected at E12.5, just following the onset of neurogenesis. Similar to our

Figure 3.2. Loss of Mcl-1 in developing neurons results in severe defects in cortical neurogenesis. (A-D) Coronal sections of E13.5 FoxG1:Cre mutant mice and littermate controls showing significant loss of cortical size and morphology. (E,F) Coronal Sections of E15.5 FoxG1:Cre mutant mice and littermate controls immunoblotted for phosphohistone H3 (PH3), an M-phase marker, to stain actively dividing cells. (G) Quantitative analysis of PH3 positive cells in the ventricular zone, 1000 μ M along the ventricle from the midline, reveal no difference in the number of proliferating cells between mutant and control mice. (H-O) Coronal sections of E12.5 FoxG1:Cre and Nestin:Cre mutant mice and littermate controls showing a dramatic reduction in cortical plate formation.



observations at E15.5, mutant embryos at E12.5 also lacked a cortical plate. In coronal sections of the Mcl-1 mutant forebrains, the ventricular zone appeared similar in size to controls, however development of layers beyond the VZ were dramatically reduced in size (Figure 3.2 M). These results strongly suggest a role for Mcl-1 following progenitor commitment to a neuronal fate.

To confirm that the defects observed with the Mcl-1 mutant were due specifically to the loss of Mcl-1 in neural progenitors, we utilized a second conditional mutant. Transgenic mice in which cre-recombinase is expressed under the control of the nestin promoter (Berube et al., 2005) (Zimmerman et al., 1994) were interbred with mice carrying a floxed Mcl-1 allele (Opferman et al., 2003). Nestin is expressed in neuronal progenitors throughout the developing nervous system at E7.5, following pre-plate formation (Dahlstrand et al., 1995). The use of two conditional mutants allowed us to examine the effect of Mcl-1 deletion within the same progenitor pool. Cre-mediated recombination of Mcl-1 throughout the developing CNS with the Nestin:Cre, resulted in embryonic lethality occurring prior to E15. Morphological analysis of coronal sections through the brains of E12.5 embryos revealed a similar cortical phenotype as the FoxG1:cre mutant embryos with a dramatic reduction in cortical plate formation (Figure 3.2 O). Deletion of Mcl-1 in neural progenitor cells by either FoxG1 or Nestin conditional mutants prevented development of the cortex. These results, therefore, strongly support a requirement for Mcl-1 in cortical neurogenesis.

3.3.3 Mcl-1 deficient neural progenitors die by apoptosis

The striking reduction of mature cortical neurons in the mutant cortex, without a defect in proliferation, suggests that a population of proliferating cells die prior to maturation. To determine if this cell loss is due to apoptosis, sections from FoxG1:cre and Nestin:cre mutant mice and control littermates at E12.5 were immunostained with antibodies to active caspase 3 and examined for nuclear condensation using Hoechst staining. Numerous active caspase 3 positive cells were present

within the VZ/SVZ of both types of mutant mice, whereas only an occasional apoptotic cell was observed in littermate controls (Figure 3.3 A-C). In the FoxG1:cre mutants, apoptotic cells, occurred in discreet pockets, whereas in Nestin:cre mutants apoptotic cells appeared more dispersed throughout the VZ/SVZ (Figure 3.3 B,C). Relative to littermate controls therefore, Mcl-1 mutants exhibit extensive neural cell loss in the developing cortex relative to littermate controls (Figure 3.3 A).

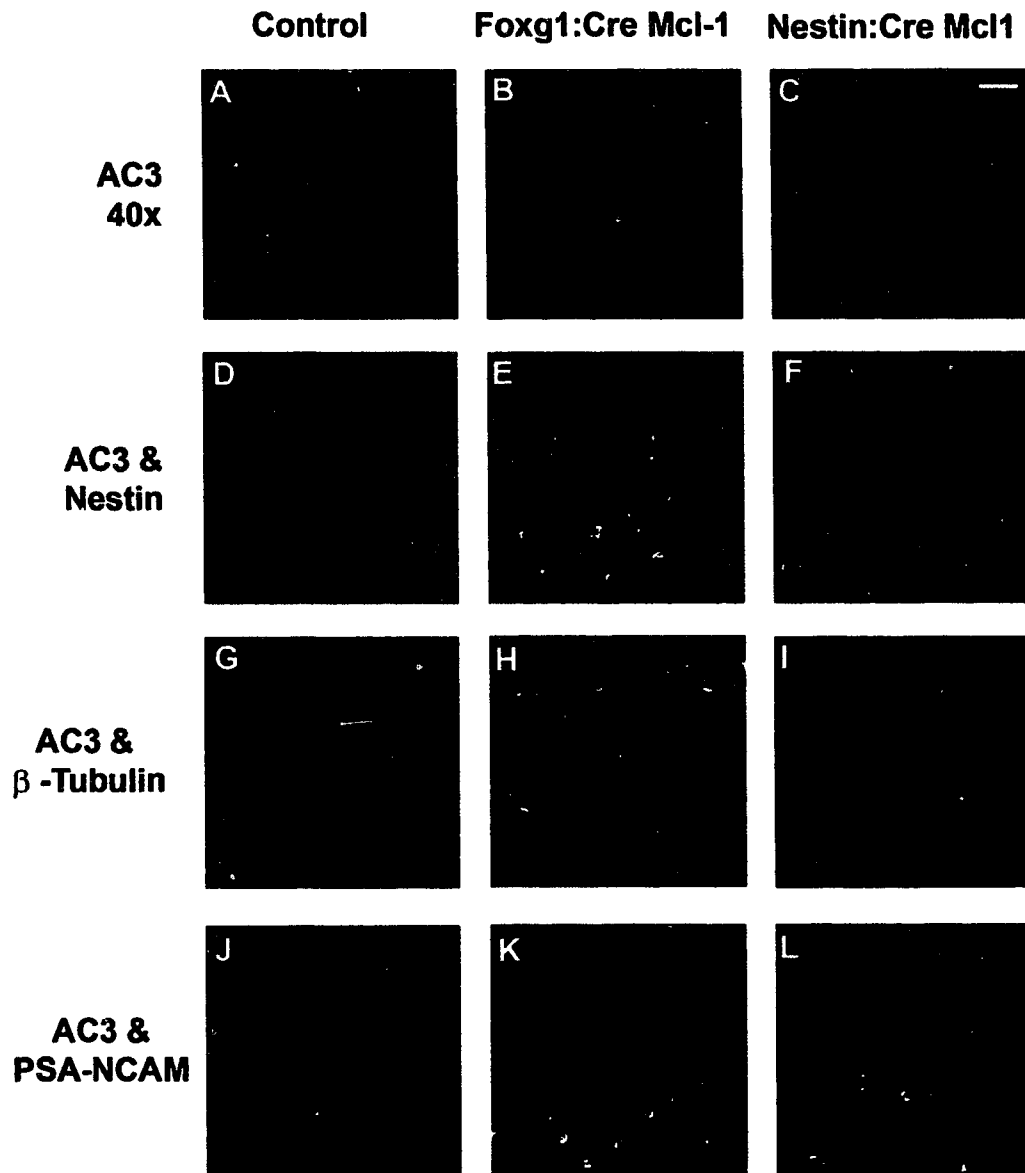
To determine if apoptosis occurs prior to or following commitment to a neuronal lineage, cells were double labeled with antibodies to active caspase 3 and stage specific markers, such as Nestin (a marker for neuronal progenitor cells (NPCs))(Figure 3.3 D-F) PSA-NCAM (a migratory neuroblast marker) (Figure 3.3 G-I) or Tuj1 (an immature neuronal marker) (Figure 3.3 J-L). Cells positive for both nestin and active caspase 3, or Tuj1 and active caspase 3 were then quantified to determine at what point in differentiation cells undergo apoptosis. Cells staining positive for active caspase 3 were predominantly nestin positive suggesting that the cells are dying as progenitors prior to commitment to a differentiated phenotype.

Mice lacking Mcl-1 in neuronal progenitors or in the developing telencephalon demonstrate that Mcl-1 is required for proper cortical development. The reduced cortical thickness observed in Mcl-1 mutant mice is due to increased apoptosis and this apoptosis occurs just prior to neuronal differentiation. These results indicate a requirement for Mcl-1 for development of the telencephalon.

3.3.4 Loss of Mcl-1 in post-mitotic neurons (PMN) results in premature death

Having determined a requirement for Mcl-1 in neuronal development, we asked if there was an important role for Mcl-1 in mature PMNs. To examine this we utilized a conditional Mcl-1 mutant system in which cre-mediated recombination occurs specifically in PMNs. Transgenic mice carrying

Figure 3.3. Loss of Mcl-1 in developing neurons results in increased apoptotic activity in neuronal progenitors. (A-C) Coronal Sections at E12.5 of FoxG1:Cre and Nestin:Cre mutant mice and representative heterozygous FoxG1:cre littermate controls were immunostained for active caspase 3, a marker of apoptotic cell death. (A) Control mice show no active caspase 3 staining. (B) FoxG1:Cre mutants show multiple pockets of active caspase 3 activity, (C) Nestin:Cre mutants show active caspase 3 activity dispersed through the VZ/SVZ. (G-I) Coronal Sections at E12.5 immunostained for or active caspase 3 and nestin, a marker for neuronal progenitor cells. (J-L) Coronal Sections at E12.5 immunostained for or active caspase 3 and Tuj1, an immature neuronal marker. (M-O) Coronal Sections at E12.5 immunostained for or active caspase 3 and PSA-NCAM, a migratory neuroblast marker.



scale bar (a-c) = 100 microns

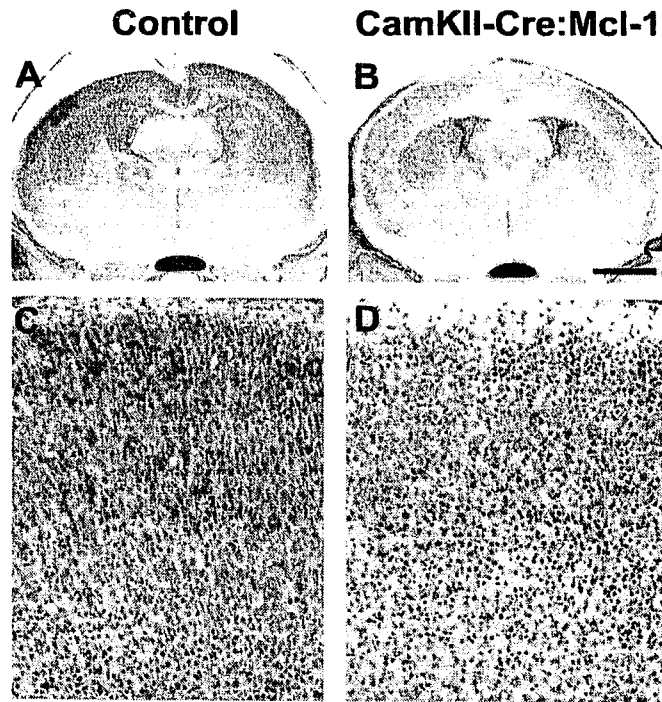
cre-recombinase driven by the CamKII α regulatory sequences on a BAC (Casanova et al., 2001) were interbred with mice carrying floxed Mcl-1 allele (Opferman et al., 2003). Since CamKII α cre expression is initiated at birth, the resulting recombination of floxed Mcl-1 occurs efficiently in post-mitotic neurons in the cortex, hippocampus, and amygdala, and at lower levels in the striatum, thalamus, and hypothalamus (Casanova et al., 2001). This conditional mutant, allowed us to determine the importance of Mcl-1, in post-mitotic neurons.

Mice homozygous for Mcl-1 [Mcl-1 fl/fl] were crossed with double heterozygous mice [CamKII α cre^{-/-} : Mcl-1 fl/-]. The crosses were allowed to come to term and the resulting litters were further examined. Newborn mutant mice looked similar to their littermate controls and were present at expected Mendelian ratios. All mutant mice reached their endpoint by 1-2 months of age, while littermate controls continue to grow to maturity.

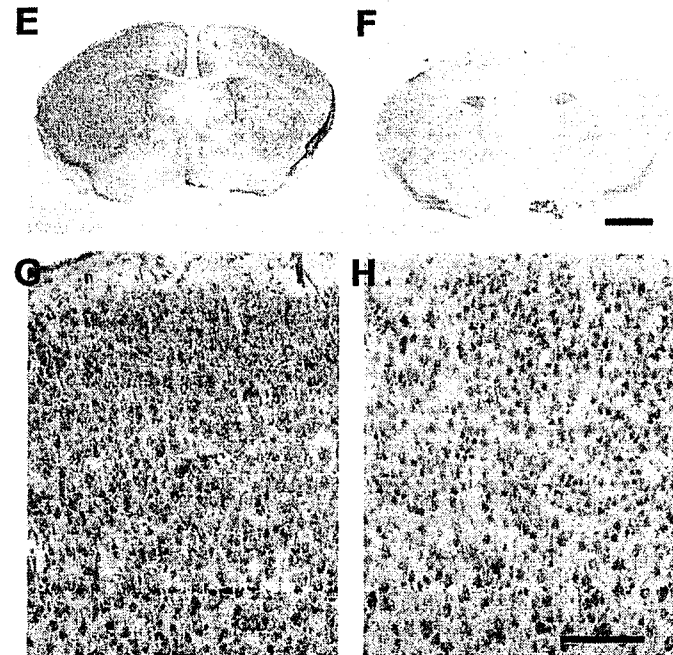
To determine the importance of Mcl-1 in the survival of PMNs, mice were collected and analyzed at several time points following birth (P1, P7, P14, 1 and 2 months). Sections from mutant and control littermate mice were stained with cresyl violet to examine cortical morphology. At birth (P1), mutant mice and their control littermates are indiscernible from one another (Figure 3.4 A-D). The loss of neurons in the cortex begins at P7 (Figure 3.4 E-H) and by P14 is readily apparent by the thinning of the cortex (Figure 3.5 A,B,E,F). At 1-2 months of age, this loss of neurons results in a complete hollowing of the brain (Figure 3.5 C,D,G,H) The progressive loss of PMNs and cortical thinning is more dramatically demonstrated when cells are labeled with an antibody against NeuN to identify mature neurons (Figure 3.5 I,J). This loss of mature PMNs indicates a requirement for Mcl-1 not only in neuronal development as seen with the FoxG1 and Nestin cre mutants, but also for the survival of mature PMNs.

Figure 3.4. Loss of Mcl-1 in post-mitotic CamKII α :Cre Mcl-1 neurons results in rapid neuronal degeneration. (A-H) Coronal sections of CamKII α :Cre mutant and littermate control mice were stained with cresyl violet at P1 and P7 to examine cortical morphology. Mutant mice exhibit smaller brain size, loss of white matter tracts and thinning of the cortical plate compared to control animals.

Postnatal day 1

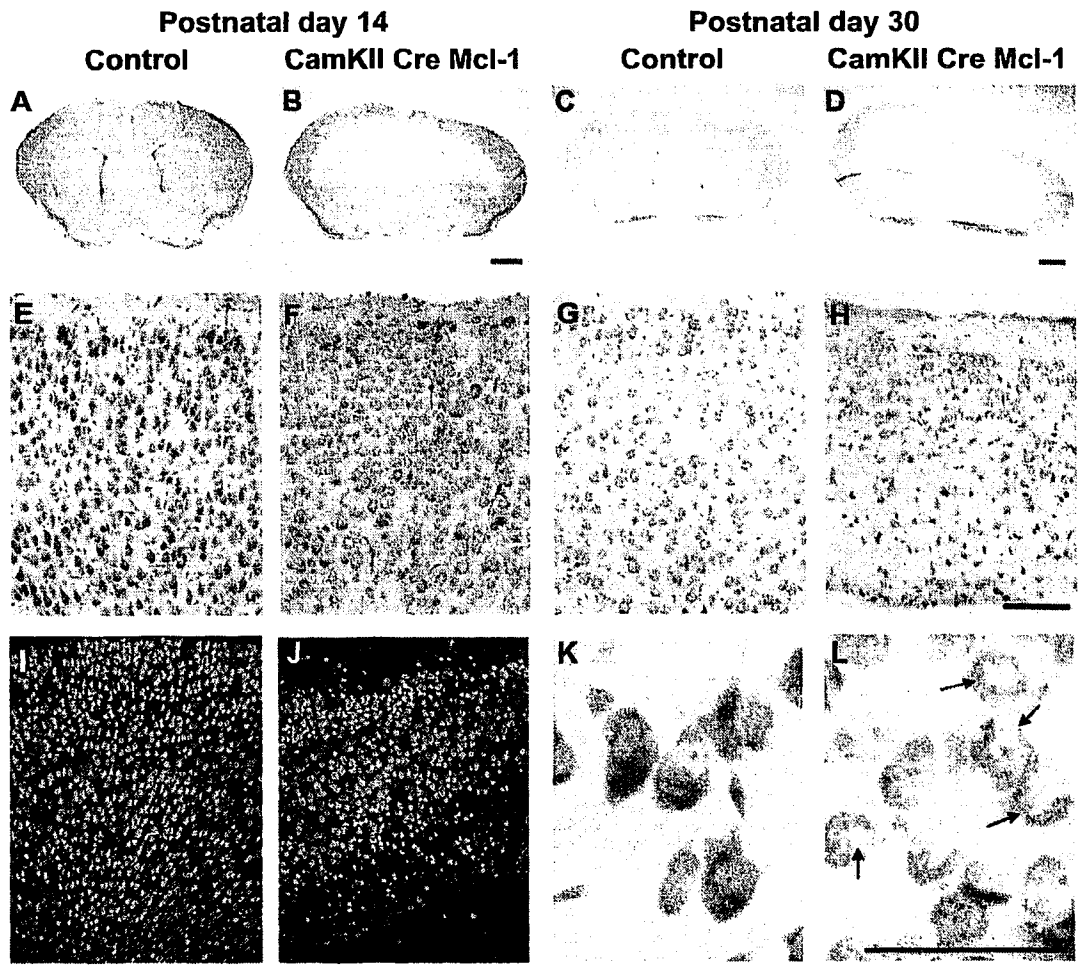


Postnatal day 7



all scale bars =100microns

Figure 3.5. Mice lacking Mcl-1 in post mitotic neurons lose cortical neurons. (A-H) Coronal sections of CamKII α :Cre mutant and littermate control mice were stained with cresyl violet at P14 and P30 to examine cortical morphology. Mutant mice exhibit smaller brain size, and signs of neuronal distress, which is exacerbated at later time points. (I,J) Coronal sections of P14 CamKII α :Cre mutant and littermate control mice were immunostained with NeuN antibody to identify neurons. (K,L) Coronal sections of mutant and control mice at P14 stained with cresyl violet. Nuclei of mutant mice show swollen nuclei.

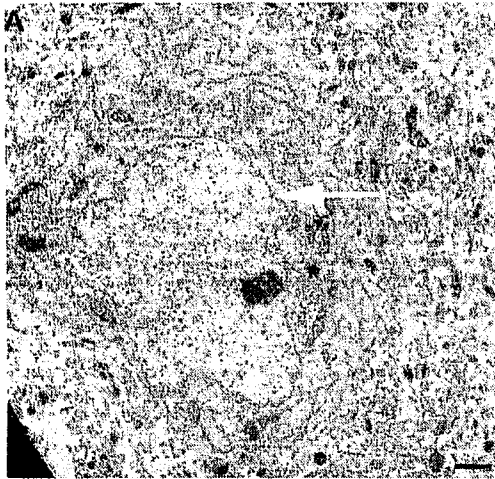


3.3.5 Loss of Mcl-1 deficient PMNs does not occur through the hallmark apoptotic pathway

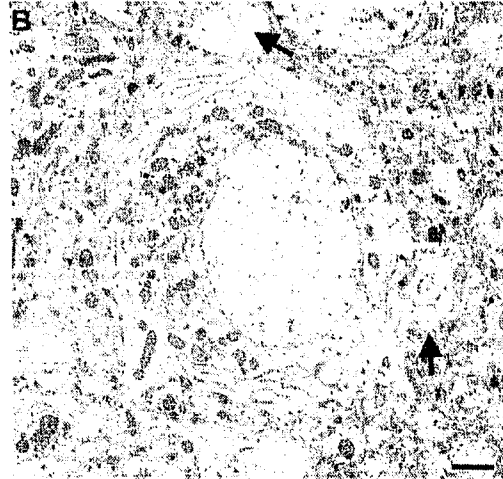
The severe loss of PMNs in the cortex prompted us to determine the mechanism by which these cells died. High magnification cresyl violet staining revealed morphological markers of cell distress including pyknotic nuclei and/or swelling and vacuolization nuclei (Figure 3.5 K,L). Although these markers of cellular distress were evident, immunostaining with active caspase 3 revealed no increases in caspase activity (data not shown). To further examine the morphological characteristics and identify this form of neurodegeneration we employed transmission electron microscopy (EM). Using EM, we were able to identify morphological characteristics exhibiting the hallmarks of autophagic cell death, including extreme tissue vacuolization, and the ultrastructural observation of double and membrane vesicles containing recognizable cytoplasmic organelles and multilamellar whorls (Larsen and Sulzer, 2002) (Figure 3.6 A-D). These results were further corroborated by increased staining for Beclin-1 a protein required for autophagy (Erlich et al., 2006; Liang et al., 1999). Our results indicate that Mcl-1 is required for neuronal survival, however, the lack of Mcl-1 does not always result in the induction of the classical apoptotic pathway as observed in neural precursors and in other cell types (Oishi et al., 2004; Opferman et al., 2005; Opferman et al., 2003; Zhang and D'Ercole, 2004). Instead, the absence of Mcl-1 in postmitotic neurons results in the activation of autophagic cell death. These results demonstrate that Mcl-1 is required for neuronal survival through the prevention of cell death through multiple cell death pathways.

Figure 3.6. Mcl-1 mutant post-mitotic neurons undergo autophagic cell death. (A-D) Transmission electron microscopy of sections from P14 CamKII α :Cre mutant and littermate control mice. (B) Mutant neuron exhibiting swollen nuclei (C,D) Mutant tissue exhibiting large numbers of autophagic vacuoles containing lamellar material and organelles. (E) Protein lysates were collected from the cortices or hippocampus of mutant and control mice at 1 month of age and were blotted for Beclin-1 expression then for actin as a loading control, showing increased Beclin-1 activity in mutant cortices.

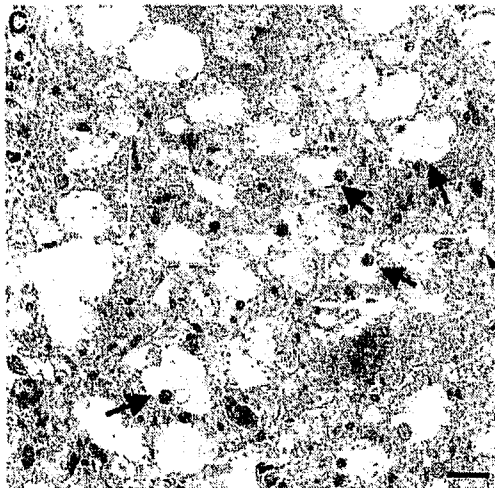
Control



CamKII Cre: Mcl-1



CamKII Cre: Mcl-1



E

	Hippo.	Cortex	
Mcl-1	+/-	+/-	-/-
CamKII α Cre	+	+	+
BECN1			
Actin			

scale bars a=10 microns

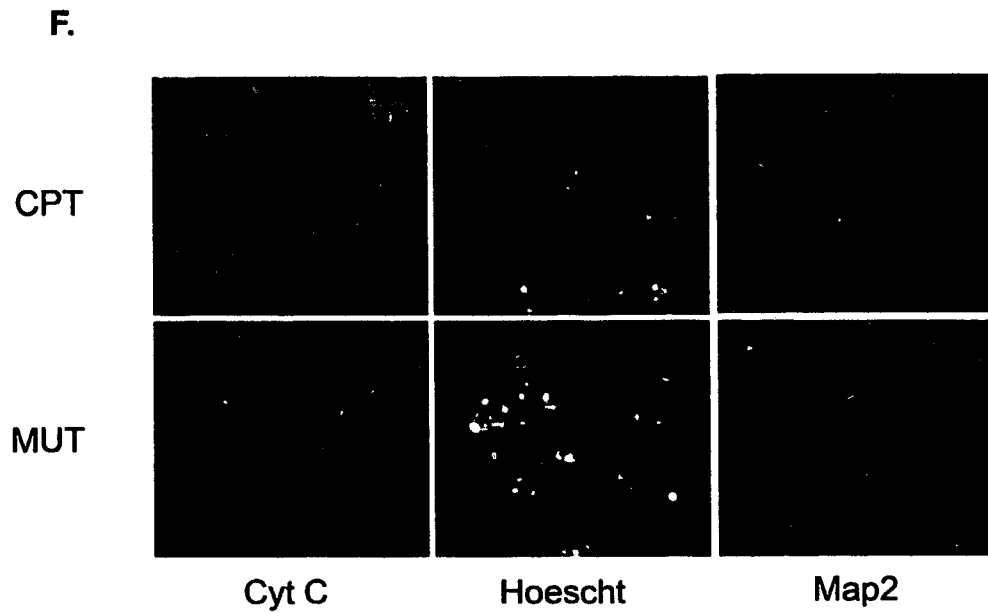
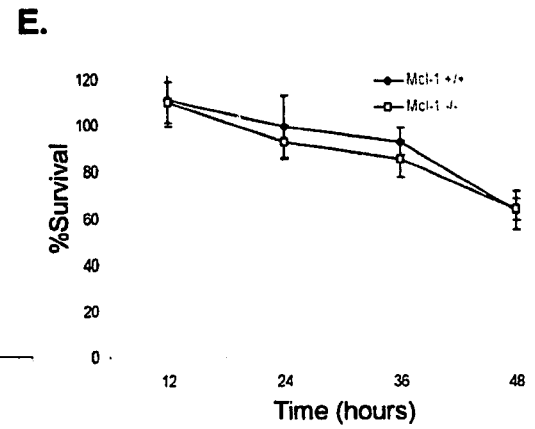
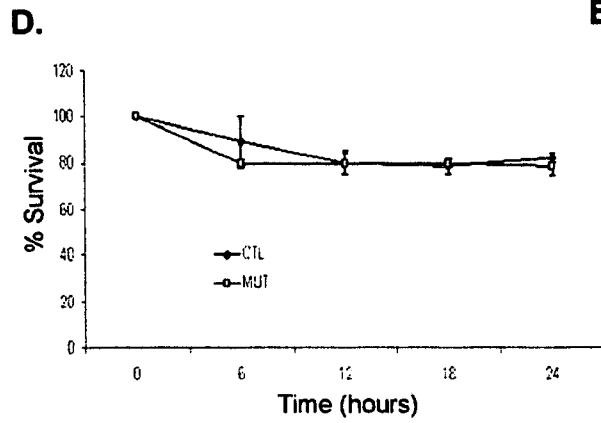
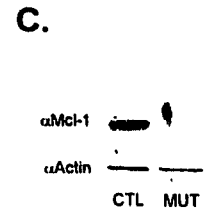
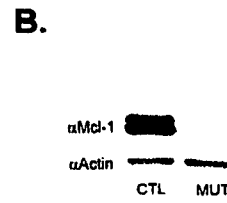
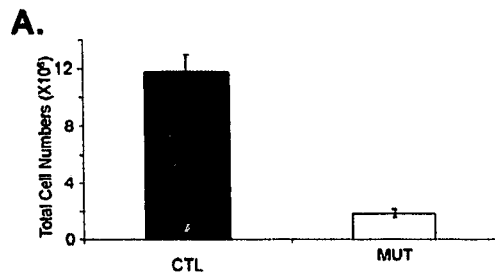
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d= 500nm

3.3.6 PMNs lacking Mcl-1 are more sensitive to DNA damage induced neuronal cell death

Our studies revealed that Mcl-1 is down-regulated following DNA damage induced neuronal cell death (Figure 3.1). Since we have shown that Mcl-1 is required for neuronal development and survival of PMN, we asked if the requirement for Mcl-1 in PMN is cell autonomous. Cortical neurons were isolated from the FoxG1 cre^{-/-} Mcl-1 fl/fl mutant mouse such that recombination occurred during development. Although there is a significant reduction in cortical size there remains a sub-population of viable neurons in the intermediate zone and cortical plate of Mcl-1 mutant mice (as seen in Figure 3.2D). Culturing cortical neurons from mutant cortices revealed a 6.4 fold reduction in viable neurons relative to their littermate controls (Figure 3.7A). Western analysis revealed that these neurons do not express Mcl-1 (Figure 3.7B), however they continue to survive *in vitro*. The mutant Mcl-1 deficient neurons express Map2 (Figure 3.7F), a pan neuronal marker, suggesting that once in culture, immature neurons are able to initiate differentiation. In contrast to our *in vivo* results with PMNs, cultured neurons lacking Mcl-1 do not die spontaneously in culture when compared to their littermate controls up to 3 DIV, as demonstrated by LIVE/DEAD assay (Figure 3.7D). Similarly, neurons were cultured from mice homozygous for the floxed Mcl-1 allele and then infected with an adenoviral vector carrying adenoviral Cre-recombinase to induced recombination *in vitro* (Figure 3.7E). Our results reveal that following cre-mediated recombination of Mcl-1 *in vitro* neurons do not undergo apoptosis but remain viable for at least 3 to 4 days. Having determined that Mcl-1 is required for development and maintenance of the nervous system, and given recent findings linking Mcl-1 to proteins involved in mitochondrial function (Maurer et al., 2006) we examined mitochondrial morphology of cultured Mcl-1 deficient neurons. Neurons were stained with an antibody against Cytochrome C, a mitochondrial protein localized to the mitochondrial inter-membrane space that is released into the

Figure 3.7. Mutant mice have fewer cells available for culture, but otherwise are similar to littermate controls. (A) Quantitative analysis of cultured cortical neurons from telencephalon specific mutant mice and control littermates shows culturable cortical neurons per mouse compared to their littermate controls (n=4). (B) Protein lysates were collected from cultured cortical neurons 48 hours after plating and were blotted for Mcl-1 expression then for actin as a loading control, showing efficient excision of Mcl-1 in mutant cortical neurons. (C) Protein lysates were collected from CGNs infected with Ad-cre or Ad-GFP (m.o.i. 50) 48 hours after plating and were blotted for Mcl-1 expression then for actin as a loading control, showing efficient excision of Mcl-1 in cultured neurons. (D) Mutant cortical neurons survive in culture similarly to cortical neurons cultured from littermate control animals. (E) CGNs excised of Mcl-1 using adenoviral-mediated cre-recombinase, survive in culture similarly to CGNs cultured from littermate control animals.

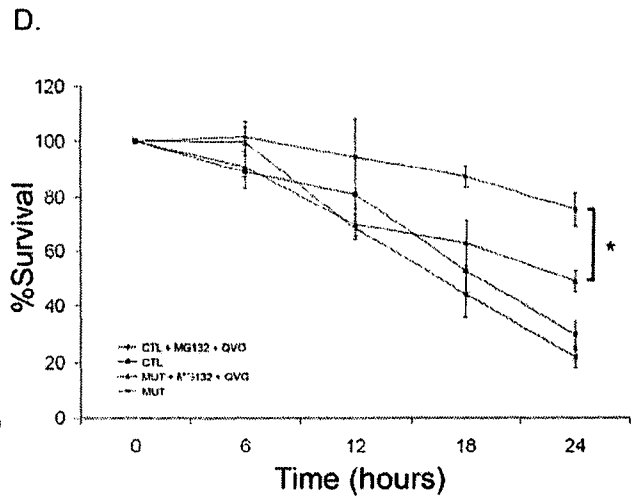
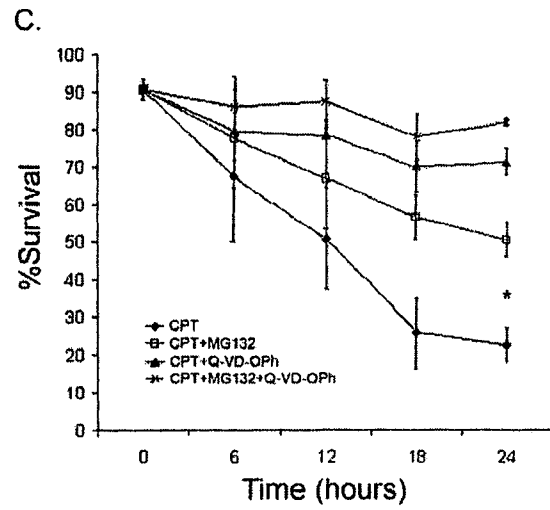
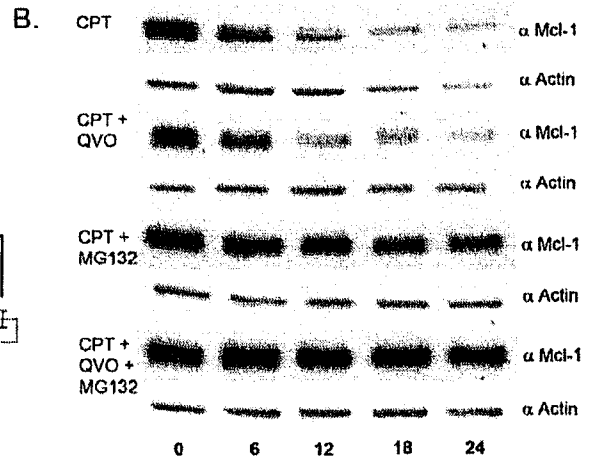
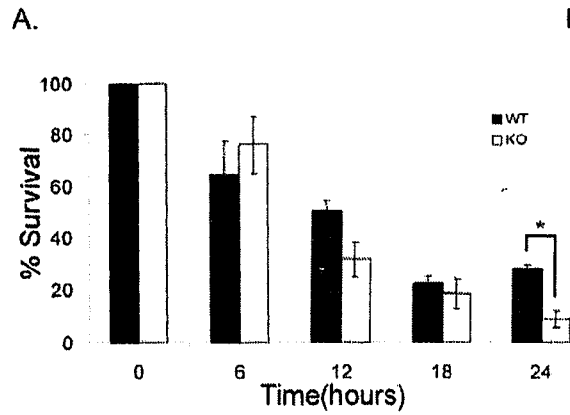


cytosol following initiation of the apoptotic program. Mitochondria were readily detectable and retained cytochrome C despite the absence of Mcl-1. In addition, mitochondria in mutant neurons appeared to be more fragmented and to have a peri-nuclear localization, in contrast to the long tubular mitochondria observed in the cell body and along the processes of control neurons (Figure 3.7F). Furthermore, although cell death was not observed in the absence of Mcl-1, these neurons were more sensitive to exposure to a DNA damaging agent, camptothecin (10 μ M) than control cells (CTL neurons 25.15 \pm 3.0% survival vs Mut 8.9 \pm 1.7% survival) (Figure 3.8A, Supplemental Figure 3.1). These results suggest that Mcl-1 may be required to maintain mitochondrial integrity in neurons. In vivo impaired mitochondrial function may result in a gradual autophagic cell death as evident in Mcl-1 deficient brains.

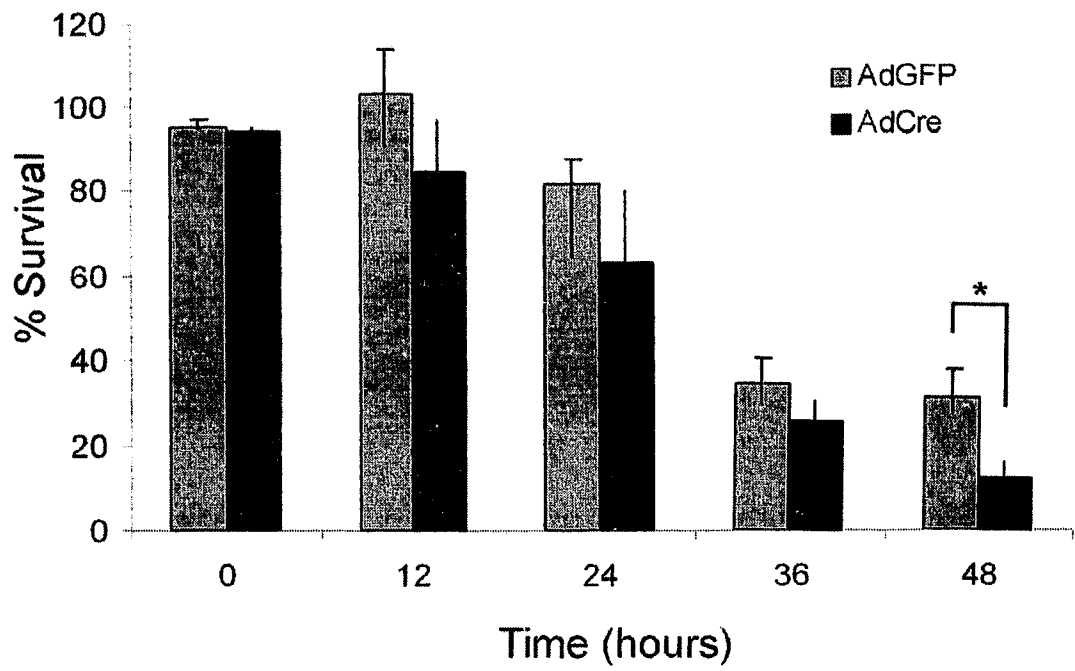
3.3.7 Mcl-1 plays an important role in injury induced neuronal cell death

It has been previously demonstrated that Mcl-1 is both cleaved by caspases (Herrant et al., 2004; Michels et al., 2004) and processed by the proteasome (Cuconati et al., 2003; Derouet et al., 2004; Nencioni et al., 2005; Nijhawan et al., 2003). With this knowledge, we asked if the same degradation pathways were maintained in neuronal cell death. We determined that inhibition of Caspases using 15 μ M QVD-OPh, a pan caspase inhibitor, and/or 5 μ M MG132, a proteasome inhibitor, in wild-type cortical neurons cultures challenged with the DNA damaging agent camptothecin (10 μ M), can stabilize Mcl-1 protein levels (Figure 3.8B) and rescue neurons from cell death (Figure 3.8C). This supports the involvement of these two processes in Mcl-1 degradation and cell death. In contrast, cortical neurons isolated from FoxG1 mutant mice, exhibit an increased sensitivity to DNA damage even when treated in combination with both caspases and proteasome

Figure 3.8. Loss of Mcl-1 in cortical neurons results in increased sensitivity to DNA damage induced cell death, and is due in part to Mcl-1 degradation by caspases and the proteasome. Cortical neurons were isolated from mutant or control mice, and treated with 10 μ M Camptothecin and collected at the times indicated. (A) Neuronal survival was measured by live/dead assay at the indicated times. Neuronal survival was determined at the indicated times by Live/Dead assay ($n = 3$). (B) Cortical neurons were isolated at E15.5 and treated with 10 μ M camptothecin as well as 5 μ M MG132 and/or 15 μ M Q-VD-OPh 48 hours post-infection. Proteins were collected from cultured cortical neurons at the times indicated following treatment and immunoblotted for Mcl-1 and then for actin as a loading control. (C) Cortical neurons were isolated at E15.5 and treated with 10 μ M camptothecin as well as 5 μ M MG132 and/or 15 μ M Q-VD-OPh 48 hours post-infection. Neuronal survival was measured by live/dead assay at the indicated times ($n = 3$). (D) Cortical neurons were isolated from mutant or control mice, and treated with 10 μ M camptothecin as well as 5 μ M MG132 and 15 μ M Q-VD-OPh 48 hours post-infection and data was collected at the times indicated. Quantitative analysis of neuronal survival was measured by live/dead assay at the indicated times ($n = 3$). * $P < 0.05$



Supplemental Figure 3.1. Loss of Mcl-1 in CGNs results in increased sensitivity to DNA damage induced cell death. (C) Wild type and Mcl-1^{fllox/fllox} CGNs were isolated at P7, infected at the time of plating with AdGFP or AdCre (MOI 75), and treated with 10 μ M camptothecin as well as 5 μ M MG132 and/or 15 μ M Q-VD-OPh 48 hours post-infection. Neuronal survival was assessed by Hoechst stained nuclei at the indicated times ($n = 3$) * $P < 0.05$.



inhibitors (CTL 75.2±6.25% vs. MUT 48.9±3.8% survival) (Figure 3.8D), indicating that the degradation of Mcl-1 by caspases and the proteasome is an important step in the initiation of cell death following a DNA damage inducing insult. These results suggest a role for Mcl-1 in maintaining neuronal survival against acute injury. Loss of Mcl-1 increases the sensitivity of PMNs to DNA damaging agents and the loss of Mcl-1 following a DNA damaging insult is in part a result of Mcl-1 degradation by caspases and the proteasome.

In these studies we have shown that Mcl-1 is required for neuronal maturation and survival *in vivo*. Loss of Mcl-1 results in different forms of cell death depending on neuronal maturity. In neuronal progenitors and immature neurons, loss of Mcl-1 results in apoptosis, whereas in post-mitotic neurons loss of Mcl-1 results in mitochondrial fragmentation and autophagic cell death, indicating that Mcl-1 sits at a critical juncture of these two pathways. Further that Mcl-1 is down-regulated in response to DNA damage-induced injury, and maintaining levels of Mcl-1 can rescue neurons. Loss of Mcl-1 also results in increased neuronal sensitivity to DNA damage insults. Mcl-1 is processed by caspases and the proteasome and that loss of Mcl-1 under injury conditions results in a *more rapid cell death*.

The results of these studies reveal that Mcl-1 is required for neuronal development and survival. Here we identify the first obligate requirement for a Bcl-2 family protein, Mcl-1, in neuronal development. Mcl-1 is also essential for maintaining the survival of post mitotic neurons in the cortex. Further, the presence of Mcl-1 protects against differing mechanisms of active cell death depending on the developmental state of the cells. Mcl-1 is required for the protection of neural progenitors and newly committed neurons from apoptosis, while PMN require Mcl-1 to inhibit autophagic cell death. Finally, we demonstrate that *in vitro*, Mcl-1 is down-regulated in response to DNA damage induced neuronal injury, and that maintaining its levels with the use of protease inhibitors or by viral-mediated

up-regulation can protect neurons against cell death. These studies identify a novel role for Mcl-1 in the regulation of autophagy and further implicate Mcl-1 as a prime therapeutic target for the protection of neurons against injury and degeneration.

3.4 Discussion

The involvement of Mcl-1 in apoptotic cell death has been well documented (as reviewed in (Craig, 2002; Michels et al., 2005), however there are still many questions regarding its role in the nervous system. Previous studies which demonstrated an increased sensitivity of heterozygous Mcl-1 neurons to injury-induced excitotoxic cell death suggested Mcl-1 has an important role in neuronal survival following injury, however, very little is known about its role in the development or survival of neurons. In this study, the striking cortical phenotypes of the three conditional mutant mice clearly demonstrate the importance of Mcl-1, an anti-apoptotic Bcl-2 family member, in cortical neurogenesis and postmitotic cortical neuron survival. The results of our studies support a number of conclusions. First, Mcl-1 is required for cortical development. Second, that Mcl-1 is required for the survival and maintenance of post-mitotic cortical neurons, and that in its absence, these neurons die through an autophagic mechanism. Third, we show that Mcl-1 is important in injury induced neuronal cell death and that under these conditions it is inactivated by caspase cleavage and proteasome degradation.

Previous studies using germline deletion of various Bcl-2 family members have found them to be largely dispensable for the development and survival of the CNS. Bcl-2 targeted deficiency studies suggest that Bcl-2 is expendable for development, however there is loss of peripheral nervous system (PNS) neurons in early post natal life, including motor, sensory and sympathetic neuron populations (Michaelidis et al., 1996). This suggests a requirement for Bcl-2 in the maintenance and survival of these post-mitotic populations. Bcl-xl germline knockout mice exhibit extensive neuronal cell death at E13.5 (Motoyama et al., 1995). This however may be a secondary effect, as has been seen in the past with germline knockouts of retinoblastoma protein (Ferguson et al., 2002). Neuron specific conditional knock out mice have shown that although Bcl-xl plays a role in neuronal development, it is dispensable for the development and survival of most neurons (Savitt et al., 2005). In these studies, Bcl-xl deletion was limited to catecholaminergic neurons, and although

they exhibit one third fewer catecholaminergic neurons than their control littermates, the mice go on to survive to adulthood healthy, active and with motor behavior similar to their littermate controls. Further, there is a large population of surviving cells lacking Bcl-xl immunoreactivity, which demonstrates that although Bcl-xl plays a role in the maintenance of certain catecholaminergic neurons it is not required for the survival of the entire population. Germline knockouts of either Bax, Bak or both of these pro-apoptotic multi-domained Bcl-2 family members show no severe neuronal abnormalities (Deckwerth et al., 1996; Lindsten et al., 2000; White et al., 1998). Although only 10% of Bax/Bak double knock out animals survive to adulthood, and have an accumulation of cells in the CNS (Lindsten et al., 2000), neither they, nor the individual knock outs exhibit an extreme phenotype, such as that seen in Caspase 3 or APAF-1 germline deletions, that one might expect if Bax, Bak or both were independently necessary for neuronal cell death (Kuida et al., 1996; Yoshida et al., 1998). The results of our studies demonstrate Mcl-1 to be independently essential for neuronal development and maintenance of homeostasis within the CNS. Loss of Mcl-1 early in neurogenesis leads to a defect in the maturation of cortical neurons, resulting in a dramatic thinning of the developing cortex, and ultimately embryonic lethality around E17. Work done previously by Oishi et al. suggested that Mcl-1 may play a role in the survival of NPCs through the Notch-1 pathway, in vitro (Oishi et al., 2004). Our results however, indicate that Mcl-1 is not required for the survival or proliferation of NPCs as the ventricular zones of the mutant mice are of identical thickness to those of their littermate controls, with similar amounts of cycling cells observed lining the ventricles. We do however; observe an increase in apoptotic cells that co-localize with Tuj1 and/or nestin positive cells. This suggests that Mcl-1 deficient cells are dying at or around the point of differentiation, prior to becoming mature neurons and migrating to the cortical plate. There does remain however a small proportion of cortical neurons that do survive, mature and migrate out in the absence of Mcl-1.

Although previous studies have shown that deletion of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xl in the nervous system results in modest losses in post mitotic neurons (Michaelidis et al., 1996; Savitt et al., 2005), none have been as dramatic as that observed in our studies with Mcl-1. Loss of Mcl-1 expression in PMN results in a rapid loss of these cells in mutant mice which is evident as early as P7, and becomes so severe that at their endpoint the animals essentially have hollow cortices. Further, as the neurons are lost there is no activation of the downstream effector caspase, caspase 3, suggesting that the cell death observed in these cells is not occurring through the classical apoptotic pathway. Studies by Rinkenberger et al., of Mcl-1 germline deficiency noted that they too were unable to detect increases in apoptosis in Mcl-1 null embryos, and that neither the addition of a p53 deletion or a Bax deletion was able to rescue the Mcl-1 mutant mice (Rinkenberger et al., 2000). The loss of neurons by non-apoptotic means suggests that Mcl-1 plays a gatekeeper role in more than one form of programmed cell death. This evidence of Mcl-1 involvement in more than one form of programmed cell death speaks to its properties as an oncogene and may prove to be an important factor in its oncogenic potential.

Both Bcl-2 and Bcl-xl have been found to regulate autophagic cell death, through the binding and inhibition of the autophagic protein Beclin-1 (Liang et al., 1998; Oberstein et al., 2007; Pattingre and Levine, 2006; Shimizu et al., 2004). Although loss of both Bax and Bak has been found to inhibit apoptotic cell death, it was unable to prevent cells from undergoing autophagy (Shimizu et al., 2004) These findings suggest that anti-apoptotic Bcl-2 family proteins also play a role in controlling non-apoptotic mechanisms of active cell death. In our studies, we demonstrate that loss of Mcl-1 results in the extreme deregulation of autophagy, resulting in significant losses of post-mitotic neurons and ultimately death of the animal. Our studies do not only identify Mcl-1 as a key anti-apoptotic during early stages of neuronal development, but also as an anti-autophagic protein in mature post mitotic neurons. This suggests that the severe phenotypes observed in these

conditional knock out mice occur as a result of the Mcl-1 protein being at the apex of two key active cell death pathways, and that its loss results in the severe deregulation of both.

The loss of developing and post mitotic cells observed *in vivo* in our novel CNS specific conditional Mcl-1 mutant mice shown here is very dramatic. Our *in vitro* data, however, shows that Mcl-1 null cortical neurons or CGNs are able to survive in culture as well as their wild type littermates. Further, in Mcl-1 null cells we observe an abundance of fragmented mitochondria with a perinuclear localization within the cell body, unlike the longer more filamentous mitochondria observed in control cells distributed throughout the processes. This phenotype is similar to that reported by Frieden et al following over-expression of hFis1, a gene involved in mitochondrial fission, in HeLa cells (Frieden et al., 2004). There is increasing evidence suggesting that mitochondrial fission is an early step leading to apoptosis (reviewed in (Perfettini et al., 2005; Scorrano, 2005; Youle and Karbowski, 2005)). The mitochondrial fission observed here however, does not correspond to an increase in apoptosis and further, cytochrome C is retained in mitochondria, in the absence of a cellular insult. It has been suggested that in some cases fragmentation of the mitochondrion prior to apoptotic stimuli may even play a protective role, by inhibiting the propagation of Ca²⁺ signaling (Szabadkai et al., 2004). Although there has been evidence of involvement of Bcl-2 family proteins in mitochondrial dynamics (Germain et al., 2005; Karbowski et al., 2002; Mathai et al., 2005), and Mcl-1 has been shown to play a role in mitochondrial Ca²⁺ signaling, more study is needed to further elucidate the role of Mcl-1 in the maintenance mitochondrial morphology (Minagawa et al., 2005). These results suggest a function for Mcl-1 beyond the regulation apoptosis within the cell.

Recent work by Mori et al. demonstrating increased neuronal sensitivity to seizures in Mcl-1 heterozygous mice (Mori et al., 2004), has prompted questions regarding the role of Mcl-1 in the regulation of injury induced neuronal apoptosis. This observed sensitivity suggests that Mcl-1 plays an important role in neuronal survival following insult; however, the mechanism by which this occurs

remains unknown. In contrast to the other anti-apoptotic Bcl-2 family members, Mcl-1 is very tightly regulated both transcriptionally and translationally (as reviewed by (Craig, 2002)). In DNA damage induced injury of primary neurons, we observe a rapid immediate down-regulation of both mRNA transcript (within 4 hours of camptothecin treatment) and protein levels. The mRNA down-regulation is immediate and complete and it is followed by a slow reappearance of the transcript over a 12 hour period; the decrease in Mcl-1 protein levels however, occurs more gradually. These divergent patterns suggest multiple levels of regulation of Mcl-1 in neurons occurring in response to the DNA-damage. By sustaining or increasing Mcl-1 protein expression we were able to maintain significant protection against neuronal cell death. Neurons deficient in Mcl-1 also show increased sensitivity to the DNA damaging agent camptothecin, further supporting its importance in the maintenance of neuronal survival.

Although there is increasing evidence of the post translational regulation of Mcl-1 in the literature, if or how this regulation occurs in the CNS has yet to be described. Here we show that in neurons, caspase cleavage and proteosomal degradation of Mcl-1 play an important role in its inactivation following injury. By treating cortical neurons with Q-VD-Oph, a pan-caspase inhibitor, and/or MG132, a proteasome inhibitor, we were able to stabilize Mcl-1 protein levels, and provide increased protection against DNA damage induced neuronal cell death. Further, we found that cortical neurons deficient in Mcl-1 are more sensitive to DNA damage injury even in the presence of both caspase and proteasome inhibitors, suggesting that Mcl-1 may be playing an important role in maintaining survival, providing the opportunity for cellular repairs to take place. These results highlight the importance of Mcl-1 in injury induced neuronal cell death, and support our *in vivo* observations that Mcl-1 plays an important role in the regulation of apoptosis.

The results of our studies show a novel requirement for Mcl-1 in the development and maintenance of neurons within the CNS. Our *in vivo* results demonstrate that Mcl-1 is required for

cortical neurogenesis and the survival of postmitotic neurons. Furthermore, our data show Mcl-1 regulates both apoptotic and autophagic modes of cell death, and is an important player in the regulation of cell death in neuronal injury. Our results implicate Mcl-1 as playing a critical role at the apex of both apoptotic and non-apoptotic cellular processes, and demonstrate an absolute requirement for Mcl-1 in neuronal survival.

3.5 Materials and Methods:

3.5.1 Mice and primary neuronal cultures

For embryonic time points, the time of plug identification was counted as embryonic (E)day 0.5. All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

Generation of Transgenic Mice

Floxed Mcl-1 mice were previously described (Opferman et al., 2003). The generation of telencephalon-specific Mcl-1 conditional mutants was accomplished by breeding floxed Mcl-1 mice with FoxG1-cre mice (Hebert and McConnell, 2000), to generate FoxG1^{cre/+}; Mcl-1^{flox/flox} mice. The generation of nestin specific conditional mutants, floxed Mcl-1 mice were crossed with Nestin-cre mice (above), to generate Nestin^{cre/+}; Mcl-1^{flox/flox} mice. The generation of the CamKII α conditional mutants, floxed Mcl-1 mice were crossed with CamKII α -cre mice (Casanova et al., 2001), to generate CamKII α ^{cre/+}; Mcl-1^{flox/flox} mice. The genotyping of these mice was performed as described previously (Casanova et al., 2001; Hebert and McConnell, 2000; Opferman et al., 2003). Mice were maintained on FVB/N and C57/BL6 mixed genetic backgrounds and littermates were used in all experiments. Cortical and cerebellar granule neurons (CGNs) were cultured as described previously (Cregan et al., 1999; Fortin et al., 2001).

3.5.2 Tissue Processing and Immunohistochemistry

Tissue fixation, and cryoprotection of embryonic tissue was performed as described previously (Ferguson et al., 2002). Pups at postnatal days 1, 7 and 14 were killed by lethal injection of sodium pentobarbital and perfused with 10 mM phosphate buffered saline (PBS) followed by cold 4% paraformaldehyde (PFA) in PBS (pH 7.4). Brains were dissected and post-fixed overnight in 4% PFA, cryoprotected in a sucrose gradient of 12, 16 and 22% sucrose (Sigma) in PBS. Tissue was frozen, and 14- μ m coronal cryosections were collected on *Superfrost Plus*TM slides (12-550-15; Fisher Scientific). Sections were stained in 0.1% cresyl violet for light microscopy/ Immunohistochemistry was done as described previously (Ferguson et al., 2002) for active caspase 3 (BD Pharmingen, 559565, 1:100), phosphohistone 3 (PH3) (Upstate Biotechnology, 06-570, 1:500), nestin (mouse monoclonal, 1:200; RDI, 21714), β III tubulin (Dave Brown; Mouse Monoclonal, 1:100), PSA-NCAM (MAB5324 Chemicon; Mouse Monoclonal 1:1000).

3.5.3 Electron microscopy

At postnatal day 14, mice were euthanized and transcardially perfused with 10mL of ice-cold 1xPBS pH 7.4 followed by a 20mL solution of 2.5% glutaraldehyde and 0.5% paraformaldehyde in phosphate buffer (pH 7.4). Brains were removed and cortices were cut into 1.5mm blocks, post-fixed overnight in 2.5% glutaraldehyde. Tissue was embedded in Epon following standard electron microscopy procedures. Ultra-thin sections were collected on copper grids and stained in 2% uranyl acetate/lead citrate and viewed with on a transmission electron microscope.

Thin sections were cut with a Leica Ultracut E ultramicrotome and counterstained with lead citrate and uranyl acetate. Digital images were taken using a JEOL 1230 TEM at 60 kV adapted with a 2K \times 2K bottom mount CCD digital camera (Hamamatsu, Japan) and AMT software.

3.5.4 Cell Culture and Recombinant Adenovirus Infection

Cortical and cerebellar granule neurons (CGNs) were cultured as described previously (Cregan et al., 1999; Fortin et al., 2001). The cDNA for Mcl-1(mouse) (Rinkenberger et al., 2000) was a gift from Dr. S. Korsmeyer. Recombinant adenoviral vectors carrying expression cassettes for Mcl-1 or Cre were constructed, purified, and titered as previously described (Cregan et al., 2000). Adenoviral vectors were added to cell suspensions immediately before plating. Multiplicity of Infection (MOI) refers to the number of plaque forming units (pfu) per cell, as calculated from viral titres (pfu/ μ l).

3.5.5 Semiquantitative RT-PCR analysis

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

3.5.6 Western Blot Analysis and Immunostaining

Western blot analysis was performed as described previously (Cregan et al., 1999) with antibodies against Mcl-1 (1:10000, Rockland Immunochemicals Inc.) (Opferman et al., 2005; Opferman et al.,

2003), Beclin 1 (Sc-10087 Santa-Cruz; Goat Polyclonal 1:200) and Actin (SC-1616, Santa Cruz Biotechnologies 1:100) as a loading control. For immunocytochemistry cells were fixed with 4% PFA in PBS for 30 minutes, permeabilized with 0.2% TritonX-100 (100ml; 789704; Boehringer. Mannheim, Indianapolis, IN) in PBS, washed with PBS and stained for GFP (Abcam Ab6556, 1:500), Cytochrome C (BD Pharmingen 556433, 1:500) or Map2 (SC-20172 Santa-Cruz; Rabbit Polyclonal 1:100). Cells were washed with PBS and then incubated for 1 hr with Alexa 488- and/or Alexa 594-conjugated secondary antibodies (Molecular Probes).

3.5.7 Camptothecin Treatment and Cell Viability Assays

For camptothecin treated cells, neurons were treated with 10 μ M camptothecin in ddH₂O with or without 15 μ M Quinoyl-valyl-O-methyl-aspartyl-[2,6-difluorophenoxy]-methyl ketone (Q-VD-OPh) (Enzyme System Products, Livermore, CA) in DMSO and/or 5 μ M MG132 (5mg; C2211; Sigma) in DMSO after 2 days *in vitro* (DIV). Cell survival was measured by the following: LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, OR) as previously described (Cregan et al., 2004), and Hoechst staining. At the times indicated, neuronal viability was determined using live/dead staining following manufacturer's instructions. For Hoescht counts, cells were fixed with 4% PFA in PBS for 30 minutes, then washed with PBS and counterstained with Hoechst. For Live/Dead assays and Hoescht counts, data represents the mean and SD from a minimum of three independent experiments.

Chapter 4: General Discussion

The Bcl-2 family proteins have been long thought to play a pivotal role in the apoptotic process. There are still many questions however regarding their importance in the nervous system. The lack of obvious phenotype demonstrated in most murine germline knockouts of individual Bcl-2 family members has lead to questions regarding the individual necessity of these proteins for the apoptotic process. To look into this matter more closely we elected to study the roles of Bcl-2 family members in a neuronal cell death paradigm, focusing on those Bcl-2 family proteins found to be involved in p53-mediated neuronal injury. The goal of this research is to demonstrate the importance of Bcl-2 family members Noxa, Puma and Mcl-1 in neuronal cell death and injury. The results presented in this dissertation support a number of conclusions: 1) that pro-apoptotic BH3-only protein Puma is required for neuronal cell death, but that Noxa, another pro-apoptotic BH3-only family member is not; 2) that the anti-apoptotic Bcl-2 family protein Mcl-1 is required for neuronal development, survival and neuronal cell death of both developing and post-mitotic neurons; 3) we also identify a novel role for Mcl-1 in the regulation of autophagic cell death.

4.1 p53-mediated neuronal cell death

P53 involvement in the regulation of neuronal cell death in neuronal injury and neurodegenerative disorders has been well studied (reviewed in Chapter 1: General Introduction). Despite its importance in neuronal cell death paradigms, many questions remain as to the exact mechanism by which p53-mediates neuronal cell death. In these studies, it was established that p53 activation domain 1 (AD1) is required for the transcriptional up-regulation of Puma following DNA damaging induced injury, and that loss of PUMA is sufficient to protect cells from DNA-damage injury. Although there is currently ample evidence indicating a role for p53 directly at the mitochondria, at the time of publication we were unable to find any evidence of this in our cell types by immunofluorescence, and were able to demonstrate that loss of transcriptional activation of p53 in

CGNs was sufficient to protect cells from DNA-damage induced injury. Recently PUMA has been shown to couple the nuclear and cytoplasmic pro-apoptotic functions of p53, through the ability of the PUMA protein to physically disrupt the interaction between Bcl-xl and p53, freeing p53 to activate Bax, and the apoptotic pathway, in MEFs (Chipuk et al., 2005). Here the authors demonstrated that loss of PUMA uncoupled these two pathways and rendered cells resistant to DNA damage induced cell death through the sequestration of p53 by Bcl-xl (upto 72 hours), but that this effect could be titrated out by exposing the cells to a second dose of the DNA-damaging UV irradiation injury (Chipuk et al., 2005). Further, deletion of p53-binding sites on the PUMA promoter region in colorectal cancer cells protected these cells from DNA-damage induced cell death, but not other stimuli (Wang et al., 2007). Although these studies only looked at the protection of cells up to 48 hours following treatment, the results further emphasize the importance of the transcriptional role of p53, and the up-regulation of PUMA for DNA-damage induced cell death paradigms. The evidence demonstrating a transcriptional independent role for p53 in neurons, suggests that although no p53 was observed at the mitochondria in the presented work, a small fraction of it may have been present but undetectable by immunofluorescence (Akhtar et al., 2006b; Dunys et al., 2007; Endo et al., 2006; Geng et al., 2007; Nair et al., 2006). The current literature however, continues to corroborate our findings, identifying PUMA as an important target for up-regulation, even under circumstances where p53 also play transcription independent roles (Chipuk et al., 2005), it is possible that there were direct protein interactions involving p53, within the cytosol or at the mitochondria of our cells that went undetected. In this case, cell death may still have been observed had we taken the time course out further, potentially allowing for the transcriptional up-regulation of the p53 protein to titrate out any interactions with anti-apoptotic Bcl-2 family proteins, resulting in apoptosis. In order to state that these exist, a transcriptional requirement for p53 in this DNA-damage induced neuronal cell death paradigm, further experiments would be required to unequivocally demonstrate a lack of p53 in the

cytosol or at the mitochondria following DNA damage stimulus, perhaps through cell fractionation, and/or immunoprecipitation experiments.

The recent generation of a transcriptionally-deficient p53 mutant mouse (QS) does however corroborate the notion of a requirement for p53 transcriptional activity following DNA damage induced cell death. Cells isolated from these mice are unable to activate apoptosis in response to double stranded DNA-damage injury (doxorubicin), or single stranded DNA-damage induced injury (UV-C irradiation), but remain able to activate apoptotic responses to other p53-mediated stimuli (Johnson et al., 2005). Regardless of an absolute requirement, the transcriptional role of p53 does however remain significantly important, if not essential, in DNA-damage induced neuronal cell death.

4.2 The roles of Noxa and Puma in neuronal cell death

The results of the studies presented here, suggest an important role for the up-regulation of PUMA for p53-mediated cell death. We do not however, find that Noxa, another BH3-only Bcl-2 family protein up-regulated by p53, plays an important role in DNA-damage induced cell death in CGNs, regardless of its transcriptional activation. Other groups have however found Noxa to be important in other neuronal cell death paradigms, specifically in axotomy induced motor neuron death (Kiryu-Seo et al., 2005). Noxa has also been found to be important in the regulation of neural precursor cell death (Akhtar et al., 2006a). These results support the theory of tissue specific functions among BH3-only family proteins, as well as the model of BH3-only proteins as potent and weak, context dependant, cell death inducers. In neurons, as in other systems, as Noxa could be characterized as a context specific inducer of apoptosis in that it is important in motor neuron injury as well as in the apoptosis of neural precursor cells, but expendable for cell death induced by DNA-damage in cerebellar granule neurons and sympathetic neurons (Akhtar et al., 2006a; Cregan et al., 2004; Kiryu-Seo et al., 2005; Wytenbach and Tolkovsky, 2006). Puma on the other hand is a potent inducer of cell death in many cell types including neurons, resulting in a strong cell death stimulus in

cerebellar granule neurons, sympathetic neurons as well as neural precursor cells (Akhtar et al., 2006a; Cregan et al., 2004; Wyttenbach and Tolkovsky, 2006). Further, Noxa mediated cell death has been found to exhibit co-operativity with BIK or BAD (Chen et al., 2005; Germain et al., 2002). These data sets support the theory that Noxa exhibits tissue specific pro-apoptotic roles, and that it is a weakly apoptotic protein able to co-operatively induce more cell death under certain circumstances. Taken together these results indicate that in neurons, the up-regulation of PUMA by p53 in neurons results in the potent induction of apoptosis. Noxa on the other hand is dispensable for p53-mediated cell death in CGNs, however, it has been found to be apoptotic within certain neuronal cell types.

4.3 The role Mcl-1 in neuronal development

Although the role of Bcl-2 family proteins in apoptosis is well established, the fact that few of the germline knockouts of the Bcl-2 family proteins exhibit any identifiable developmental phenotype suggests that there is little individual requirement for, or significant redundancy among Bcl-2 family proteins, in developmental apoptosis. Bcl-xl and Mcl-1 are the only exceptions to this, with Bcl-xl mice displaying embryonic lethality at E13, with extensive tissue damage in the hematopoietic compartment and the nervous system (Motoyama et al., 1995), and Mcl-1 deficient mice being peri-implantation lethal (Rinkenberger et al., 2000). Conditional mutants of Bcl-xl however have shown it to be largely dispensable for neuronal development and survival (Savitt et al., 2005). Mcl-1 is the only member of the Bcl-2 family to display an absolute importance in development. Not only are the germline knockout peri-implantation lethal, suggesting an important role in early embryonic development, but targeted Mcl-1 deletions have further shown Mcl-1 to play an obligate role in early hematopoieses and development of the immune system (Opferman et al., 2005; Opferman et al., 2003). The data presented here identifies a novel role for Mcl-1 in the developing CNS. In two separate targeted deletion models of neuronal development, one in the developing telencephalon, under the control of the FoxG1 promoter (Hebert and McConnell, 2000), and the second in neuronal

progenitor cells under the control of the nestin promoter (Berube et al., 2005), we show that the presence of Mcl-1 is absolutely required for the proper development of the cortical plate. The increase in apoptosis observed also suggests that this may be occurring through deregulation of apoptosis as a result of the loss of Mcl-1 in developing neurons. This makes Mcl-1 the only Bcl-2 protein to be required for proper development of the CNS, with no other conditional or germline deficient mutants showing such a severe phenotype. It is clear from this work that Mcl-1 plays an obligate role in CNS development, and although this is likely due in large part to its role as an anti-apoptotic protein, this does not negate the possibility that Mcl-1 may also play other roles in the control of cell survival and the maintenance of cellular homeostasis.

4.4 The role of Mcl-1 in the survival of post-mitotic neurons

In addition to its role in CNS development, this data further identifies Mcl-1 as necessary for the maintenance of post-mitotic neuronal populations. Excision of Mcl-1 in post mitotic neurons, at post-natal day 0 (P0), results in a slow progressive loss of post-mitotic neurons, through an autophagic cell death mechanism. Although both Bcl-2 and Bcl-xl have also been implicated in autophagic regulation via binding of Beclin-1, loss of either of these proteins does not result in the extreme autophagic phenotype witnessed in mice where Mcl-1 is conditionally deleted in post-mitotic neurons as seen here (Michaelidis et al., 1996; Motoyama et al., 1995; Pattingre et al., 2005; Shimizu et al., 2004). Loss of Bcl-2's ability to bind to Beclin-1 does however result in over-active autophagy in certain cell types, leading to the theory that the Bcl-2 family of proteins may be involved in a second cell death rheostat, one controlling autophagy (Pattingre and Levine, 2006; Pattingre et al., 2005). This rheostat theory does not seem to require the activity of pro-apoptotic proteins Bax and Bak however, as studies show that cells deficient in both of these proteins are resistant to apoptosis, yet still undergo autophagic cell death (Shimizu et al., 2004). In these studies, the authors were also able to show that this autophagic cell death could be further modulated through the manipulation of

expression levels of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xl. Contrary to our findings however, in MEFs, they found that over-expression of either protein resulted in increases in autophagic cell death, while loss of Bcl-xl, but not Bcl-2, was able to decrease levels of autophagic cell death in Bax/Bak deficient cells (Shimizu et al., 2004). These results also contrast with findings from the Levine lab that show that Bcl-2 inhibits starvation induced autophagy in mouse cardiac muscles, and that Beclin-1 mutants that are unable to bind to Bcl-2 show increased autophagic activity (Pattingre et al., 2005). Pattigre et al. also found that Bcl-2 localized to the ER, but not to the mitochondria was able to inhibit autophagy, which suggests that the regulation of autophagy may occur independently of the mitochondria. The Bcl-xl protein can be localized to the cytosol, MOM inserted or loosely associated to the MOM and although its absence from integration into the ER does not preclude its involvement in autophagy, it may indicate a different role to that of either Mcl-1 or Bcl-2 as may be further substantiated by the differences in their autophagic involvement observed by the Tsujimoto lab (Schinzel et al., 2004; Shimizu et al., 2004). Although Mcl-1 is predominantly associated with mitochondrial functions, Mcl-1 is also ER associated; whether it's role in autophagic cell death is ER specific however remains to be determined (Schinzel et al., 2004).

The data presented here not only identify a novel role for Mcl-1 in the autophagic cell death pathway, but also demonstrate that the roles of Mcl-1 in apoptosis and autophagy are able to be dissociated. We see that under certain circumstances, i.e. neuronal development that loss of Mcl-1 results in a default apoptotic pathway, whereas; in post-mitotic neurons loss of Mcl-1 results in cell defaulting to autophagy. More work will be necessary to determine what cellular cues prompt some cells lacking Mcl-1 to default to apoptosis while others default to autophagic forms of cell death.

4.5 The role of Mcl-1 in autophagy: far reaching implications in the regulation of cell death

Mcl-1 was identified as a gene activated in the human monocytic cell line ML-1, and found to have sequence similarity to Bcl-2 (Kozopas et al., 1993). Elevated levels of Mcl-1 in leukemia cells

correlate with their resistance to chemotherapeutic drugs (Kaufmann et al., 1998), and Mcl-1 transgenic mice exhibit a high incidence of B-cell lymphomas (Zhou et al., 2001). Although a great deal of research indicates an important role for Mcl-1 in cancer, this role has been predominantly associated with its ability to inhibit apoptosis (Craig, 2002; Kitada and Reed, 2004; Le Gouill et al., 2004). In this dissertation, I describe a previously unknown role for Mcl-1 in autophagic cell death. The involvement of autophagy in cancer is not a new concept (reviewed in (Botti et al., 2006)). Nor is the involvement of anti-apoptotic Bcl-2 family members in either cancer or autophagic cell death (Pattingre and Levine, 2006). The findings presented here, in co-ordination with the existing research, provide additional insight into the oncogenic properties of anti-apoptotic Bcl-2 family members. In the absence of available apoptotic pathways, cells appear to default to alternate active or passive cell death pathways which may be dependant on the available energy stores (Kiffin et al., 2006; Skulachev, 2006; Vandenabeele et al., 2006). In the case of cancer, autophagy is able to function as a tumor suppressor, in conjunction with apoptosis, through the restriction of necrosis and inflammation that is associated with accelerated tumor growth (Degenhardt et al., 2006). Loss of autophagy alone can result in increased tumorigenesis as seen in Beclin-1 heterozygous mice (Qu et al., 2003). The intersection of these two pathways at the point of anti-apoptotic Bcl-2 family proteins may help to explain the impressive oncogenic properties observed in this family of proteins and help to further elucidate important therapeutic targets for the treatment of this debilitating and often fatal disease.

4.6 Mcl-1 and mitochondrial fission?

Another striking observation made in the course of our research into the role of Mcl-1 in neuronal cell death, has been that cortical neurons lacking Mcl-1 display a mitochondrial fragmentation and perinuclear clustering similar to that observed in HeLa cells over-expressing hFis-1 (Frieden et al., 2004). Work done by other groups has implicated Bcl-2 family member involvement in fission/fusion

activities; however Mcl-1 has never before been studied in this context. Pro-apoptotic Bax and Bak have been shown to localize at mitochondrial scission sites co-localized with DRP1 (Karbowski et al., 2002). Over expression of Bax is sufficient to induce apoptosis, which proceeds through a mitochondrial fission step and can be inhibited by a dominant negative form of DRP1 (Frank et al., 2001). Inhibition of mitochondrial fission pathway however is not sufficient to inhibit Bax/Bak dependant cell death (Parone et al., 2006). Although Both Bcl-2 and Bcl-xl have been shown to inhibit apoptosis mediated by excessive fission, it is difficult to determine at what stage this inhibition takes place and what, if any, role these proteins are playing in the fission/fusion process (James et al., 2003; Kong et al., 2005). Further, although our studies do show increased susceptibility of Mcl-1 deficient cells to undergo DNA-damage induce cell death; untreated cells do not undergo spontaneous cell death even though they exhibit fragmented mitochondria. It is possible to hypothesize that anti-apoptotic Bcl-2 family proteins may regulate intracellular calcium stores both at the mitochondria and at the ER, and not necessarily modulate cell death through the direct interaction with the fission/fusion machinery of the cell (Baffy et al., 1993; Chen et al., 2004; Oakes et al., 2005; Pinton et al., 2000; Pinton et al., 2002; Pinton and Rizzuto, 2006; White et al., 2005). Apoptosis dependant on ER-mediated calcium release, mitochondrial calcium uptake and mitochondrial fusion regulated by DRP1 has been previously observed (Breckenridge et al., 2003b; Germain et al., 2005). The BH3-only protein BIK was shown, under these circumstances, to act at the ER to induce cell death through the release of ER calcium stores (Germain et al., 2005). It is conceivable that this occurs through the ability of BIK to antagonize anti-apoptotic Bcl-2 family proteins, thus disrupting their ability to regulate intracellular calcium homeostasis. Further, cells in which mitochondrial fission has taken place prior to the cellular insult (by over-expression of the DRP1 protein), were significantly protected from the propagation of the signal resulting from the release of ER calcium stores, suggesting an important role for the mitochondria in ER-mediated cell death (Szabadkai et al., 2004).

These results however do not explain why cells lacking Mcl-1 would exhibit this particular mitochondrial phenotype. The only 2 other Bcl-2 family proteins shown to have any direct effect on the mitochondrial structure include tBID and BIK (Germain et al., 2005; Mathai et al., 2005; Scorrano et al., 2002). It is difficult to infer a role for Mcl-1 from these studies however as BH3-only proteins share only the BH3 domain in common with Mcl-1, and are otherwise unique. Furthermore, the cristae remodeling activity of tBID occurs independent of its BH3 domain (Scorrano et al., 2002). Further, BIK mediated mitochondrial remodeling has been shown to be dependant on calcium signaling occurring between the ER and the mitochondria resulting, in a cell death signal that precedes the mitochondrial fission (Germain et al., 2005; Mathai et al., 2005). In our studies, however, fission is observed in the absence of apoptosis. There has been some evidence of Mcl-1 involvement in calcium signaling at the mitochondria, it is unclear whether this would have any effect on mitochondrial dynamics (Minagawa et al., 2005). Although the exact role of Bcl-2 family proteins in the interplay between ER and mitochondrial apoptosis, and the role mitochondrial fusion plays in this dynamic transaction, is unclear; these data suggest a thoroughly integrated series of interconnected pathways that communicate with one another through intracellular signals, each of which is intimately sensitive to perturbations in cellular homeostasis.

4.7 Mcl-1 in p53 mediated neuronal cell death

Mcl-1 plays an important role in p53-mediated neuronal cell death. Its immediate down-regulation following DNA damage induced injury in neurons suggests an important role in the mediation of neuronal survival. Although Mcl-1 deficient cells are more susceptible to DNA damage induced neuronal injury, it is not quite as significant as one might have expected based on the *in vivo* data presented. There is a possibility however that the increased sensitivity of these cells is masked by the fact that the mitochondria in Mcl-1 deficient cells exhibit fragmentation and as such may be less susceptible to the amplification of the apoptotic signal that might otherwise occur through the

mitochondrial network. Mitochondrially propagated waves of depolarization have been found to play an important role in the distribution of the apoptotic signal, and the maintenance of mitochondrial network integrity allows for the efficient propagation of these signals (Pacher and Hajnoczky, 2001; Szabadkai et al., 2004). Thus the fragmented state of the mitochondrial network in the Mcl-1 deficient cells may be result in decreasing efficiency of the apoptotic signal propagation through the mitochondrial network of these cells, as these cells display an obvious phenotype *in vivo*.

Mcl-1 is highly post-translationally regulated (reviewed in (Craig, 2002)). This tight post translational regulation provides the cell with a brief window of opportunity for intracellular damage control, while inhibiting the potential for oncogenesis. In these studies I demonstrate the ability of caspase inhibitors coupled with proteasome inhibition to protect cortical neurons form DNA damage induce stimuli. This protection is no longer effective in Mcl-1 deficient cells, supporting the model that Mcl-1 is required in these cells to increase their window of viability allowing cellular repair processes to take place. If the cell is unable to repair itself within the fixed time, Mcl-1 is degraded and the cell undergoes apoptosis and/or autophagy.

4.8 Significance of Research

Neuronal injury and neurodegenerative diseases such as stroke, AD, PD and HD have a significant impact, particularly amongst the growing aging population. Research aimed at identifying the molecular mechanisms involved in the progression of these neurodegenerative disease states will allow us to identify potential therapeutic targets for their treatment and prevention. Although many clinical trials relating directly to stroke research have been done, and many are currently underway, only 1 of 178 therapies in clinical trial has actually been adopted in the treatment of stroke injury (Cheng et al., 2004; Kidwell et al., 2001). Lack of success at the level of clinical trials further emphasizes the need for continued research and innovation in the identification of the key factors involved in neuronal injury and degenerative diseases.

The research presented in this dissertation underlines the importance of Bcl-2 family members in p53-mediated neuronal cell death, and further, highlights a requirement for Mcl-1 in the development and survival of the CNS. The involvement of p53 has been implicated in many neurodegenerative diseases and as such, recognizing its transcriptional targets and method of activity has become the focus of much research. Here I show that the regulation of Bcl-2 family members is important in p53 mediated neuronal cell death. This research demonstrates that the transcriptional up-regulation of the pro-apoptotic BH3-only protein PUMA is important for the regulation of neuronal cell death. Further, although BH3-only protein PUMA plays a key role in neuronal cell death, Noxa, another BH3-only protein transcriptionally up-regulated by p53, is expendable in this process.

I also show that Mcl-1 is the only multi-domain Bcl-2 protein found to be differentially regulated following p53-mediated neuronal injury. This discovery prompted further investigation into its role in neuronal survival and cell death. Not only does loss of Mcl-1 sensitize cells to DNA damage injury, it is also required *in vivo* for the proper development of the CNS and the survival of post mitotic neurons. It is clear that Mcl-1 plays multiple roles in various cell death processes, including apoptosis as seen in its requirement for neuronal development, and also autophagic cell death seen in its requirement for the survival of post-mitotic neurons. This research has implications; not only in the field of neuroscience, but in cellular biology as a whole, as it identifies a novel role for Mcl-1 in autophagy, that can be differentiated from its role in apoptosis depending on the circumstances. These results potentially identify an important crossroad in the regulation of apoptotic and autophagic cell death pathways.

- Responsible for high level technical support
- Involved in human resource management

Winter 2004

Laboratory Demonstrator/Marker, 1st Organic CHM1320

Department of Chemistry, University of Ottawa, Ottawa, ON

- Assisted and supervised weekly laboratory sessions
- Answered student questions and demonstrated laboratory equipment/techniques
- Evaluating laboratory performance
- Marked weekly lab reports
- Proctored final examinations

Fall 2003-2004

Teaching Assistant, 1st Year General Chemistry CHM1310

Department of Chemistry, University of Ottawa, Ottawa, ON

- Prepared and presented weekly tutorial session
- Answered student questions regarding course material
- Marked Midterm and final examinations
- Held additional exam preparation tutorial sessions
- Proctored final examinations

Fall 2000-2002

Laboratory Demonstrator/Marker, 1st Year General Chemistry CHM1310

Department of Chemistry, University of Ottawa, Ottawa, ON

- Assisted and supervised weekly laboratory sessions
- Answered student questions and demonstrated laboratory equipment/techniques
- Evaluating laboratory performance
- Marked weekly lab reports
- Proctored final examinations

Winter 2002
BCH 3

Laboratory Demonstrator/Marker, 3rd Year Physical Biochemistry

Department of Chemistry, University of Ottawa, Ottawa, ON

- Assisted and supervised weekly laboratory sessions
- Answered student questions and demonstrated laboratory equipment/techniques
- Evaluating laboratory performance
- Prepare marking scheme for laboratory reports
- Marked weekly lab reports

Winter 2001

Laboratory Demonstrator/Marker, 2nd Year Biochemistry Lab BCH 2

Department of Chemistry, University of Ottawa, Ottawa, ON

- Assisted and supervised weekly laboratory sessions
- Answered student questions and demonstrated laboratory equipment/techniques
- Evaluating laboratory performance
- Prepare marking scheme for laboratory reports
- Marked weekly lab reports

Peer Reviewed Publications

A. Fortin, J. G. MacLaurin, N. A. Arbour, S. P. Cregan, N. Kushwaha, S. M. Callaghan, D. S. Park, P. R. Albert and R. S. Slack (2004) THE PROAPOPTOTIC GENE SIVA IS A DIRECT TRANSCRIPTIONAL

TARGET FOR THE TUMOR SUPPRESSORS P53 AND E2F1 - Journal of Biological Chemistry, 279(27):28706-14.

*My contribution was to carry out some of the cell culture work, and to perform some *in situ* hybridization work that was not included in the manuscript. This amounted to 20% of the total experimental work.

S. P. Cregan^Y, **N. A. Arbour^Y**, J. G. MacLaurin, S. M. Callaghan, A. Fortin¹, E. C. C. Cheung, A. E. Mackenzie, D. S. Park and R. S. Slack (2004) P53 ACTIVATION DOMAIN 1 IS ESSENTIAL FOR PUMA UPREGULATION AND P53-MEDIATED NEURONAL CELL DEATH – Journal of Neuroscience. 2004 Nov 3;24(44):10003-12.

^YThese authors contributed equally to this work

*My contribution was to carry out 50% of the experimental work, including cell culture and all of the Noxa and Puma data except for the final Puma deficiency data. I completed 100% of the writing of the text with help with revisions from the co-authors.

Selected Abstracts

Arbour, N. A., C. Coffill, D. Barnes, and A. E. MacKenzie (2001)
Neuronal Apoptosis Inhibitory Protein (NAIP) Expression and its Effects on Cell Cycle Progression. Canadian Genetic Diseases Network, St Sauveur, Quebec. April 2001

Arbour, N. A., S. P. Cregan, J. G. MacLaurin, S. M. Callaghan, A. E. MacKenzie, D. S. Park and R. S. Slack (2002)
P53 Up-regulates Noxa, a BH3 only member of the Bcl-2 Family, which Promotes Neuronal Cell Death in a p53 and Bax Dependant Fashion. Cold Spring Harbor Laboratory-Cancer Genetics and Tumor Suppressors Meeting, Cold Spring Harbor, NY. August 2002

Arbour, N. A., S. P. Cregan, J. G. MacLaurin, S. M. Callaghan, A. E. MacKenzie, D. S. Park and R. S. Slack (2003) P53 Up-regulates Noxa and PUMA, BH3 only members of the Bcl-2 Family, Promoting Neuronal Cell Death in a p53 and Bax Dependant Fashion. Keystone Symposia--Molecular Mechanisms of Apoptosis (B6), Fairmont Banff Springs Hotel, Banff, Alta. February 8th-13th 2003

Arbour, N. A., S. P. Cregan, J. G. MacLaurin, S. M. Callaghan, D. S. Park, A. E. MacKenzie and R. S. Slack (2003) Involvement of BH3 only members of the Bcl-2 Family in p53-Mediated Neuronal Cell Death and Ischemic Injury. Society for Neuroscience, New Orleans, LA. November 8th – 12th, 2003 USA

Arbour, N. A., S. P. Cregan, E.C.C. Cheung, J. G. MacLaurin, S. M. Callaghan, D. S. Park and R. S. Slack (2004) Involvement of Mcl-1, an Anti-Apoptotic Member of the Bcl-2 Family, in Neuronal Cell Death. Society for Neuroscience, San Diego, CA. October 23rd-27th, 2004, USA

Arbour, N.A., G. Iyirhiaro, E.C.C. Cheung, J.G. MacLaurin, S.M. Callaghan, D. S. Park, and R. S. Slack (2005)
Involvement of Mcl-1, an Anti-Apoptotic Member of the Bcl-2 Family, in Neuronal Cell Death. Cellular Senescence and Cell Death. Keystone, CO, Mar 3rd-9th 2005

Arbour, N. A., G.O. Iyirhiaro, E.C.C. Cheung, J. G. MacLaurin, S. M. Callaghan, D. S. Park and R. S. Slack (2005)

Mcl-1 Protects Against Neuronal Cell Death through Interactions with BH3-Only Proteins. Cold Spring Harbor Laboratories Programmed Cell Death Meeting, Cold Spring Harbor, NY. September 21st-25th, 2005

Arbour, N. A., G.O. Iyirhiaro, E.C.C. Cheung, J. G. MacLaurin, D. S. Park and R. S. Slack (2006) Differential Regulation of Mcl-1 Occurs in DNA Damage and Hypoxic Models of Neuronal Cell Death, 6th International Cell Death Symposium on "The Mechanisms of Cell Death", Angra Dos Ries, Brazil. June 2nd-5th, 2006

Arbour, N.A., E.C.C. Cheung, J.L. Vanderluit, J.G. MacLaurin, D. S. Park, J. T. Opferman and R. S. Slack (2006) Mcl-1 is Required for Neuronal Survival, 14th Euroconference on Apoptosis "Death or Survival? Fate in Sardinia.", Chia, Sardinia, Italy, September 29-October 4, 2006

Community and Volunteer Service

University of Ottawa, Enrichment Mini-Courses-Department of Biochemistry 2000

- Lead a mini-course activity aimed at teaching students to understand DNA coding
- Showed them how to read a sequencing gel and use the blast function of Pubmed

University of Ottawa Enrichment Mini-Courses-Department of Chemistry 2001-2005

- Lead various mini-course activities aimed at teaching students about chemistry

University of Ottawa Department of Chemistry "Celebration of Science/Sciences en Fête" 2001-2005

- Lead activities aimed at promoting a love of science in younger children

Volunteer Writer-CRAM Science, a scientific website for teens. August 2006-present

- Writing about currently relevant science in a way that can be understood by teens
 - <http://www.cramscience.ca/es.php?a=60>

University Service

Graduate Student Association(GSAED) Board of Directors

Director Without Portfolio
2004-2005

- Elected by GSAED general council from within the membership
- Responsible as director for overseeing the financial workings of the GSAED and reports to council-as is BOD mandate.

BMI Graduate Student Council

Vice-president Communications
2004-2005

- Involved in the initiation of a published website
- Lobbied the Dean of Medicine with CMM/NSC council for inclusion of graduate students in the faculty award of excellence ceremony
- Initiated the adaptation of the CMM/NSC council annual survey to students for recruiting new councilors and gauging opinion of students

The BMI Bulletin-Departmental Newsletter

Editor-in-Chief
2005-Present

- Initiated and major contributing author of The BMI Bulletin- a biweekly newsletter highlighting the accomplishments of students and faculty, intended to

inform the department of coming events and promote a sense of community

Graduate Student Association(GSAÉD)
BMI Departmental Councilor 2004-2006

- Representing the interests of graduate students to the university wide graduate student community.
- Informing the GSAED of activities and events within our department, and promoting co-operation between local and university wide Graduate Student Associations.

GSAÉD Bulletin Committee
Assistant Editor and Committee Member 2005-present

- Assistant editor responsible for proof reading of English text.
- Contributing author and committee member offering input on campus wide electronic graduate student newsletter.

GSAED Capital Building Fund Committee
Committee Member 2005-present

- Contributing committee member offering input on applications for the GSAED Capital Building Fund.

GSAED Café Nostalgica Committee
Committee Member 2005-present

- Contributing committee member offering input on the running of Café Nostalgica, the Graduate Student run Café.

Faculty of Medicine, Graduate Studies Open House Committee
Committee Member 2005-April 2007

- Contributing committee member responsible for helping to organize the Faculty of Medicine Grad Studies Open House, including preliminary planning, organizing volunteers and making sure everything runs smoothly on the day of.
- Initiated first ever official BMIGSA elections

BM! Graduate Student Council
President
2006-2007

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Appendix 2: Letters of Permission to Reprint Published Materials

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