RESTRICTION OF APOPTOSIS IN MATURE NEURONS BY MULTIPLE REDUNDANT BRAKES

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ABSTRACT

Ryan Patrick Annis: Restriction of Apoptosis in Mature Neurons by Multiple Redundant Brakes
(Under the Direction of Mohanish Deshmukh)

Apoptotic cell death is a key part of normal nervous system development, as a process that allows for the controlled removal of developing neurons that fail to properly integrate into the nervous system. However, once the nervous system is established, the production of new neurons is halted in most areas of the nervous system, meaning that neurons remaining after the establishment of the nervous system must last for the lifetime of the organism. Aberrant or accidental activation of the apoptotic pathway would be deleterious for this long-term neuronal survival, and neurons have been found to restrict the apoptotic pathway as they mature. However, while the resistance of mature neurons to apoptosis is well documented, the precise molecular mechanisms underlying this resistance have remained unclear.

Recent findings from our lab found that one way neurons can restrict Bax activation is by upregulating the members of the miR-29 family of microRNAs, which can inhibit Bax activation by blocking the upregulation of multiple redundant members of the BH3-only family of proteins. While overexpression of miR-29 has been found to be sufficient to render young neurons resistant to apoptosis, it was unclear whether the

miR-29 family of miRNAs was the only brake restricting Bax activation in mature neurons, or if other brakes on Bax activation exist.

The work presented in this dissertation addresses this question by examining the status of the apoptotic pathway in neurons from mice in which all three members of the miR-29 family have been deleted. I found that in neurons lacking miR-29 expression, the apoptotic pathway remains restricted. I identified miR-24 as a microRNA that is also upregulated with neuronal maturation, which can act redundantly with miR-29 by repressing a similar repertoire or BH3-only domain genes. Additonally, I found that mature neurons are more resistant to Bax activation induced by direct injection of BH3-only peptides, and that mature neurons may also block apoptosis by restricting the phosphorylation and activation of the pro-apoptotic transcription factor c-jun. My findings also suggest that mature neurons are able to restrict the apoptotic pathway in the soma while maintaining a permissive environment in their axons.

To my Mom and Dad

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LIST OF ABBREVIATIONS

Aβ: Amyloid Beta

AD: Alzheimer's Disease

Ago: Argonaute

ALS: Amyotrophic Lateral Sclerosis

APAF-1: Apoptosis Associated Factor 1

APP: Amyloid Precursor Protein

BACE1: β-Site APP-Cleaving Enzyme 1

BH: BCL-2 Homology

BCL: B-Cell Lymphoma

CARD: Caspase Activation and Recruitment Domain

C. elegans: Caenorhabditis elegans

CNS: Central Nervous System

Cyt *c*: Cytochrome *c*

dATP: Deoxyadenosine Triphosphate

DED: Death Effector Domain

DIV: Days *in vitro*

DLK: Dual Leucine Zipper Kinase

DRG: Dorsal Root Ganglia

GABA: *Gamma*-Aminobutyric Acid

HD: Huntington's Disease

HSP: Heat Schock Protein

FOXO: Forkhead Box

IAP: Inhibitor of Apoptosis Protein

JNK: c-Jun N-Terminal Kinase

LNA: Locked Nucleic Acid

MCAO: Middle Cerebral Artery Occlusion

MCL- Mantle Cell Lymphoma

miR/miRNA: microRNA

MLK: Mixed Lineage Kinase

MOMP: Mitochondrial Outer Membrane Permeablization

MPP+: 1-methyl-4-phenylpyridinium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA: Messenger RNA

NFT: Neurofibrillary Tangle

NGF: Nerve Growth Factor

NMDA: N-Methyl-D-Aspartate

P: Postnatal Day

Pol II: RNA Polymerase II

PD: Parkinson's Disease

PI3K: Phosphoinositide 3-kinase

PNS: Peripheral Nervous System

qRT-PCR: quantitative RT-PCR

RISC: RNA-Induced Silencing Complex

RNA: Ribonucleic Acid

SNc: Substantia Nigra

SOD1: Superoxide Dismutase 1

UTR: Untranslated Region

XAF1: XIAP Associated Factor 1

XIAP: X-linked inhibitor of Apoptosis Protein

CHAPTER 1: INTRODUCTION

1.1 Apoptosis: A Brief History

Death is an unavoidable and important aspect of life, but death is something that all organisms, including humans, instinctually avoid in most cases. However, in most eukaryotes, decisions about life and death are constantly taking place at a cellular level, and are crucially important for both the development and health of complex organisms like mammals. The work of many researchers throughout the years has uncovered a process of voluntary, genetically programmed self-destruction that takes place in mammalian cells, called apoptosis.

The term apoptosis was first coined by the researchers Kerr, Wyllie, and Curry in 1972. They described a process of “controlled cell deletion” that opposed the process of mitosis, or cell division, in mammalian cells for the purposes of regulating the size of cell populations (Kerr, Wyllie et al. 1972). Early observations in developing chick embryos found that the same populations of cells would undergo cell death at the same time-point during embryonic development, suggesting that cell death might be a normal, voluntary, and perhaps genetically programmed part of development (Hinchliffe and Johnson, 1980).

The molecular mechanisms underlying apoptosis remained a mystery until 1986, when Robert Horvitz of MIT, working in the nematode worm caenorhabditis elegans, (*C.*

elegans). *C. elegans* was an ideal model system to explore programmed cell death, because every worm consists of exactly 1090 cells, of which 131 are lost during development in a highly reproducible fashion (Sulston and Horvitz 1977). Using a forward genetic screen in these worms, Horvitz identified the first “death genes”, called *ced-3* and *ced-4*. Horvitz showed that functional *ced-3* and *ced-4* genes were necessary for the execution of the cell death program (Ellis and Horvitz 1986). In 1988, researchers identified the B-Cell Lymphoma 2 (BCL-2) oncogene, which promoted survival in mammalian B-Cells (Vaux, Cory et al. 1988). A gene with similar function, *ced-9*, was discovered in *C. elegans* in 1992 (Hengartner, Ellis et al. 1992), and later that same year, it was discovered that expression of human BCL-2 in C*.elegans* was able to inhibit cell death, providing strong evidence that BCL-2 performed an evolutionarily conserved function of opposing cell death in both worm and mammalian cells (Vaux, Weissman et al. 1992). Indeed, later work would find that BCL-2 and ced-9 are actually the same evolutionarily conserved gene (Hengartner and Horvitz 1994).

The evidence that an evolutionarily conserved programmed cell death pathway existed between worms and humans set researchers to searching for other homologues conserved between the two organisms. In 1993, a mammalian homologue of *ced-3* was discovered, which would become the first member of the family of proteins known as cysteine aspartic proteases, or caspases, now known to be essential for the execution of mammalian apoptosis (Yuan, Shaham et al. 1993). In 1997, a mammalian homologue of *ced-4* was discovered, named Apoptosis Activating Factor 1, or Apaf-1 (Zou, Henzel et al. 1997) In 1998, EGL-1, a protein which in *C. elegans* opposes the action of *ced-9* to promote the execution of apoptosis, was identified (Conradt and

Horvitz 1998). In 1996, the central role played by the mitochondria in apoptosis was established by the discovery that translocation of the electron transport chain protein cytochrome *c* (cyt *c*) was essential for the activation of the apoptotic program. (Liu, Kim et al. 1996).

The connection between cyt *c* release from the mitochondria and BCL-2 was established by the finding that BCL-2 overexpression is able to inhibit apoptosis by preventing cyt *c* release (Kluck, Bossy et al. 1997). It was subsequently discovered that cyt *c* release is governed by complex interactions between multiple members of what came to be known as the BCL-2 family of proteins. Named after the founding member of the family (described above), the BCL-2 family is now known to consist of subgroups of multiple anti- and pro-apoptotic members that serve redundant functions(Danial and Korsmeyer 2004). The first pro-apoptotic member of the family, Bax, was discovered in 1993 based on its interaction with BCL-2 (Oltvai, Milliman et al. 1993). It was subsequently found that Bax and BCL-2 have opposing functions in the regulation of apoptosis, with Bax promoting the release of cyt *c* from the mitochondria and BCL-2 acting to inhibit this process (Jurgensmeier, Xie et al. 1998; Rosse, Olivier et al. 1998).

The BCL-2 Family

The BCL-2 family is now divided into three groups defined by function and by homology of BH1-4 domains within the family (Figure 1.1). The first group, the anti-apoptotic BCL-2 members, consists of BCL2, BCL-XL, MCL-1, A1, and BCL-W (Boise, Gonzalez-Garcia et al. 1993; Kozopas, Yang et al. 1993; Choi, Boise et al. 1995).

These members are characterized by conservation of BH1-4 domains and act to inhibit cell death.

The second group, known as multidomain proapoptotic BCL-2 family proteins, consists of Bax and Bak. These members exist as inactive monomers in the cytosol, and undergo conformational changes that result in their translocation to the mitochondria to promote the release of cyt *c* and subsequently apoptosis. Bax and Bak exhibit conserved expression of BH1-3 domains and serve as key gatekeepers on the apoptotic pathway, as loss of Bax and Bak expression is sufficient to render cells resistant to all known intrinsic apoptotic stimuli (Lindsten, Ross et al. 2000; Wei, Zong et al. 2001).

The third and largest group of BCL-2 family proteins consists of the pro-apoptotic BH3-only domain proteins, which bear strong structural homology with the pro-death c. elegans protein EGL-1(Conradt and Horvitz 1998). This family promotes Bax activation and apoptosis by acting upstream of the mitochondria, and is regulated at both the transcriptional level and by post-translational modification (Lomonosova and Chinnadurai 2008). BH3-only domain proteins promote the activation of Bax and apoptosis, but there is some controversy over the precise mechanism by which this is accomplished. Two non-mutually exclusive models have been proposed, called the direct and indirect activation models.

In the direct activation model, “activator” BH3 proteins, a group thought to be comprised of Bim, Puma, and Bid, are held in check by binding to the anti-apoptotic proteins BCL-2, BCL-xl, BCL-W and MCL-1. In this model, the direct binding of

activators to Bax itself is essential for apoptosis to proceed. In the indirect activation model, Bax and Bak are constitutively active and held in check by binding to the anti-apoptotic BCL-2 family proteins. In this model, the BH3-only domain proteins promote apoptosis by liberating active Bax from BCL-2 family proteins and permitting the translocation of Bax to the mitochondria, where it promotes apoptosis (Shamas-Din, Brahmbhatt et al. 2011). These models are not mutually exclusive and requirements for the induction of apoptosis may vary based on the endogenous expression levels of the various BCL-2 family genes in different cell types.

Caspases

The ultimate execution of the apoptotic pathway in mammalian cells is carried out by caspases (Figure 1.2). Caspases are a family of cysteine proteases that can be divided into two functional groups: effectors and initiators. Effector caspases, a group comprised of Caspases-3, -6, and -7, exist as inactive zymogens in the cytosol of mammalian cells, and remain inactive until cleaved by initiator caspases, a group comprised of Caspase-2, -9, -8, and -10. Caspase-8 and-10 act in the extrinsic pathway of apoptosis, and contain a Death Effector Domain (DED), while Caspase-2 and -9 act in the intrinsic apoptosis pathway, and contain Caspase Activation Recruitment Domains (CARDs) (Riedl and Shi 2004).

The activation of the initiator caspase, Caspase 9, is governed by a large protein complex called the apoptosome. The apoptosome is formed when cyt *c*, after its release from the mitochondria, binds to the CED-4 homologue Apaf-1, along with dATP, to form a large protein complex known as the apoptosome, which mediates the catalytic

cleavage and activation of Caspase-9. Caspase-9 then cleaves and activates executioner caspases such as Caspase-3/7, which cleave a variety of downstream substrates, resulting in the final death of the cell (Li, Nijhawan et al. 1997; Zou, Henzel et al. 1997).

1.2 Apoptosis in Development and Disease

Defects in the apoptotic program are associated with a wide range of disorders, most notably cancer (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Indeed, the anti-apoptotic BCL-2 gene was discovered based on its overexpression in follicular lymphoma (Vaux, Cory et al. 1988). Since this discovery, abundant evidence has accumulated that many cancer types exhibit mutations or changes in gene expression that interfere with the proper function of the apoptotic pathway. For example, the well-known tumor suppressor P53 is mutated in 50% of human cancers, and is known to induce apoptosis through transcriptional upregulation of the BH3-only domain proteins Puma and Noxa (Oda, Ohki et al. 2000; Nakano and Vousden 2001). Mutations in P53 that interfere with its function prevent the induction of the cell death program and allow cancer cells to continue to survive and proliferate.

The apoptotic pathway also plays a key role in the development of many organisms. For example, over the course of normal limb development, many organisms possess interdigital webbing that is normally lost through apoptosis. In mice where genes in the apoptotic pathway are mutated or removed, this webbing persists long after it would normally have been lost (Lindsten, Ross et al. 2000). The main subject of

this study, however, will focus on neurons, a population of cells unique in many ways from others in the body, not least of which is their ability to precisely spatially and temporally regulate the apoptotic pathway.

1.3 Apoptosis in Neurons

Neurons are the building blocks and key functional units of the nervous system. They are unique among cells in their ability to form connections, called synapses, with muscles, glands, and other neurons, through which they transmit signals via the release of chemicals known as neurotransmitters. The development of the nervous system is governed by a variety of complex and overlapping processes. Neurons differentiate from neuronal precursor cells, and must migrate to the appropriate location and form connections with the correct targets. These processes often occur simultaneously at the cellular level during development (Figure 1.3). Regulation of these processes in various populations of neurons is the subject of intensive ongoing research that is beyond the scope of this dissertation. Instead, we will focus on the apoptotic pathway in neurons, which is employed in the developing nervous system to remove neurons that fail to integrate into the nervous system properly as a result of defects in differentiation, migration, or target innervation.

A noteworthy characteristic of neurons compared to other populations of cells is that neurons are terminally differentiated cells that, with few exceptions, do not undergo division in the mature nervous system. Therefore, once a neuron is born and integrated into the nervous system, that neuron remains there for the lifetime of the organism, and

if lost, cannot easily be replaced. One might expect, given the requirement that neurons survive for the lifetime of an organism, that these cells would not possess an active apoptotic pathway. On the contrary, however, apoptosis plays a key role in nervous system development and function. Indeed, mice deficient for key proteins in the apoptotic pathway exhibit craniofacial defects and early embryonic lethality stemming from overproliferation of neuronal precursors (Figure 1.4).

The observation that neurons undergo cell death as a part of normal development was first made by Dr. Erich Kallius in 1926 (Ernst 1926). Many of the key early observations in the neuronal cell death field, however, were made by Dr. Rita Levi Montalcini and Dr. Viktor Hamburger beginning in 1949. In 1960, Dr. Montalcini injected neonatal mice with a neutralizing antibody to the newly-discovered protein Nerve Growth Factor, or NGF, and observed almost total degeneration in the mouse sympathetic nervous system (Levi-Montalcini and Booker 1960). This observation represented both a key discovery in the role of cell death in neuronal development, as well as the birth of the sympathetic neuron model of neuronal apoptosis, which has proven to be one of the most informative models of neuronal cell death to date.

Sympathetic neurons are a population of neurons in the peripheral nervous system (PNS) that are perhaps the best understood model of neuronal apoptosis. Mouse sympathetic neurons undergo extensive cell death as a part of normal development in the first week of postnatal life in mice (Wright, Cunningham et al. 1983). During this time period, these neurons are acutely dependent on Nerve Growth Factor, or NGF, for their survival (Levi-Montalcini and Booker 1960). NGF is secreted in limiting amounts from target organs, glands, and muscles, and binds the TRKA receptor in

sympathetic neuron terminals to promote cell survival. Neurons that properly innervate their targets and obtain NGF survive and mature, while neurons that fail to innervate their targets are eliminated by apoptosis during the first postnatal week (Glebova and Ginty 2005).

Sympathetic neurons are an attractive model for the study of apoptosis because they can be isolated from neonatal mice and maintained in culture by adding exogenous NGF to the cell culture media. Switching cells to NGF-free media results in neuronal death within 48-72 hours, and early studies noted that this death features the hallmarks of apoptosis, including chromatin condensation, DNA fragmentation, declines in RNA and protein synthesis, and membrane disruption. This death was also found to depend on protein synthesis, as pharmacological inhibition of protein synthesis is able to significantly delay cell death. This requirement for active protein synthesis highlights the voluntary “self-destructive” nature of apoptosis in neurons (Martin, Schmidt et al. 1988; Martin, Ito et al. 1992; Deckwerth and Johnson 1993; Edwards and Tolkovsky 1994).

NGF deprivation has been found to induce the intrinsic apoptosis pathway in sympathetic neurons (Figure 1.5). In this pathway, an apoptotic stimulus such as NGF deprivation leads to both the upregulation of the transcription factor c-jun and its phosphorylation by JNK family kinases. Inhibition of c-jun with neutralizing antibodies or expression of a dominant-negative c-jun mutant inhibits apoptosis (Estus, Zaks et al. 1994; Ham, Babij et al. 1995). Phosphorylated c-jun translocates to the nucleus, and promotes the transcriptional induction of multiple pro-apoptotic BH3-only genes. The balance between these pro-apoptotic BH3-only genes and anti-apoptotic the BCL2 family genes BCL2, BCL-XL and MCL-1 is thought to govern Bax activation. Indeed,

overexpression of BCL2 inhibits cell death and cyt *c* release in NGF-deprived sympathetic neurons, and neurons from BCL2- deficient animals die faster than their wild-type counterparts (Garcia, Martinou et al. 1992; Greenlund, Korsmeyer et al. 1995). Similarly, overexpression of BCL-xL and MCL-1 have been found to promote neuronal resistance to apoptosis (Gonzalez-Garcia, Garcia et al. 1995; Arbour, Vanderluit et al. 2008).

Since the BH3-only proteins govern the activation of Bax and the release of cyt *c* from the mitochondria, their roles in neurons have been the subject of considerable study. It is now known that NGF-deprivation in sympathetic neurons leads to the transcriptional upregulation of four BH3-only proteins: Bim, Puma, BMF, and HRK (Kristiansen, Menghi et al. 2011). Overexpression of these genes is sufficient to induce or accelerate cell death in neurons; however, knockout of any of these genes on their own is at best only partially protective from apoptosis in neurons, suggesting that these proteins may have overlapping functions (Whitfield, Neame et al. 2001; Wyttenbach and Tolkovsky 2006; Coultas, Terzano et al. 2007; Pfeiffer, Anilkumar et al. 2014). Indeed, it has been found that knockout of Bim and Puma together provides significantly enhanced protection from apoptosis in the cerebellar granule neuron model of apoptosis compared to individual knockout of either gene alone, and additional deletion of Bid to generate Bim/Puma/Bid knockout neurons phenocopies knockout of Bax/Bak in the cerebellum (Ren, Tu et al. 2010).

The regulation of the transcriptional induction of BH3-only genes is the subject of ongoing study. Early experiments described above established the importance of the transcription factor c-jun in neuronal apoptosis. C-jun has been found to bind to the

promoter of the BH3-only gene Bim and participate in its transcriptional upregulation (Whitfield, Neame et al. 2001). It has subsequently been discovered that the activation of Bim in NGF-deprived sympathetic neurons requires the participation of a number of other factors in addition to c-jun, including the Myb and Forkhead Box (FOXO) transcription factors, which bind to the Bim promoter and first intron (Gilley, Coffer et al. 2003; Biswas, Shi et al. 2007). More recent studies discovered binding sites for the transcription factor NF-Y in the Bim promoter, and found that NF-Y forms a complex with FOXO3A and CBP/P300 proteins to promote increased Bim transcription. It has been hypothesized that CBP/P300 may integrate the contributions of various pro-survival and death signals at the Bim promoter to regulate Bim induction (Hughes, Kristiansen et al. 2011).

The various transcription factors that control Bim induction in sympathetic neurons undergoing apoptosis are regulated by several different pathways. C-jun phosphorylation is known to be regulated by the MLK/JNK pathway. During apoptosis, MLKs become active and phosphorylate JNKs, which in turn phosphorylate c-jun. Phospho-c-jun translocates to the nucleus, where it acts as a pro-death transcription factor (Davis 2000). Activity of the FOXO transcription factors is governed by their phosphorylation. It has been found that the AKT pathway, which is stimulated by NGF, phosphorylates FOXO transcription factors, resulting in their sequestration in the cytoplasm by 14-3-3 proteins. In response to NGF deprivation, the activity of the PI3K-AKT pathway declines, leading to the dephosphorylation of FOXOs, which then translocate from the cytoplasm to the nucleus to stimulate pro-apoptotic gene expression (Gilley, Coffer et al. 2003; Zareen, Biswas et al. 2013). Another pro-survival

pathway stimulated by NGF, the Raf-MEK-ERK pathway, is thought to promote survival by phosphorylating and activating the transcription factor RSK, which promotes survival by phosphorylating and activating the pro-survival factor CREB (Riccio, Ahn et al. 1999). More recently, ERK has been found to directly repress Bim gene induction by direct binding to the 3'UTR of Bim RNA, although to date the mechanism remains unclear (Hughes, Gilley et al. 2011).

Other pro-death transcription factors, such as the Myb proteins, are thought to be regulated by cell cycle induction. It has been found that neurons stimulated to undergo apoptosis upregulate cell-cycle genes like Cyclin D1 and reenter the cell cycle, and that pharmacological inhibitors of cell cycle progression, such as the CDK inhibitors Roscovitine and Flavopiridol, are potent inhibitors of neuronal apoptosis (Freeman, Estus et al. 1994; Liu and Greene 2001).

1.4 Apoptosis in Nervous System Pathologies

The discovery of a pathway mediating programmed neuronal cell death spurred considerable interest in exploring its involvement in neuropathological conditions. The mechanism by which neurons die in these disorders is of particular interest to this dissertation because many of these conditions afflict older individuals, whose neurons would be resistant to apoptosis under normal conditions. Unfortunately, despite many years of intensive study, the evidence for the contribution of apoptosis is mixed, and varies considerably between different disorders.

Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by memory problems, dementia, and progressive neuronal death, which was estimated to afflict 5.1 million Americans as of 2013. The chief risk factor for AD has been found to be age, and the number of patients is expected to grow rapidly as the population ages, according to the US centers for Disease Control.

The pathological hallmarks of AD are neurofibrillary tangles composed of the cytoskeletal associated protein Tau and extracellular plaques composed of the protein Amyloid-β (Aβ), as well as progressive loss of neurons and synapses (Serrano-Pozo, Frosch et al. 2011). The extent to which apoptosis contributes to the loss of neurons and synapses during AD is controversial. Some studies have reported widespread expression of apoptotic markers, while others have reported small or absent changes in expression of apoptotic markers between diseased brains and age-matched controls (Su, Anderson et al. 1994; Troncoso, Sukhov et al. 1996; Selznick, Holtzman et al. 1999; Su, Zhao et al. 2001; Woodhouse, Dickson et al. 2006). Characterizing cell death in post-mortem samples from degenerating human brains is challenging, mainly because neurons that die are cleared by microglial phagocytosis (Takahashi, Rochford et al. 2005). Since cell death in humans suffering from AD occurs gradually over the course of many years, at any given time, only a small number of neurons would be caught in the act of dying during any given "snap shot", and any dead neurons would be quickly removed and thus impossible to detect.

Interestingly, while the evidence that apoptosis contributes to neuron loss in AD patient brains is controversial, substantial evidence exists that Beta-Amyloid (Aβ), one of the proteins that make up the hallmark lesions found in the AD brain, has a pro-apoptotic effect on neurons. Incubation of cortical neurons with purified Aβ has been found to induce robust cell death (Morishima, Gotoh et al. 2001). Aβ-induced death has been found to be Bax dependent and dying neurons were found to exhibit cleaved caspase 3, but pharmacological inhibition or genetic knockout of caspase 3 failed to prevent cell death of Aβ treated telencephalic neurons. This suggests that arrest of apoptosis after the point of cyt *c* release is not able to prevent neurons from dying in reponse to Aβ, but inhibition prior to the release of cyt *c* can promote survival (Selznick, Zheng et al. 2000). More recently, it has been found that exposure to Aβ causes increases in the levels of Bim and active Bax, while decreasing the levels of BCL-2, in organotypic hippocampal slice cultures or in the hippocampi of 2-3 month old mice injected with Aβ intrahippocampally (Kudo, Lee et al. 2012). Aβ was subsequently found to upregulate Bim *via* a Foxo3a-dependent mechanism in cultured cortical neurons (Sanphui and Biswas 2013).

While the evidence for Aβ toxicity is substantial, the role that Tau, plays in AD pathology is less clear. Tau has been well established to exist in a hyper-phosphorylated state in the brains of AD patients, but how hyper-phosphorylated Tau contributes to the progression of the disease, and whether the presence of hyper-phosphorylated Tau reflects a cause or a consequence of AD is still unclear. Tau is a protein that typically associates with microtubules and promotes their polymerization and assembly. Under pathological conditions, Tau becomes hyperphosphorylated and

no longer associates with microtubules, and accumulates in the cytosol in the form of neurofibrillary tangles (NFTs).

It is thought that a combination of loss of endogenous Tau function as well as pathological gain-of-function in hyperphosphorylated Tau contributes to neuronal dysfunction in AD (Ballatore, Lee et al. 2007). For example, in mouse models of tauopathy, neurons exhibit defects in axonal transport that can be rescued by treatment with microtubule stabilizing drugs (Zhang, Maiti et al. 2005). Interestingly, hyperphosphorylation of Tau has been found in several studies to be neuroprotective and to inhibit apoptosis, suggesting that the toxic effects of Tau may be due to indirect effects or toxic intermediates rather than a direct toxic effect of the hyperphosphorylated Tau present in NFTs (Li, Wang et al. 2007; Liu, Liao et al. 2010; Duan, Chai et al. 2013). A recent finding also found that Tau expression is important for the clearance of Aβ plaques in cultured hippocampal neurons and mouse brain, suggesting that there may be crosstalk between Tau and Aβ during AD pathogenesis (Lonskaya, Hebron et al. 2014)

Parkinson's Disease

Parkinson's Disease (PD) is the second most common neurodegenerative disease in the US, afflicting some 1 million Americans. It is characterized by progressive motor deficits including tremor, slowed movement, muscle rigidity, impaired posture, and loss of automatic movements. It also features nonmotor symptoms, including sleep disturbance, sensory abnormalities, and cognitive decline.

The reason for the classical motor symptoms of PD is the progressive loss of dompaminergic neurons in the Substantia Nigra (SNc) region of the brain. PD is also associated with the presence of insoluble protein aggregates known as Lewy Bodies, which are made up of aggregated α-synuclein protein, among other proteins. In addition to the Nigro-Striatal pathway, other brain nuclei have been found to degenerate with Lewy Body pathology. The degeneration of these nuclei is believed to contribute to many of the non-motor symptoms of PD (Jellinger 2012; Dexter and Jenner 2013).

Similar to AD, the contribution of apoptosis to the progression of PD is controversial. Evidence from animal and cell culture models of PD suggested that apoptosis played a prominent role in PD. PD has been modeled *in vitro* and in mouse models using inhibitors of mitochondrial complex I, namely the toxins MPP+, MPTP, and rotenone. These toxins cause energy failure and oxidative stress in cells, and their injection into mice causes a Parkinson's-like pathology (Dauer and Przedborski 2003). Injection of these compounds into mice was found to cause increased apoptosis in the Substantia Nigra (SNc) region of the brain (Tatton and Kish 1997; Viswanath, Wu et al. 2001).

MPTP administration was shown to cause cell death in the brains of mice in a JNK dependent manner (Xia, Harding et al. 2001; Saporito, Hudkins et al. 2002). Further evidence for the involvement of apoptosis in PD pathology came from the finding that active Bax is present in the brain tissue of MPTP-treated mice, 6OHDA lesioned mice (an alternative model of PD) and the brains of human PD patients (Hassouna, Wickert et al. 1996; Tatton 2000; Hartmann, Michel et al. 2001; Vila, Jackson-Lewis et al. 2001; Perier, Bove et al. 2012). Ablation or inhibition of Bax was

found to be protective in mouse models of PD (Vila, Jackson-Lewis et al. 2001; Perier, Bove et al. 2007). Overexpression of BCL-2 was also found to be protective in both *in vitro* and *in vivo* models of PD, although the degree of protection varies depending on the dosage regimen of MPTP (Offen, Beart et al. 1998; Yang, Matthews et al. 1998; Vila and Przedborski 2003).

The abundance of evidence for the involvement of apoptosis in preclinical models of PD led to the initiation of clinical trials examining whether a drug that inhibits neuronal apoptosis could improve patient outcomes in PD. The drug tested, CEP-1347, is a Mixed Lineage Kinase Inhibitor, which inhibits apoptosis at an early point in the pathway by preventing MLK-JNK activation. CEP-1347 was found to inhibit neuronal apoptosis in multiple models of developmental and pathological cell death (Wang, Ma et al. 2004). Unfortunately, the CEP-1347 trial was stopped early when an interim analysis of the data determined that it would be "futile" to continue the trial due to the drug's lack of efficacy (Parkinson Study Group PRECEPT Investigators, 2007). The reason for the failure of promising preclinical data to translate to the clinic is unclear, but may reflect inadequacy of the current animal and cellular models to accurately depict human disease, or the presence of other "back-up" cell death pathways, such as necrotic or autophagic cell death, that are engaged when apoptosis is inhibited (Venderova and Park 2012).

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis, or ALS, also known as Lou Gehrig's Disease, is a degenerative disorder affecting the motor neurons of the spinal cord. Its symptoms

include muscle weakness, atrophy, and spasticity. Approximately 5-10% of ALS cases are familial, and have been found to be caused by specific mutations, most notably a toxic gain-of-function mutation in the Superoxide Dismutase 1 (SOD1) gene. Familial ALS can also be caused by hexanucleotide repeat expansion of the C9ORF72 gene, and mutations in the RNA processing gene TARDBP.

The pathology of ALS involves progressive loss of motor neurons in the anterior horns of the spinal cord and in the motor nuclei of the brainstem. Denervation of muscles after motor neuron death leads to muscle atrophy. Most ALS patients die within 2-3 years of the onset of symptoms, usually of respiratory muscle paralysis.

The role of apoptosis in ALS was recently examined by crossing mice bearing the SOD^{G93A} mutation, which is known to lead to familial ALS in humans. Apoptosis was inhibited in these animals by crossing them with a mouse line lacking CNS expression of Bax and Bak, two genes which are required for activation of the mitochondrial apoptotic pathway. Double deletion of Bax and Bak was found to delay neuronal loss, symptom onset, and preserve neuronal function in these mice, as well as increasing lifespan by 21%. These findings suggest that apoptosis plays a role in the progression of ALS, but also highlight that apoptosis alone is not responsible for the observed symptoms in this disease (Reyes, Fisher et al. 2010). Consistent with this finding, inhibition of apoptosis with caspase inhibitors or BCL-2 overexpression also delays, but does not prevent, the onset and progression of ALS symptoms (Kostic, Jackson-Lewis et al. 1997; Azzouz, Hottinger et al. 2000).

As the research summarized above demonstrates, while there is evidence that apoptosis contributes to the progression of some neurodegenerative diseases, it has been difficult to translate even the most promising preclinical data into successful treatments in humans. While improved animal models of neurodegeneration would no doubt aid in this process, it appears at this point that inhibition of apoptosis will not serve as a “magic bullet” in the treatment of neurodegenerative disorders.

1.5: Introduction to microRNAs

MicroRNAs (miRNAs) are small (~22 nucleotide) single-stranded RNAs that act as regulators of gene expression. miRNAs are typically transcribed from genomic DNA by RNA Polymerase II (Pol II) to produce a primary transcript, or pri-miRNA. These pri-miRNAs include stem-loop structures that will eventually be processed into mature miRNAs, as well as varying amounts of surrounding RNA. In the next step of miRNA biogenesis, these stem-loop structures are cleaved from the surrounding RNA by the microprocessor complex, which includes the proteins DGCR8 and Drosha. The product of this cleavage event is a ~65 nucleotide hairpin structure known as a pre-miRNA. Following this cleavage, pre-miRNAs are exported from the nucleus to the cytoplasm via the nuclear pore complex. The most important protein for this export process is the nuclear transport receptor Exportin-5 (Ha and Kim 2014).

Following nuclear export, the pre-miRNA hairpin structure is further processed in the cytoplasm near the terminal loop, resulting in a small RNA duplex consisting of two strands of ~22 nucleotides in length. This duplex is loaded into a protein complex, the

RNA-Induced Silencing Complex (RISC), at which point one strand, the passenger strand, is removed by cleavage or unwinding. The strand remaining in the RISC, known as the guide strand, is able to participate in RNA silencing at this point, while the removed passenger strand is typically degraded rapidly. Argonaute proteins, especially Argonaute 2 (Ago2), are critical for miRNA silencing activity (Huntziner et al., 2011). miRNAs bind primarily to the 3' untranslated regions (3'UTRs) of target mRNAs in a sequence-dependent manner and mediate their silencing through translational inhibition or RNA degradation (Ha and Kim 2014). A schematic of this process is shown in Figure 1.6. Target selection is mediated by a short sequence within each miRNA known as the seed sequence, with miRNAs within families (e.g. miR-29a, b, and c) sharing conserved seed sequences.

1.6 MicroRNAs in Nervous System Development and Disease

Since their discovery in the 1990s, miRNAs have been implicated in a wide variety of biological processes and pathologies. Indeed, it has been estimated that more than 60% of genes in the human genome are targeted by at least one miRNA. The importance of miRNAs for proper mammalian development is strongly supported by data from mouse knockout studies. Germline knockout of Drosha or Dicer leads to embryonic lethality in mice by embryonic day 7.5 (Park, Choi et al. 2010), while knockout of DGCR8 leads to arrest early in embryonic development, with DGCR8-deficient stem cells exhibit defective proliferation and differentiation (Wang, Medvid et al. 2007).

miRNAs have also been found to play crucial roles in neuronal development and disease. For example, deletion of Dicer using the cerebral cortex-specific *Emx1-Cre* line results in a range of defects, including decreased survival and differentiation (De Pietri Tonelli, Pulvers et al. 2008; Kawase-Koga, Otaegi et al. 2009). Deletion of Dicer using the CAMKII promoter, which is specific to postmitotic neurons of the cortex and hippocampus, results in decreased cortical size, enhanced neuronal cell death, defects in axon pathfinding, and impaired dendritic branching (Davis, Cuellar et al. 2008). Certain aspects of these Dicer-deletion phenotypes have been attributed to specific miRNAs. miRNAs, including miR-124, miR-9, and Let-7 have been found to promote neuronal differentiation, neurite outgrowth, and synaptogenesis (Bian and Sun 2011; McNeill and Van Vactor 2012). miR-128 was found to be highly expressed in adult neurons and to regulate neuronal excitability and mouse motor behavior. Knockdown of miR-128 in the brain was found to lead to neuronal hyperexcitability and fatal epilepsy, while overexpression of miR-128 was found to suppress motor activity and alleviate motor abnormalities associated with Parkinson's-like disorders in mice (Tan, Plotkin et al. 2013).

microRNAs have also been implicated in various neurodegenerative disease states and in cases of acute neuronal injury, such as stroke. Selective ablation of Dicer in adult forebrain neurons was found to result in cellular shrinkage, neurodegeneration, and abnormal phosphorylation of the cytoskeletal protein Tau, which is a hallmark of Alzheimer's Disease (Hébert, Papadopoulou et al. 2010).

Screens performed in age-matched human patient samples as well as mouse models of neurodegenerative disease have yielded a number of miRNA candidates that

may play a role in the etiology of neurodegenerative disorders (Table 1.1). While the candidates summarized in Table 1.1 were selected for analysis based primarily on their ability to target disease-specific genes, such as α-synuclein in Parkinson's disease or β-Site APP-Cleaving Enzyme 1 (BACE1) in Alzheimer's Disease. However, disruption of other miRNA functions, such as the ability of miR-29 to regulate apoptosis, could also play an important role in the progression or onset of neurodegenerative disease, and merits further investigation (Kole, Swahari et al. 2011). The effects of these non-disease-specific functions of miRNAs will likely become clearer as more miRNA-specific knockout organisms are generated. For example, in *Drosophila*, knockout of miR-34 has been shown to lead to late-onset brain degeneration, decreased lifespan, and a gene-expression profile consistent with accelerated aging. Conversely, overexpression of miR-34 extended lifespan and mitigated neurodegeneration induced by overexpression of a pathogenic (poly-glutamine) ataxin-3 protein (Liu, Landreh et al. 2012).

1.7 Increasing Resistance to Apoptosis with Neuronal Maturation

Early in the development of the mammalian nervous system, neurons are acutely sensitive to apoptosis. As referenced above, when Dr. Rita Levi-Montalcini injected neonatal mice with an antigen that neutralized the activity of nerve growth factor *in vivo*, she observed widespread cell death in the sympathetic ganglia, with more than 99% neuron loss in most cases ((Levi-Montalcini and Booker 1960), Figure 1.7). However, Montalcini and colleagues later discovered that in adult mice treated with the same neutralizing antibody, a significant proportion of sympathetic neurons were able to survive. Further experiments discovered that sympathetic neurons of the adult nervous

system did undergo changes in response to inhibited NGF signaling, including decreases in metabolism and catecholamine synthesis, these changes were largely reversible once NGF signaling was restored (Figure 1.5) (Angeletti, Levi-Montalcini et al. 1971; Goedert, Otten et al. 1978; Otten, Goedert et al. 1979; Gorin and Johnson 1980).

Interestingly, this phenomenon of increased neuronal resistance to apoptosis with maturation can be recapitulated *in vitro*. Neurons isolated from neonatal mice and cultured in the presence of NGF for 5 days will undergo robust apoptosis in response to NGF deprivation, with virtually 100% of cells dying by 72 hours post-NGF-deprivation. In contrast, neurons maintained in culture for 28 days are remarkably resistant to NGF deprivation, with >90% of cells surviving for an extended period of time in the absence of NGF. These mature neurons were found to undergo some of the initial steps of the apoptosis pathway in response to NGF-deprivation, including phosphorylation of the transcription factor c-jun, but never progressed to the point of Bax activation and mitochondrial outer membrane permeablization (MOMP) (Lazarus, Bradshaw et al. 1976; Chun and Patterson 1977; Deshmukh and Johnson 1997; Easton, Deckwerth et al. 1997). These findings were also recapitulated in another peripheral neuron population, the Dorsal Root Ganglia (DRG) neurons, which, similar to sympathetic neurons, are dependent on NGF for survival early in development and lose this dependence as they mature (Lindsay 1988; Kimpinski, Campenot et al. 1997; Vogelbaum, Tong et al. 1998). Similar cases have also been observed in the central nervous system (CNS), where neurons of the anteroventral cochlear nucleus (AVCN), which stimulate the cochlea, will undergo robust apoptosis if the cochlea is removed

during a critical early postnatal period, but these neurons persist if the cochlea is removed later in development (Tierney, Russell et al. 1997; Mostafapour, Cochran et al. 2000; Mostafapour, Del Puerto et al. 2002; Harris and Rubel 2006).

In addition to developmental cues, enhanced survival of mature neurons has also been observed following pathological or traumatic stimuli. For example, nerve transection has been found to cause widespread cell death in multiple neuronal populations in young mice, but significantly reduced cell death in neurons of older mice subjected to the same insult (Rich, Luszczynski et al. 1987; Yan and Johnson 1988; Yu 1988; Snider and Thanedar 1989; Sendtner, Kreutzberg et al. 1990; Kuzis, Coffin et al. 1999; McKernan, Caplis et al. 2006).

In the case of hypoxic-ischemic brain injury, it has been found that widespread caspase-dependent death occurs in the neonatal brain *in vivo* when mice are subjected to hypoxic ischemic insult. This death exhibits classical hallmarks of apoptosis, including DNA fragmentation, TUNEL staining, and the presence of apoptotic morphology detected by electron microscopy (Cheng, Deshmukh et al. 1998). In contrast, older mice subjected to the same insult show marked decreases in the number of cells exhibiting apoptotic morphology, as well as decreased Caspase 3 activation (Hu, Liu et al. 2000).

Neuronal resistance to apoptosis may also play a role in the response to viral infection in mammals. Many neurotropic viruses, such as rhabdoviruses, reoviruses, bunyaviruses, alphaviruses, herpesviruses, and flavivviruses, have been found to cause more severe infections and neurological symptoms in young patients when compared to older patients (Griffin, Levine et al. 1994; Fazakerley and Allsopp 2001; Kimberlin

2004). While the outcome of a viral infection depends on many factors, such as immune response, changes in cell-surface receptors, development of anatomical barriers, etc., there is evidence that increased neuronal resistance to apoptosis may also play a role in this acquired resistance to viral infection with increasing age. For example, infection of 1-day old mice with Sindbis virus leads to death of 100% of the infected mice within 8 days, and widespread neuronal cell death in the CNS as determined by histology and TUNEL staining. In contrast, virus-infected 4-week old mice displayed no mortality or clinically apparent disease, and no increase in CNS cell death (Labrada, Liang et al. 2002). Importantly, overexpression of the anti-apoptotic protein BCL-2 in the brains of neonatal mice infected with Sindbis virus significantly increased the survival of the infected mice (7.8% mortality vs. 72.1 and 78.1% mortality in mice infected with control viruses) and decreased apoptosis in infected brains (Levine, Goldman et al. 1996).

The observation that older neurons are more resistant to apoptosis may also have clinical relevance in the field of anesthesiology. Studies have found that commonly used anesthetic agents cause widespread apoptosis in the brains of neonatal mice, and that this leads to behavioral deficits in these mice later in life (Jevtovic-Todorovic, Hartman et al. 2003; Zhou and Ma 2014) Correlative studies examining learning and behavioral problems in cohorts of human patients have produced mixed results, however several researchers have found evidence of increased learning and behavioral problems in children who have undergone anesthesia early in life, particularly in those children who have been anesthetized multiple times (Wilder, Flick et al. 2009; DiMaggio, Sun et al. 2012).

The precise mechanism through which anesthetics cause apoptosis in the young brain is has not been definitively determined. It is thought that the ability of most anesthetics to antagonize excitatory N-Methyl-D-Aspartate (NMDA) receptors and potentiate inhibitory *Gamma*-Aminobutyric Acid (GABA) receptor activity in the developing brain plays a role (Chiao and Zuo 2014). Indeed, neurons in the young brain have been found to be acutely sensitive to either excitation or inhibition of NMDA receptor activity. Excitation of NMDA receptors through direct injection of NMDA, or inhibition of NMDA receptors by direct injection of the NMDA antagonist MK-801 into the brains of developing mice were found to cause widespread apoptosis in neonatal rodent brains, which was decreased with increasing age (McDonald, Silverstein et al. 1988; Ikonomidou, Bosch et al. 1999). It is likely that changes in the relative levels of NMDA and GABA receptors in the brain, as well as changes in the cellular response to manipulation of GABA/NMDA receptor activity, play a role in this response. However, developmental changes in the apoptotic machinery likely also have an effect.

1.8 Molecular Mechanisms Underlying Increased Neuronal Resistance to Apoptosis

Post-Mitochondrial Apoptotic Brakes

Maturing neurons across the nervous system have been found to restrict cell death by regulating the apoptotic pathway at various points before and after mitochondrial outer membrane permeablization (MOMP). After MOMP, cyt *c* is released from the mitochondria and binds to the pro-apoptotic protein Apaf-1. Cyt *c* and Apaf-1 then oligomerize with the activator caspase, Caspase 9, to form the apoptosome

complex. This complex then cleaves and activates executioner caspases, most notably Caspase 3/7, which cleave a large number of substrates, resulting in the ultimate death of the cell.

Evidence that apoptosis is restricted has been definitively demonstrated in the sympathetic neuronal model of apoptosis. As previously described, young neurons have been found to undergo robust cell death in response to apoptotic stimuli, while mature neurons have been found to be markedly resistant to apoptosis induction. To investigate whether mature neurons restrict apoptosis at a checkpoint after cyt *c* release, experiments were conducted utilizing microinjection of purified cyt *c* directly into the cytosol of sympathetic neurons. While cyt *c* injection is insufficient to induce death in young sympathetic neurons maintained in NGF, young neurons can be induced to develop so-called “competence to die” by depriving cells of NGF in the absence of Bax or the presence of inhibitors of protein synthesis, which arrest the apoptotic pathway prior to the point of cyt *c* release (Deshmukh and Johnson 1998). Development of competence depends on the inactivation and/or degradation of X-Linked Inhibitor of Apoptosis Protein (XIAP). XIAP is known to be able to bind to Caspase 3 and Caspase 9 and inhibit their activity (Riedl, Renatus et al. 2001; Shiozaki, Chai et al. 2003). NGF deprivation leads to downregulation of XIAP levels, and neurons from XIAP-deficient animals are acutely sensitive to cytosolic injection of cyt *c* (Potts, Singh et al. 2003).

In contrast to young sympathetic neurons, mature neurons are not sensitive to cyt *c* microinjection, even when subjected to the same treatments that induce competence to die in young neurons (Putcha, Deshmukh et al. 2000). It was found that mature sympathetic neurons restrict apoptosis post-mitochondrially by restricting the

expression of Apaf-1 at the chromatin level, preventing apoptosome formation and subsequent caspase activation to arrest the cell death pathway (Wright, Smith et al. 2007). Interestingly, Apaf-1 levels have been found to decline during development in multiple neuronal tissues, including the cerebral cortex, cerebellum, and retina (Yakovlev, Ota et al. 2001; Donovan and Cotter 2002; Ota, Yakovlev et al. 2002; Johnson, Huang et al. 2007). This suggests that restriction of Apaf-1 expression is a conserved mechanism to restrict apoptosis during the maturation of multiple neuronal populations.

While maturing sympathetic neurons and dorsal root ganglion neurons have been found to specifically downregulate Apaf-1 while maintaining the levels of Caspase 3 and Caspase 9, other neuronal populations have been found to restrict apoptosis by downregulating the expression of caspases as well. Decreases in Caspase 3 expression with maturation have been observed in cortex, cerebellum, photoreceptors, and neurons of the AVCN in the brainstem (Yakovlev, Ota et al. 2001; Donovan and Cotter 2002; Harris, Hardie et al. 2005; Johnson, Huang et al. 2007). In the motor neurons of the spinal cord, it was found that the ratio of XIAP to XIAP Associated Factor 1 (XAF1) increases with maturation. XAF1 is a protein which acts to promote apoptosis by restricting the ability of XIAP to inhibit caspases, and increasing the XIAP/XAF1 ratio enhanced the ability of XIAP to inhibit cell death and contributed to the enhanced resistance of mature motor neurons to axotomy when compared with younger neurons subjected to the same insult. Indeed, overexpression of XAF1 in mature spinal cord neurons was able to abrogate the resistance of these neurons to axotomy-induced apoptosis (Perrelet, Perrin et al. 2004).

Heat shock protein 27, or HSP27, has been found to be a critical determinant in the survival of sensory and motor neurons in response to axotomy. In young mice subjected to nerve transection, it was observed that the majority of neurons in the transected ganglia die, but a subset survive, and the surviving neurons were found to express HSP27. In adult mice subjected to the same insult, a much greater percentage of neurons survive, and 100% of neurons were found to express HSP27, suggesting that the enhanced survival of DRG or motor neurons in response to axotomy is due to the increased ability of adult neurons to upregulate HSP27 in response to this insult (Costigan, Mannion et al. 1998). Overexpression of HSP27 was found to promote survival of young DRG neurons when delivered *in vivo*, while knockdown of HSP27 in mature DRGs was found to sensitize them to death in response to axotomy (Benn, Perrelet et al. 2002).

Interestingly, HSP27 may promote survival *via* different mechanisms in different cell types. In neurons, it was found that HSP27 prevents caspase 3 activation but not cyt *c* release (Benn, Perrelet et al. 2002). Consistent with this observation, it has been found that HSP27 can directly bind to and sequester cyt *c* and caspase 3 (Bruey, Ducasse et al. 2000; Concannon, Orrenius et al. 2001). However, a separate study in cancer cells found that HSP27 was able to indirectly prevent Bax activation by promoting pro-survival AKT signaling (Havasi, Li et al. 2008).

Pre-Mitochondrial Apoptotic Brakes

Similar to other aspects of apoptosis regulation during neuronal maturation, the characterization of pre-mitochondrial apoptotic brakes has been most thoroughly performed in the model of mouse sympathetic neurons. The early stages of apoptosis in mouse sympathetic neurons deprived of NGF involve changes in the phosphorylation status of its receptor, TRKA. In response to NGF stimulation, the TRKA receptor becomes phosphorylated, and exerts pro-survival and trophic effects through stimulation of a several downstream pathways, most notably the Ras-Raf-Mek-ERK and PI3-Kinase pathways (Kaplan and Miller 2000).

Upon NGF deprivation, levels of phospho-TRKA decline, leading to diminished stimulation of downstream pro-survival pathways and increased stimulation of pro-death pathways, such as the MLK-JNK pathway described above. Interestingly, while phospho-TRKA levels decline rapidly in young neurons deprived of NGF, phospho-TRKA levels have been found to decline much more slowly in mature neurons. It has been suggested that this increased persistence of TRKA signaling in the absence of NGF could contribute to the resistance to apoptosis observed with neuronal maturation (Tsui-Pierchala and Ginty 1999).

While preservation of TRKA signaling in mature neurons deprived of NGF may indeed play a role in the resistance of mature sympathetic neurons to apoptosis, evidence in the literature suggests that it is not the dominant mechanism that mature neurons employ to restrict cyt *c* release. Other studies that investigated the status of NGF-deprived mature neurons found that while death was arrested, a number of the

early events associated with NGF deprivation still proceeded as they did in young neurons. For example, both young and mature neurons exhibit a decrease in soma diameter and protein synthesis in response to NGF deprivation and both young and mature neurons display decreases in their rates of glucose uptake, RNA synthesis, and protein synthesis within the first 24 hours of NGF deprivation (Easton, Deckwerth et al. 1997).

When examining the components of the apoptotic pathway more directly, it was found that c-jun phosphorylation, known to be a key event in the induction of apoptosis in sympathetic neurons, still occurred within 6 hours in mature neurons deprived of NGF, and persisted out to 48 hours in culture. Furthermore, induction of several genes found to be induced with NGF deprivation in young neurons, including cyclophilin, c-jun, and c-fos, still occurred in mature neurons deprived of NGF. Taken together, these results suggested that the early events of the apoptotic pathway in mature neurons were able to proceed as in young neurons, but neurons failed to die (Easton, Deckwerth et al. 1997). A subsequent study examined the status of Bax and cyt *c* in mature neurons induced to die by NGF deprivation, and found that while Bax localized to the mitochondria and induced cyt *c* release in young neurons, both of these events failed to occur in mature neurons, consistent with the findings of the study discussed above (Putcha, Deshmukh et al. 2000). Taken together, these two studies provided the first direct mechanistic evidence for the existence of apoptotic brakes upstream of Bax activation in mature neurons. Importantly, neither study found that mature neurons had changes in the expression level of Bax itself. The efficacy of restricting Bax activation as a mechanism for inhibiting death in mature neurons is underscored by the finding that

Bax-knockout neurons are capable of surviving long-term in the absence of any trophic stimulation (Deckwerth, Elliott et al. 1996).

In contrast to sympathetic neurons, some other neuronal populations appear to repress Bax activation as they mature. Bax expression has been found to decline during the maturation of cerebellum, dorsal root ganglia, and forebrain neurons (Vekrellis, McCarthy et al. 1997; Vogelbaum, Tong et al. 1998; Polster, Robertson et al. 2003). Interestingly, in the case of forebrain neurons, it was found that a percentage of Bax is present at the mitochondrial outer membrane of purified mitochondria in the absence of any apoptotic stimulus, and that the amount of Bax present at the outer membrane declined with maturation. This correlated strongly with decreasing ability of purified mitochondria to release cyt *c* in response to incubation with purified Bax-BH3-only peptide, and suggests that Bax may exist at the mitochondria of young neurons in a "primed" state that increases the sensitivity of cells to apoptosis.

The findings summarized above highlight the increasing resistance of diverse populations of neuronal cells to apoptosis as they mature. The fact that different populations of neurons restrict the pathway in different ways (e.g. repressing Bax vs expressing Bax with maturation) suggests that apoptotic proteins may perform other functions. Indeed, it has been found recently that activation of caspase 3 in a limited fashion is required for Long-Term Depression (LTD) and AMPA receptor internalization in hippocampal neurons (Li, Jo et al. 2010) . Thus, different populations of neurons may restrict apoptosis in different ways depending on their specific stresses and requirements for apoptotic proteins to perform non-apoptotic functions.

1.10 Introduction to miR-29

The miR-29 family of miRNAs are of particular interest to the study of neuronal maturation and apoptosi. The miR-29 family is composed of 3 members: miR-29a, miR-29b, and miR-29c. These family members are transcribed from two separate chromosomes in humans and mice. miR-29a and miR-29b1 are cotranscribed from chromosome 7, while miR-29b2 and miR-29c are cotranscribed from chromosome 1. miR-29b1 and b2 are identical in sequence and are collectively referred to as miR-29b in their mature form. The three family members share a conserved seed sequence, which is thought to be important for target selectivity and specificity.

The miR-29 family is widely expressed and is one of the more extensively studied microRNAs to date. It has been identified as playing a role in regulating a diverse range of cellular pathways, including innate/adaptive immune response (Ma, Xu et al. 2011) (Ma et al., 2011), lipid metabolism (Kurtz, Peck et al. 2014), and the regulation of various extracellular matrix components, such as collagen proteins, in the fibrosis of various tissues such as lung (Xiao, Meng et al. 2012; Montgomery, Yu et al. 2014)(Montgomery et al., 2014; Xiao et al., 2012), kidney (Qin, Chung et al. 2011), and heart (van Rooij, Sutherland et al. 2008). Of particular interest to this dissertation, however, is the role that miR-29 has been found to play in the regulation of apoptosis.

The ability of miR-29 to regulate apoptosis was first discovered in 2007, when it was found that the anti-apoptotic BCL-2 family protein MCL-1 contained a putative miR-29 binding site in its 3 ’UTR. It was found that miR-29b was highly expressed in a healthy bile-duct epithelium cell line, while malignant bile-duct epithelium cells, known as cholangiocarcinoma cells, miR-29b levels were decreased. Enforced expression of

miR-29 in malignant cells was found to reduce MCL-1 protein levels and sensitize these cells to Tumor Necrosis Factor-induced apoptosis, while transfection of non-malignant cells with a Locked-Nucleic Acid (LNA) designed to inhibit miR-29 activity resulted in increased MCL-1 levels and decreased sensitivity to apoptosis (Mott, Kobayashi et al. 2007). Consistent with a pro-apoptotic role for miR-29 in cancer, miR-29 has been found to be suppressed or deleted in a number of cancer types, including Acute Myeloid Leukemia (Eyholzer, Schmid et al. 2010), Mantle Cell Lymphoma (Zhang, Zhao et al. 2012), and B-Cell Chronic Lymphocytic Leukemia (Pekarsky, Santanam et al. 2006). miR-29 was also found to promote p53 expression and apoptosis in HeLa cells by downregulating P85-alpha and CDC42, which are two proteins that negatively regulate p53 (Park, Lee et al. 2009).

miR-29 miRNAs as Regulators of Neuronal Apoptosis

Recently, it has been found that mature sympathetic neurons fail to upregulate BH3-only domain genes in response to NGF deprivation (Kole, Swahari et al. 2011). The same study found that maturing neurons of multiple neuronal populations upregulate members of the miR-29 family of microRNAs, miR-29a, miR-29b, and miR-29c. It was found that each of the BH3-only genes that are typically upregulated with NGF deprivation in sympathetic neurons, namely Bim, BMF, Puma, and HRK, possessed a sequence that was complimentary to the miR-29 seed sequence in its 3'UTR. Luciferase reporter assays, performed by cloning the 3'UTRs of these genes into a luciferase reporter vector and measuring the ability of transfected miRNA mimics to inhibit luciferase transcription, validated the ability of miR-29 to bind these sites *in vitro*. Most importantly, it was found that overexpression of the miR-29 family member

miR-29b in young neurons was able to provide significant protection for those neurons against diverse apoptotic insults, including NGF deprivation, DNA damage, and Endoplasmic Reticulum stress (Kole, Swahari et al. 2011). microRNAs, with their ability to simultaneously target multiple different genes in the same pathway, provide an attractive candidate for regulation of the redundant members of the BH3-only domain proteins in maturing neurons.

Studies performed in other labs further strengthened the case for the miR-29 family as promoters of neuronal survival. miR-29b was found to be decreased in the infarct area of mice subjected to brain ischemia by middle cerebral artery occlusion (MCAO), and exogenous expression of miR-29b mimics resulted in a significant decrease in infarct size. Furthermore, mice treated with miR-29b mimics prior to MCAO exhibited improved sensorimotor function and behavioral outcome post-stroke (Khanna, Rink et al. 2013). A separate study examined the regulation of the miR-29 family in the ischemic hippocampus and found that miR-29a was selectively downregulated, and that overexpression of miR-29a was able to preserve astrocyte survival and function during ischemia by inhibiting Puma induction (Ouyang, Xu et al. 2013). miR-29 levels have also been reported to be decreased in injured mouse spinal cord, and that addition of miR-29 mimics could partially rescue the apoptosis that occurred in response to spinal cord injury. This apoptosis could be completely abolished if the miR-29 mimics were co-transfected with inhibitors to miR-20a, a pro-apoptotic miRNA that increased in the lesioned spinal cord (Liu, Zheng et al. 2015).

miR-29 may also regulate neuronal apoptosis through mechanisms independent of its ability to target BH3-only genes. A recent study examining the effects of ethanol on cell death in the developing cerebellum found that mir-29 levels were decreased in neurons exposed to ethanol, and that exogenous expression of miR-29 could dramatically decrease cell of cerebellar granule neurons treated with ethanol. Interestingly, the authors noted that they observed no effect of miR-29 mimic expression on BH3-only gene levels in cerebellar granule neurons, and that this protection instead was mediated by inhibition of an SP1-RAX-PKR cascade by miR-29 (Qi, Zhang et al. 2014)(Qi and Zhang et al., 2014). Additionally, a recent study identified Aquaporin 4 as another potential target of miR-29 that at least partially mediates its neuroprotective effects during ischemia (Wang, Huang et al. 2015). The apparently opposite effects of miR-29 upregulation on apoptosis in cancer cells and neurons may be due to differential dependence of these different cell types on MCL-1 for survival. They may also be due to the effect of miR-29 on downregulating cell cycle gene expression, such as CDK6 (Zhao, Lin et al. 2010). Inhibition of cell-cycle progression can lead to cell-cycle arrest and apoptosis in mitotic cells, but has been found to be protective in neurons.

The findings summarized above raise many interesting questions. Firstly, miR-29 upregulation is the only brake identified so far that neurons can employ to block apoptosis upstream of cyt *c* release. Is miR-29 the only brake that blocks this event in mature neurons? Do neurons lacking miR-29 survive the maturation process? Are mature neurons lacking miR-29 more vulnerable than wild-type neurons to apoptotic insults? Are other miRNAs upregulated during nervous system maturation, and can they play a role in regulating apoptosis? What regulates the up/downregulation of various

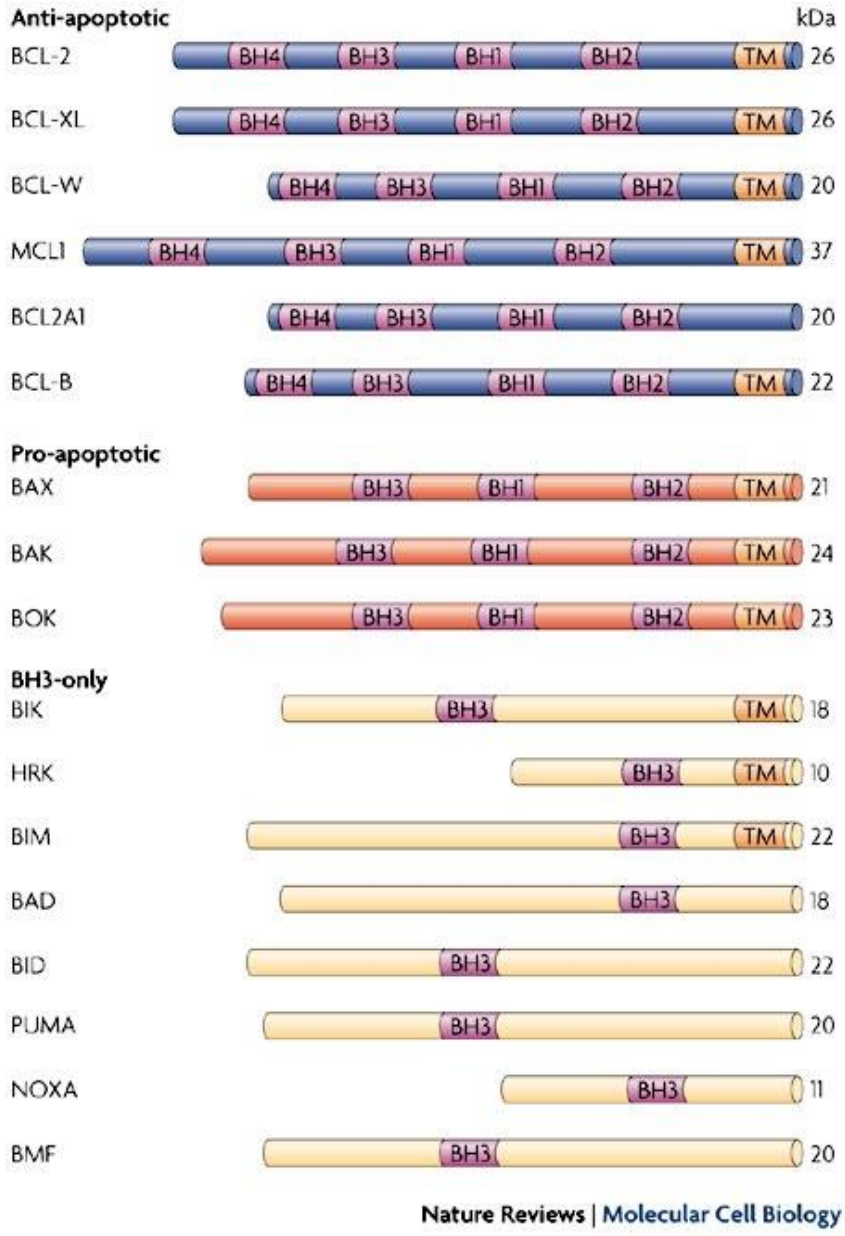
apoptotic components that change their expression level to render mature neurons more resistant to apoptosis? This dissertation supplies answers to some of these questions, and raises new questions that will remain under investigation as the study of neuronal apoptosis continues.

1.11 Figures and Legends

Figure 1.1: The BCL-2 Family of Proteins

The BCL-2 family can be broken down into three subgroups based on structure and function. The anti-apoptotic members of the family contain BH1-BH4 domains. The pro-apoptotic members can be further subdivided into the multidomain pro-apoptotic proteins and the BH3-only domain proteins. The multidomain pro-apoptotic proteins Bax and Bak lack BH4 domains and are essential for the permeablization of the mitochondrial outer membrane to release cytochrome *c*. BH3-only domain proteins , as their name suggests, express only BH3 domains and promote apoptosis by either activating Bax and Bak or inhibiting the activity of the anti-apoptotic BCL-2 family members.

Figure 1.1: The BCL-2 Family of Proteins

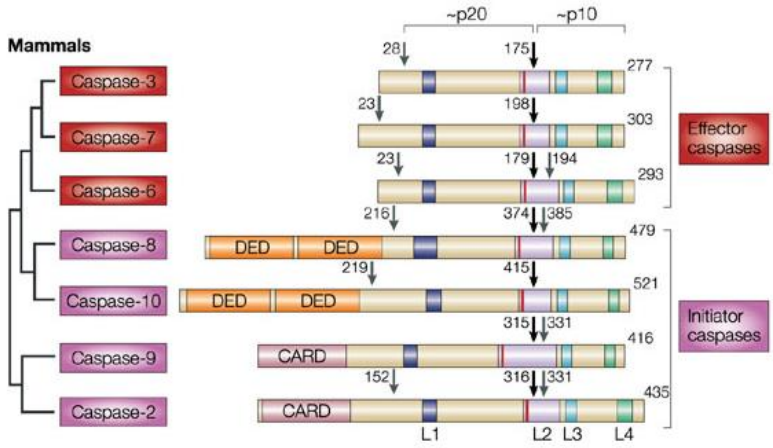


Adapted from (Taylor, Cullen et al. 2008)

Figure 1.2: Effector and Initiator Caspases

Domain structure of effector caspases (Caspase 3, 6, 7) and initiator caspases (Caspase 2, 8, 9, 10). Cleavage sites are indicated by black arrows, while alternate cleavage sites, thought to be important for caspase regulation, are indicated with gray arrows. Catalytic cysteines are indicated in red. Death Effector Domains (DED) are important for participation in the extrinsic apoptotic pathway, while Caspase Activation and Recruitment Domains (CARD) are important for participation in the intrinsic apoptotic pathway. Members of the caspase family thought to be more important for inflammatory cell death (e.g. Caspase 1) are not pictured.

Figure 1.2: Effector and Initiator Caspases

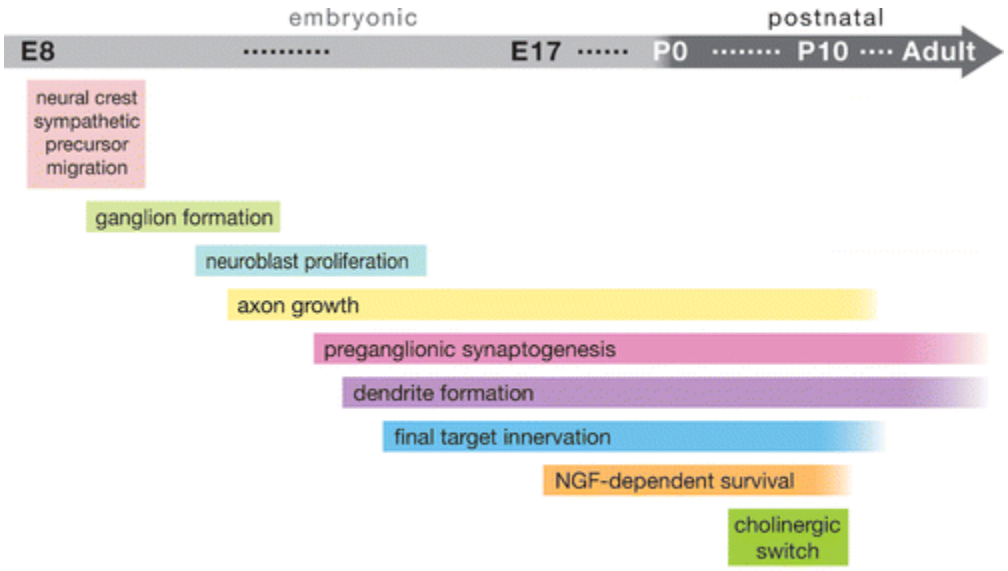


Adapted from (Riedl and Shi 2004)

Figure 1.3: Timecourse of Developmental Milestones in Sympathetic Neuron Development

Figure 1.3 shows the timecourse of the key events in the formation of the sympathetic nervous system. Proper formation of the nervous system requires neurons to properly migrate, differentiate, and make connections with the proper targets, often simultaneously, with cells that fail do any of the above pruned from the population by apoptosis. Adapted from (Glebova and Ginty 2005).

Figure 1.3: Timecourse of Developmental Milestones in Sympathetic Neuron Development

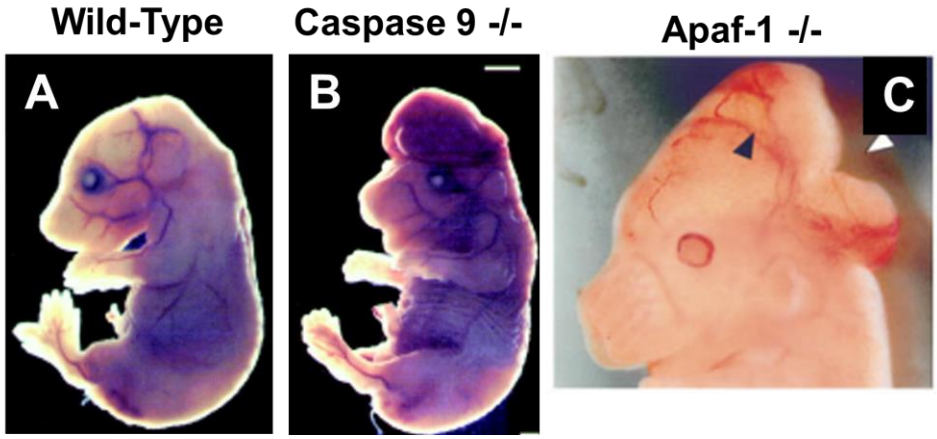


Glebova, NO and Ginty, DD. 2005
Annu. Rev. Neurosci. 28: 191–222

Figure 1.4: Apoptosis is Essential for Proper Brain Development

Inhibition of apoptosis during development leads to defects in brain morphology and embryonic lethality. Mice deficient in Caspase-9 (Panel B) or Apaf-1 (Panel C) exhibit brain and craniofacial defects during embryonic development when compared to wild-type mice. Caspase 9 knockout embryos are age E16.5, while Apaf-1 knockout embryos are age E13.5.

Figure 1.4: Apoptosis is Essential for Proper Brain Development



Adapted from (Kuida, Haydar et al. 1998; Yoshida, Kong et al. 1998)

Figure 1.5: The Intrinsic Apoptotic Pathway in Sympathetic Neurons

In sympathetic neurons, apoptotic stimuli, such as growth factor deprivation, lead to the activation of Mixed Lineage Kinases (MLKs). MLKs then phosphorylate and activate members of the c-Jun N-Terminal Kinase (JNK) family of proteins, which phosphorylate the transcription factor c-Jun. Upon phosphorylation, c-Jun translocates from the cytoplasm to the nucleus, where it cooperates with other transcription factors to upregulate multiple redundant members of the pro-death BH3-only domain family of proteins. Increased levels of BH3-only proteins promote apoptosis both by directly activating Bax and neutralizing the anti-apoptotic members of the BCL-2 family (BCL-2, BCL-XL, BCL-w, and MCL-1) which oppose Bax activation. Once activated, Bax undergoes conformational changes, resulting in its oligomerization and translocation to the mitochondria, where it mediates the permablization of the outer mitochondrial membrane and the release of the electron transport chain protein cytochrome *c* (cyt *c*) into the cytoplasm. Once in the cytoplasm, cyt c forms the apoptosome complex with Apaf-1 and procaspase 9, resulting in procaspase 9 cleavage and activation. Active Caspase 9 then cleaves Caspase 3, which in turn cleaves a large number of downstream substrates, ultimately resulting in the death of the cell.

Figure 1.5: The Intrinsic Apoptotic Pathway in Sympathetic Neurons

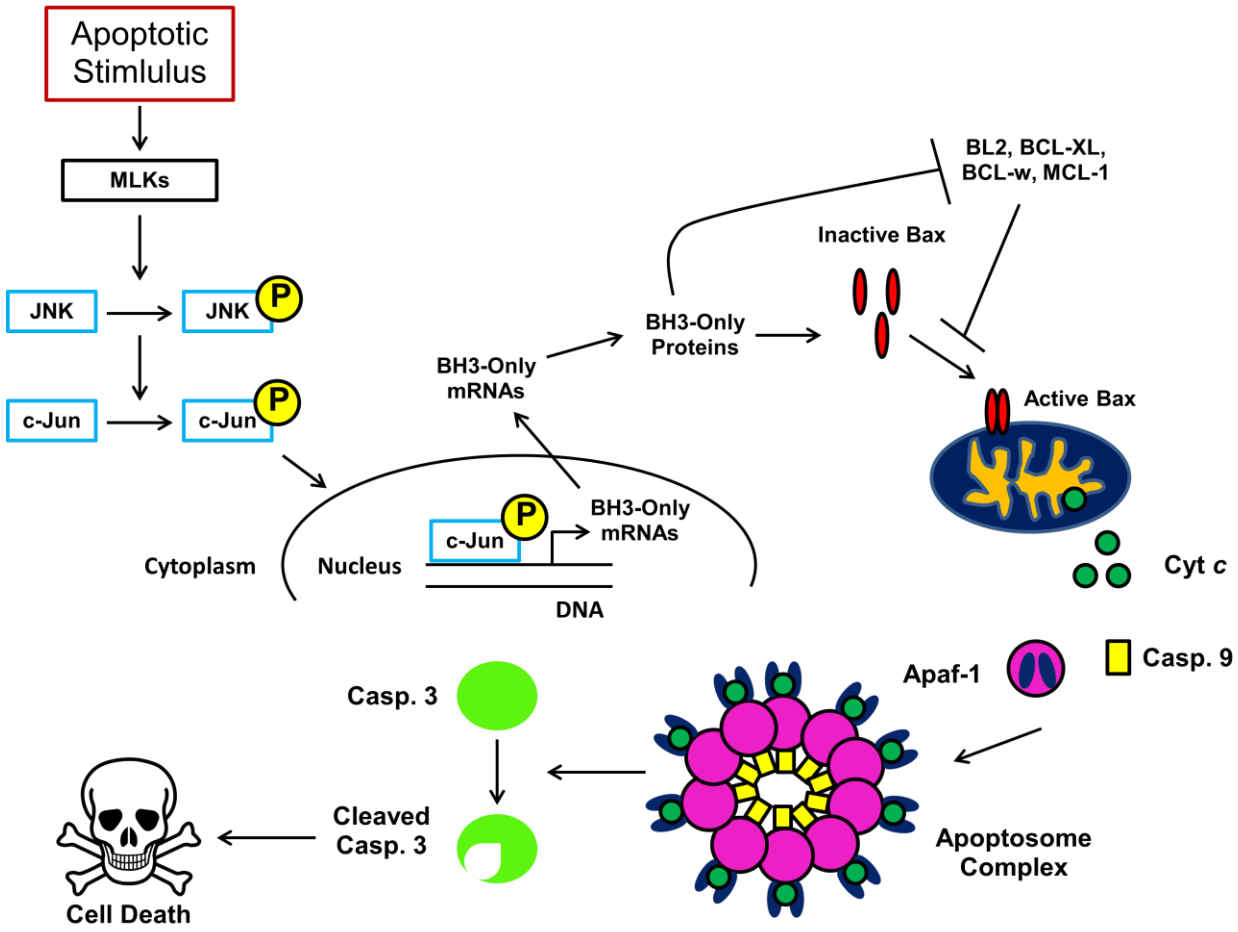
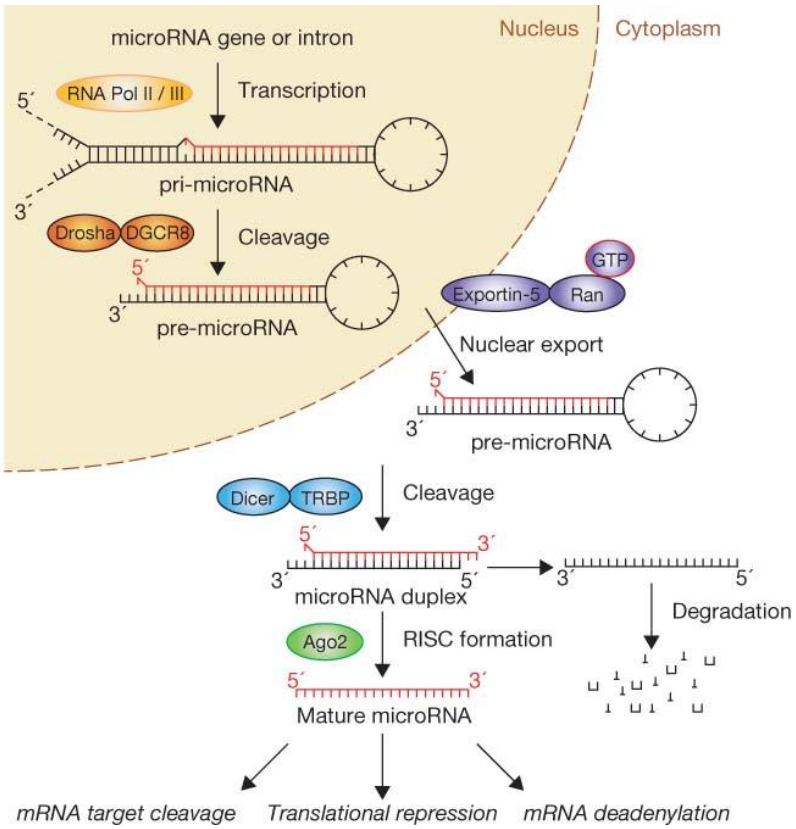


Figure 1.6: Overview of miRNA Biogenesis and Function

miRNAs are transcribed from genomic DNA by RNA Polymerase II to generate pri-miRNAs, which are then cleaved by Drosha and DGCR8 to generate pre-miRNAs. Pre-miRNAs are in turn exported from the nucleus via an Exportin-5-dependent process, where they are further cleaved by Dicer to generate mature miRNAs. These miRNA duplexes are loaded into the RISC Complex, after which one strand, the passenger strand, is removed and degraded, leaving the other strand, the guide strand, bound to the RISC complex. The miRNA in the RISC complex binds to target mRNAs and mediates their post-transcriptional repression via RNA degradation or translational repression.

Figure 1.6: Overview of microRNA Biogenesis

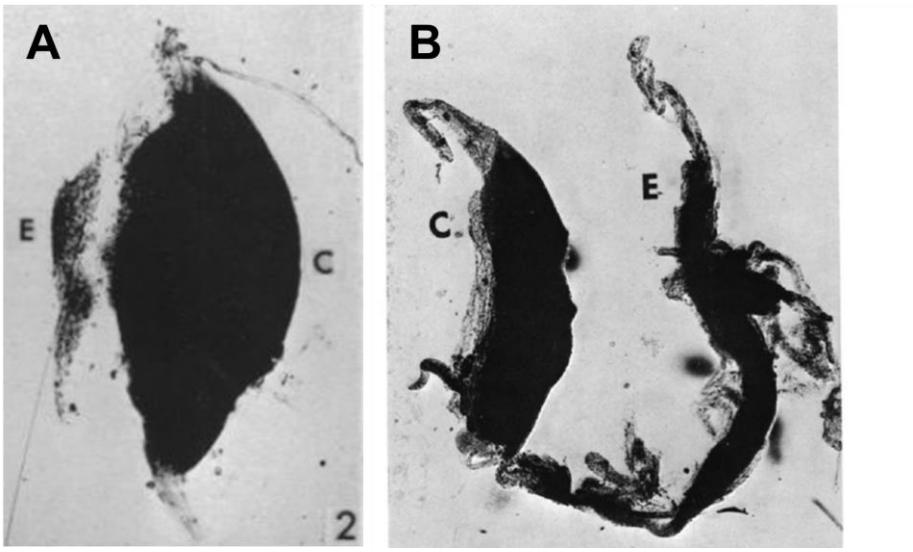


Adapted from (Winter, Jung et al. 2009)

Figure 1.7: Resistance of Sympathetic Neurons to Apoptosis with Maturation

Neonatal mice injected with a neutralizing antibody to NGF (Panel A, Ganglion E) exhibit dramatic atrophy compared to control-injected mice (Panel A, Ganglion C). In contrast, adult mice injected with a neutralizing antibody to NGF (Panel B, Ganglion E) display markedly reduced atrophy compared to ganglia from control injected mice (Panel B, Ganglion C).

Figure 1.7: Resistance of Sympathetic Ganglia to Apoptosis with Maturation



Adapted from Levi-Montalcini and Booker, 1960 and Angeletti et al., 1970

1.14 Tables

Table 1.1: miRNAs in Neurodegeneration and Stroke

Disorder	miRNA	Status	Mechanism	Citation
Alzheimer's Disease	**miR-107**	**Decreased**	**Targets BACE1 to inhibit Beta-Amyloid Production**	**Wang et al., 2008**
			Targets Cofilin	Yao et al., 2010
	miR-29	Decreased	Targets BACE1 to inhibit Beta-Amyloid Production	Hebert et al., 2008
	miR-298	Decreased (Mouse)	Targets BACE1 to inhibit Beta-Amyloid Production	Boissonneault et al., 2009
	miR-328	Decreased (Mouse)	Targets BACE1 to inhibit Beta-Amyloid Production	Boissonneault et al., 2009
	miR-15a	Decreased	Targets ERK1, which is can phosphorylate Tau.	Hebert et al., 2010
	miR-153	Decreased	Represses expression of APP.	Long et al., 2012
Huntington's Disease	miR-9/9*	Decreased	Represses expression of REST/CoREST.	Packer et al., 2008
Parkinson's Disease	miR-7	Decreased	Targets α-Synuclein	Junn et al., 2009
	miR-34	Decreased	Deletion leads to neurodegeneration in *Drosophila.*	Minones-Moyano et al., 2011
	miR-433	Target Mutated	Increased FGF20 Expression leads to increased α-Synuclein expression.	Wang et al., 2008
ALS	miR-206	Increased	Slows ALS progression by inhibiting HDAC4 and FGF Pathways.	Williams et al., 2009
Stroke	miR-21	Increased	Targets and downregulates FASLG.	Buller et al., 2010
	miR-124a	Increased	Mimic delivery decreases infarct area and apoptosis	Sun et al., 2013
	miR-29	Decreased	miR-29 mimic delivery decreases infarct size and improves behavioral outcomes.	Khanna et al., 2013

CHAPTER 2: MATURE NEURONS DYNAMICALLY RESTRICT APOPTOSIS VIA REDUNDANT PRE-MITOCHONDRIAL BRAKES

2.1: Introduction

In recent years, it has become increasingly clear that the threshold to undergo apoptosis can be markedly different in different cell types. For example, primary mitotic cells are sensitive to apoptotic insults, whereas postmitotic cells such as neurons, cardiomyocytes and myotubes have acquired mechanisms for restricting apoptosis (Benn, Perrelet et al. 2002; Potts, Singh et al. 2003; Sanchis, Mayorga et al. 2003; Mayorga, Bahi et al. 2004; Nam, Mani et al. 2004; Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005; Wright, Smith et al. 2007; Vaughn and Deshmukh 2008; Smith, Huang et al. 2009; Kole, Swahari et al. 2011; Xiao, Ferry et al. 2011; Gama, Swahari et al. 2014; Oláh, Szczesny et al. 2015). Such differences in the regulation of apoptosis are physiologically important because while mitotic cells are at continual risk of becoming cancerous and need to maintain their ability to die rapidly, this risk is significantly lower in terminally differentiated postmitotic cells. Indeed, the ability of organisms to maintain the long-term survival of postmitotic cells such as neurons is critical for normal physiological functions (Wright et al., 2002).

What is less appreciated is that even within the same cells, the apoptotic pathway can sometimes undergo dynamic changes during and after development. A cell type that exemplifies this phenomenon is neurons, where the apoptotic pathway becomes

highly restricted as young neurons become mature (Kole, Annis et al. 2013). Apoptosis plays an important role in the developing nervous system, where it is estimated that more than 50% of neurons that are initially produced will die by apoptosis as a part of normal neuronal development (Oppenheim 1991). However, once the nervous system is fully formed and neurons are appropriately wired, it is physiologically important for these neurons to survive long term. Indeed, the apoptotic pathway becomes highly restricted with neuronal maturation, but the molecular details are not completely understood.

Mouse sympathetic neurons provide an excellent model system for studying the regulation of the apoptotic pathway during neuronal maturation. Young P5 (postnatal day 5) neurons can undergo apoptosis in response to multiple stimuli including nerve growth factor (NGF) withdrawal, DNA damage, and Endoplasmic Reticulum stress. In contrast, those same neurons after 4-5 weeks (P28) become remarkably resistant to the same apoptotic stimuli (Kole et al., 2011; Easton et al., 1997; Putcha et al., 2000). Apoptotic stimuli in neurons are known to transcriptionally upregulate multiple redundant members of the pro-apoptotic BH3-only family of proteins (Kristiansen et al., 2011). These proteins activate Bax, which then permeablizes the mitochondria to induce the release of cytochrome *c* (cyt *c)* into the cytosol (Putcha, Deshmukh et al. 2000; Ham, Towers et al. 2005). Once in the cytosol, cyt *c* binds to Apaf-1 (Apoptotic Activating Protease 1) and forms the apoptosome complex with procaspase-9. Autoactivation of caspase-9 on the apoptosome can then activate caspase-3 to ultimately trigger cell death (Wang 2001).

We and others have previously shown that one mechanism by which mature neurons become resistant to apoptosis is via the epigenetic silencing of Apaf-1(Wright et al., 2007; Donovan and Cotter, 2002; Madden et al., 2007; Yakovlev et al., 2001). However, mature neurons induced to undergo apoptosis with NGF deprivation fail to release cyt *c*, (a process that is unaffected by Apaf-1 levels), despite maintaining Bax levels (Easton et al., 1997; Putcha et al., 2000). Interestingly, mature neurons still undergo the initial steps in the apoptotic pathway, such as c-jun phosphorylation, after NGF deprivation (Easton et al., 1997). This suggested the presence of one or more brakes upstream of cyt *c* release but downstream of c-jun phosphorylation in mature neurons. Indeed, mature neurons were found to have markedly elevated levels of the microRNA (miRNA) miR-29, which targets and represses multiple redundant members of the BH3-only family of proteins (Kole et al., 2011). A neuroprotective role for miR-29 is further supported by the results of *in vivo* models of neuronal insult, which have found that overexpression of miR-29 is able to reduce cell death in ischemic stroke (Khanna, Rink et al. 2013; Ouyang, Xu et al. 2013; Pandi, Nakka et al. 2013), spinal cord injury (Liu et al., 2015), and ethanol-induced toxicity (Qi et al., 2014). However, it is unclear at this point whether miR-29 induction is the only brake employed to inhibit cyt *c* release in mature neurons, or if other redundant brakes also exist.

We find that deletion of all three miR-29 family members fails to re-sensitize mature neurons to apoptosis, with mature miR-29 knockout neurons remaining significantly resistant to cyt *c* release induced by NGF deprivation. Here we report that another miRNA, miR-24, is also upregulated with neuronal maturation and is capable of acting redundantly with miR-29 to inhibit cyt *c* release by targeting a similar subset of BH3-only

genes. Our results highlight the ability of mature neurons to engage multiple, redundant mechanisms to restrict the apoptotic pathway and help ensure their long-term survival.

2.2 Results

Maturing neurons simultaneously restrict apoptosis both pre- and post-mitochondria

In previous studies of neuronal maturation, the mechanisms by which mature neurons become resistant to apoptosis have been investigated in post-natal day 28 neurons. At this time-point, neurons engage multiple mechanisms to restrict apoptosis both up-stream and down-stream of mitochondrial permeablization. However, the exact timing for when these brakes are initiated during the maturation process is not known. Therefore, to investigate the time course and mechanisms by which neurons become resistant to apoptosis with maturation, we matured neurons for increasing lengths of time and assessed their susceptibility to apoptotic stimuli. Sympathetic neurons were isolated from neonatal (postnatal day 0-1) mouse pups and maintained in culture for 5-25 days. Apoptosis was then induced by deprivation of Nerve Growth Factor (NGF). While neurons maintained in culture for 5-7 days remained vulnerable to NGF deprivation-induced apoptosis, marked resistance to apoptosis was observed by as early as 9 days in vitro (DIV) (Figure 2.1A, B).

Neurons have been previously shown to restrict apoptosis both before and after the mitochondrial checkpoint. To more precisely define whether these two separate brakes were engaged simultaneously or sequentially, we specifically assessed apoptosis

restriction at the pre- and post-mitochondrial checkpoint. First, to assess the post-mitochondrial resistance to apoptosis, we microinjected maturing neurons with purified cyt *c*. These experiments were conducted in neurons from XIAP-deficient mice as XIAP (X-Linked Inhibitor of Apoptosis) is known to inhibit cyt *c*-induced apoptosis even in young neurons (Potts et al., 2003). As reported earlier, neurons ($XIAP^{-/-}$) maintained for 5 DIV exhibited massive cell death in response to cytosolic cyt *c* injection, with virtually 100% of neurons dying within 24 hours of injection. Approximately 50% of neurons became resistant to cyt *c* injections by 10 DIV, with greater than 80% exhibiting resistance by 15 DIV (Figure 2.1C).

Second, to assess pre-mitochondrial resistance to apoptosis, we subjected maturing neurons to NGF deprivation and assessed whether cyt *c* was maintained at the mitochondria or released into the cytosol by immunofluorescence. As mitochondrially-released cyt *c* is rapidly degraded in neurons, loss of cyt *c* signal is an established indicator of its release from mitochondria in these cells (Gama et al., 2014). Consistent with the results of our neuronal survival experiments, neurons by 10 DIV exhibited significant resistance to NGF-deprivation induced cyt *c* release, with approximately 50% of neurons maintaining cyt *c* after 48 hrs of NGF deprivation. Nearly complete resistance at this pre-mitochondrial checkpoint was seen in 15 DIV neurons (Figure 2.1D, E). Together, these results show that both pre- and post-mitochondrial brakes are engaged concurrently in maturing neurons, resulting in resistance to apoptosis within 2 weeks in culture.

Loss of miR-29 is not sufficient to re-sensitize mature neurons to apoptosis

Recent studies in our lab have identified the miR-29 family as regulators of apoptosis in maturing neurons. miR-29 is induced during neuronal maturation (Figure 2.2A), and can inhibit the induction of multiple members of the BH3-only family of proteins to prevent Bax activation and apoptosis (Kole et al., 2011). We hypothesized that loss of miR-29 expression may render mature neurons sensitive to apoptosis. miR-29 has three family members (miR-29a,b,c) that are expressed from two separate genomic loci. Thus, to completely delete miR-29 in sympathetic neurons, mice floxed at both miR-29 loci were crossed with a mouse line expressing Cre recombinase under a tamoxifen inducible promoter (ER-Cre). Neurons isolated from these mice were then treated with 4-hydroxy-tamoxifen *in vitro* to induce recombination and generate miR-29 deficient neurons. Loss of miR-29 expression was verified by RT-qPCR comparing cre-positive (hereafter referred to as miR-29 KO) and Cre-negative (hereafter referred to as miR-29 WT) littermates, with mature miR-29 KO neurons exhibiting virtually no detectable miR-29 as compared to WT controls (Figure 2.2B).

miR-29 WT and miR-29 KO neurons were matured in culture until 35 DIV and subjected to NGF deprivation to induce apoptosis. Since miR-29 is known to inhibit apoptosis upstream of the mitochondria (and mature neurons are known to engage an additional brake downstream of mitochondria), we specifically examined mitochondrial release of cyt *c* as a readout for these experiments. We were surprised to find that

mature miR-29 KO neurons still exhibited negligible cyt *c* release in response to NGF deprivation (Figure 2.2C, D).

Since miR-29 is known to be able to target multiple pro-apoptotic BH3-only domain genes and inhibit their induction, we next assayed BH3-induction in young wild-type, mature wild-type, and mature miR-29 knockout neurons. Consistent with our findings that mature miR-29 knockout neurons fail to release cyt *c*, we found that miR-29 knockout neurons also failed to substantially induce BH3-only domain genes in response to 48 hours of NGF deprivation when compared to young neurons as measured by RT-qPCR (Figure 2.2E). These results indicate that loss of miR-29 expression alone is not sufficient to re-sensitize mature neurons to apoptosis, and led us to hypothesize that other brakes could be acting redundantly with miR-29 to the induction of BH3-only genes, cyt *c* release, and apoptosis in mature neurons.

Other microRNAs with predicted targets in the apoptotic pathway are upregulated during neuronal maturation

To identify other miRNAs that are induced during maturation and capable of acting redundantly with miR-29 to inhibit cyt *c* release and apoptosis, we utilized two approaches (Figure 2.3A). First, we used TargetScan software (www.targetscan.org) to identify predicted miRNAs that could target the BH3-only genes. Second, we reviewed existing literature on miRNAs that are known to increase with brain maturation, in addition to performing small-RNA-Seq on maturing cerebellum, to identify miRNAs that were upregulated in maturing neurons. While the fold increase in miR-29 was the most

striking (Figure 2.2A, Table 2.1), we identified three other miRNAs upregulated more than 5-fold in maturing neurons that were predicted to target the 3' UTRs of multiple BH3-only family genes: miR-24, miR-124, and miR-128 (Figure 2.3B). We confirmed the upregulation of these miRNAs during neuronal maturation using RT-qPCR in developing cerebellum (Figure 2.3C-E) and sympathetic neurons (Figure 2.3F-H). These results identify miR-24, miR-124, and miR-128 as potential candidates that could act redundantly with miR-29 to restrict apoptosis in mature neurons.

Overexpression of miR-24 in Young Neurons Inhibits Cyt c Release and Cell Death

To determine whether any of the selected miRNAs were capable of inhibiting BH3-only gene induction and cell death in a manner similar to miR-29, we overexpressed mimics to the selected miRNAs in young neurons using single-cell microinjection. Neurons isolated from neonatal mice were injected at 3 DIV with selected mimics or negative control miRNA and, after 48 hours, were deprived of NGF to induce apoptosis. As a positive control, we also injected neurons with a mimic to miR-29b which, as reported previously, inhibited cyt *c* release in young neurons. Among the candidate miRNAs, we found only miR-24 overexpression to be capable of inhibiting cyt *c* release in neurons deprived of NGF (Figure 2.4A, B). The ability of miR-24, but not miR-124 and miR-128, to inhibit cyt *c* release is likely because miR-24 is predicted to target more members of the redundant BH3-only family than either miR-124 or miR-128 (Figure 2.3B). Importantly, we also examined whether miR-24 expression could inhibit

apoptosis. Consistent with the observed effect on cyt *c* release, we found that miR-24 significantly inhibits apoptosis in NGF-deprived young neurons (Figure 2.4C).

miR-24 Can Inhibit Bim and Puma Induction in NGF-Deprived Neurons

While previously published reports have validated miR-24 binding sites in the Bim 3'UTR, and miR-24 has been previously shown to downregulate Bim expression and inhibit apoptosis in cardiac tissue (Qian et al., 2011), its potential role in regulating neuronal apoptosis has not been investigated. To examine if miR-24 could inhibit Bim induction in neurons, young (P3 equivalent) sympathetic neurons were injected with mimics to miR-24, miR-29 (positive control), or a negative control miRNA, and were then subjected to NGF deprivation to trigger Bim induction. Just as seen with miR-29, miR-24 expression potently suppressed Bim induction in NGF-deprived neurons (Figure 2.5A, B).

We next sought to determine if miR-24 could also target Puma, another BH3-only family member that has been found to act redundantly with Bim to induce neuronal apoptosis (Ren et al., 2010). Puma has two conserved miR-24 sites in its 3'UTR (Figure 2.5C). To validate these sites, we cloned the 3'UTR of Puma into a luciferase reporter vector. We then cotransfected this vector with either a negative control miRNA or a miR-24 mimic into HEK293T cells. We found that miR-24 was indeed able to significantly repress the expression of luciferase in cells expressing the Puma 3'UTR compared to a negative control (Figure 2.5D).

To test the functional ability of miR-24 expression to inhibit Puma, we took advantage of the fact that in sympathetic neurons, cyt *c* release and apoptosis in response to DNA damage is known to be entirely dependent on Puma expression (Wyttenbach and Tolkovsky 2006). Thus, we examined whether miR-24 expression was able to effectively inhibit cyt *c* release in young neurons treated with the DNA damaging agent etoposide. Consistent with our observation that miR-24 is able to target Puma, we found that the release of cyt *c* was inhibited in young neurons expressing miR-24 in response to etoposide treatment (Figure 2.5E, F). Together, these results identify miR-24 as a miRNA that is not only induced with neuronal maturation, but as seen with miR-29, also targets multiple BH3-only genes and inhibit neuronal apoptosis.

Inhibition of miR-24 in miR-29-deficient neurons leads to partial restoration of apoptotic response

Given our observation that miR-24 is induced in mature neurons and is able to target similar BH3-only genes as miR-29, we hypothesized that miR-24 and miR-29 may act redundantly to inhibit the release of cyt *c* in mature neurons. Thus, we sought to determine if inhibition of miR-24 function in mature miR-29 KO neurons would render these cells more sensitive to apoptosis. Mature miR-29 KO neurons were injected with a locked nucleic acid (LNA) targeting miR-24 designed to inhibit its activity, or a negative control LNA. Neurons were then deprived of NGF and the status of cyt *c* release was quantified using immunofluorescence. Inactivation of miR-24 and miR-29 together rendered mature neurons more sensitive to NGF deprivation than seen with miR-29 deficiency alone (Figure 2.6 A, B). However, the majority of mature neurons still failed to release cyt *c*, raising the possibility that yet more redundant brakes likely exist to

restrict the apoptotic pathway in mature neurons. Indeed, when we examined whether mature sympathetic neurons express nuclear phospho-c-jun, an early marker for engagement of the apoptotic pathway, we found that while the vast majority of young neurons exhibit nuclear phospho-c-jun at this timepoint, only a small fraction mature neurons did (Figure 2.6 C, D).

2.3: Discussion

The ability of maturing neurons to dynamically switch the apoptotic pathway from a permissive to a restrictive state is of critical importance. This allows young neurons to permit physiological apoptosis during nervous system development, but once established, to highly restrict apoptosis to promote the long term survival of mature neurons. Previous studies had identified miR-29-mediated inhibition of BH3-only genes and the epigenetic silencing of the Apaf-1 promoter as mechanisms by which apoptosis is restricted pre- and post-mitochondria in mature sympathetic neurons (Wright et al., 2007; Kole et al., 2011). Our results now highlight the finding that the apoptotic pathway is even more restricted in adult neurons than previously appreciated, with mature neurons engaging redundant brakes at the pre-mitochondrial checkpoint.

Our previous results brought focus on the neuroprotective capability of miR-29 in mature neurons. Not only is miR-29 markedly induced with neuronal maturation but expression of miR-29 alone is sufficient to inhibit apoptosis in young neurons by its ability to target the BH3-only gene family. Delivery of miR-29 can also confer neuroprotection in models of stroke and spinal cord injury and alcohol-induced toxicity *in*

vivo (Ouyang et al., 2013; Khanna et al., 2013; Pandi et al, 2013; Liu et al., 2015; Qi et al., 2014). Our results now show that while overexpression of miR-29 promotes neuronal survival, loss of miR-29 failed to restore the ability of mature neurons to release cyt *c* in response to NGF-deprivation. Thus, endogenous miR-29 does not seem to be the only brake restricting apoptosis at the pre-mitochondrial checkpoint in mature neurons. These results prompted us to identify other brakes that function redundantly with miR-29 to effectively inhibit apoptosis in mature neurons.

Our finding that miR-24 is upregulated in maturing cerebellum and sympathetic neurons is consistent with recent findings that miR-24 expression is up-regulated in maturing cerebral cortex (Chen, Xu et al. 2014). miR-24 is also a miRNA that has been found to be a key regulator of apoptosis in ischemic heart muscle through its ability to target Bim, a BH3-only protein also known to be important for neuronal apoptosis (Qian, Van Laake et al. 2011).The observation that the upregulation correlates with an increase in resistance to apoptosis made miR-24 an attractive candidate as a molecule that may act redundantly with miR-29 to inhibit apoptosis. Indeed, our results show that expression of mir-24 in young sympathetic neurons can target the BH3-only proteins Bim and Puma, which are thought to be the two most important BH3-only proteins for neuronal cell death (Ren et al., 2010), and inhibit apoptosis at the level of cyt *c* release. It is also interesting to note that miR-24 is expressed in two separate clusters with two other miRNAs, miR-23a/b and miR-27a/b. Recent studies have found that miR-23a/b and miR-27a/b are also capable of inhibiting neuronal apoptosis in cases of traumatic brain injury or ischemia (Chen, Xu et al. 2014; Sabirzhanov, Zhao et al. 2014). Thus,

miRNAs of this cluster may work synergistically to inhibit the apoptotic pathway in the adult brain.

Our finding that inhibition of miR-24 in miR-29 knockout neurons has only a marginal effect on the ability of neurons to release cytochrome *c* in response to NGF deprivation suggests the presence of further redundant brakes in mature neurons that restrict apoptosis. Our finding that c-jun-phosphorylation is decreased in mature neurons is consistent with this. This decrease in c-jun-phosphorylation may be due to prolonged TRK-A phosphorylation in mature neurons (Tsui-Pierchala and Ginty 1999), or may represent another, as yet uncharacterized apoptotic brake engaged in mature neurons. These results differ from those previously published in Rat sympathetic neurons (Easton, Deckwerth et al. 1997), and may reflect interspecies differences in the regulation of apoptosis in mature neurons.

Increased resistance to apoptosis with maturation has been observed in many populations of neurons in response to diverse insults. For example, the neonatal brain is far more vulnerable to hypoxia ischemia-induced apoptosis or traumatic brain injury than the adult brain (Hu et al., 2000). Likewise, greater numbers of neurons survive nerve crush or axotomy if the injury is done on 3-week-old mice as compared to 1-week-old mice (Snider et al., 1992). Our work provides mechanistic insight into the multiple brakes engaged by mature neurons that allow these cells to withstand diverse stresses and survive long-term.

An interesting question here is why do mature neurons not shut down all the components of the apoptotic pathway? Mature sympathetic neurons repress Apaf-1 but

continue to express many apoptotic proteins including Bax, caspase-9 and caspase-3 (Wright et al., 2007). A potential explanation for this has come from recent studies that have found these proteins to have functions outside of the canonical apoptotic pathway. For example, Bax is known to regulate mitochondrial dynamics and caspases-9 and -3 have essential roles in synaptic plasticity and axon pruning, which are events that are important for optimal neuronal function and plasticity (Unsain and Barker 2015). Thus, maintaining these proteins, albeit with increased regulation, permits mature neurons to utilize these proteins while simultaneously limiting the risk of apoptosis.

Despite the mechanisms described here that provide neurons with improved capacity to survive injury and apoptosis, adult neurons can still be vulnerable and undergo cell death in situations of acute brain injury or neurodegenerative disease. It is possible that even the partial removal of these apoptotic brakes that could occur with injury or chronic neurodegeneration could increase the vulnerability of adult neurons. Indeed, consistent with a role of miR-29 in neuroprotection, its levels are reduced in Alzheimer's disease, Huntington's disease, and during hypoxic-ischemic brain injury (Khanna et al., 2013; Shioya et al., 2010; Johnson et al., 2008). Restoring these brakes on the apoptotic pathway via overexpression of miR-29 or miR-24 could be an effective therapeutic strategy that promotes long-term neuroprotection. A previous study reported that mice partially deleted for miR-29 (deletion of the miR-29a/b1 loci) had grossly normal brains but exhibited an ataxic phenotype (Papadopoulou, Serneels et al. 2015). Our findings that apoptosis is not increased in miR-29 knockout neurons support the hypothesis that observed phenotypes in these studies are more likely to be due to the effect of miR-29 on neuronal function rather than survival.

Together, our results identify miR-29 as sufficient but not solely responsible for the inhibition of the apoptotic pathway in mature neurons, and highlight the redundant brakes employed by mature neurons to inhibit apoptosis and promote long-term survival.

2.4 Materials and Methods

Cell Culture

For young neurons, sympathetic ganglia were isolated and cultured from neonatal CD1 mice unless otherwise indicated as described previously (Potts et al., 2003). All animal handling and protocols were carried out in accordance with established practices as described in the National Institutes of Health Guide for Care and Use of Laboratory Animals and as approved by the Animal Care and Use Committee of the University of North Carolina (UNC).

Neurons were then maintained in culture for 5 days. For mature neurons, ganglia from P5-P12 mice were isolated and cultured as previously described until 28-35 DIV (Wright et al., 2007). NGF deprivation experiments were performed by washing cells three times with media containing no NGF, then refeeding cells in NGF-free media containing NGF-neutralizing antibody. For DNA damage experiments, cells were treated with 20 µM Etoposide (Sigma). For luciferase assay experiments, HEK293T cells were grown in DMEM/F12 medium supplemented with 10% FBS, 100u/mL penicillin, and 100µg/mL streptomycin.

RNA Isolation and RT-qPCR

RNA was isolated from tissue or cultured cells using the Zymo Research Direct-zol RNA MiniPrep kit (Genesee) according to the manufacturer's instructions. Mature miRNA expression was determined using hydrolysis probe based-miRNA assays (Taqman/Life Technologies). RT primers specific for miR-29a, miR-29b, miR-29c, miR-24, miR-124, miR-128 and U6 were used to amplify the indicated genes from 10 ng of

isolated RNA using the Superscript III Reverse Transcriptase system (Life Technologies) according to manufacturer instructions. cDNA was amplified using TaqMan universal PCR master mix (Life Technologies) on an ABI 7500 Real-Time PCR system. Relative quantification and statistical comparisons were performed using the delta-delta-ct method. Samples were internally normalized to U6 SnoRNA expression. For non-miRNA gene expression, cDNA libraries were prepared using 50-100 ng RNA. RNA was pre-treated with RQ1 DNase (Promega) for 30 minutes at 37°C followed by 10 min incubation with DNAse Stop Solution at 65°C for 10 min. cDNA was reverse-transcribed using random hexamers (Invitrogen) and the Superscript III Reverse Transcriptase System according to manufacturer instructions. cDNA was diluted 1:20 in each qPCR reaction, along with 400 nM of forward and reverse primers and Power SYBR Green PCR master mix (Applied Biosystems). BH3-only gene and GAPDH primers have been previously published (Kole et al., 2011). Reactions were amplified in an ABI 7500 Real-Time PCR system. Relative quantification and statistical comparisons were performed using the delta-delta CT method. Samples were internally normalized to GAPDH expression.

Small RNA sequencing library production and mapping

Libraries for Illumina sequencing were prepared using a modification of the TruSeq protocol. Briefly, 1 ug total RNA was ligated to 3 pmol of the 3' linker using T4 RNA ligase 2. RNA size fractions corresponding to 35- 70 nucleotides (insert plus linker) were gel isolated and ligated to 3 pmol of the 5' linker. Products were reverse transcribed, PCR amplified to mid-log phase, and size isolated. Libraries were barcoded using indexed 5' linkers. Libraries were sequenced on an Illumina HiSeq

2000. These libraries were aligned to the mm9 genome. miRNA annotations were downloaded from miRBase r18.

Immunofluorescence Staining

Immunofluorescence was carried out as previously described (Potts, Singh et al. 2003). The primary antibodies used were as follows: anti-cyt *c* (BD Biosciences #556432); anti-Tom20 (Santa Cruz sc11415); anti-Bim (Cell Signaling #2189); Anti-phospho-c-jun (Cell Signaling #9261) Secondary antibodies were anti-mouse Cy3 (The Jackson Laboratory) or anti-rabbit Alexa Fluor 488 (Life Technologies). Nuclei were stained with Hoechst 33258 (Molecular Probes). For analysis of cyt *c* release, neurons treated with apoptotic stimuli in the presence of 25 μM Q-VD-OPH to inhibit downstream caspase activation and preserve cells for staining. Images were acquired using an ORCA-ER digital B/W charge-coupled device camera (Hamamatsu) mounted to a DMI6000 microscope (Leica) using Metamorph 7.6 software and processed using Adobe Photoshop. For fluorescence intensity measurements, average pixel intensities were measured in individual injected neuronal cell bodies using Metamorph 7.6 software and were normalized to neighboring uninjected cell bodies on the same plate.

Assessment of Neuronal Survival

Neuronal survival was assessed by the presence of intact, phase bright cell bodies at the indicated time after treatment. Survival was quantified as the percentage of healthy cells at the indicated time point compared to immediately prior to treatment. This method of assessing survival correlates well with independent methods of measuring cell death such as trypan blue exclusion and calcein AM staining (Potts et al., 2003).

Cloning of Puma 3'UTR and Luciferase Assays

A 719-bp segment of the Puma 3'UTR was amplified from mouse genomic DNA and cloned into a modified PGL3-control plasmid (Promega), in which the multiple cloning site was placed downstream of the firefly luciferase gene. For luciferase assays, 60,000 HEK293T cells were plated into each well of a 12-well plate and transfected with 1.5 µg of PGL3-3'UTR reporter construct or empty vector, 100 ng phRL renilla luciferase (Promega) and 100 nM of either miR-24-3p or cel-mir-67 MIRIDIAN mimic (GE Dharmacon) as a negative control. Transfections were performed using lipofectamine 2000 according to manufacturer's instructions. 48 hours post transfection, cells were lysed and firefly- and renilla- luciferase intensities were measured using Promega Dual-Luciferase Reporter system on a Fluorscan Ascent Type 379 fluorescence plate reader (Thermo). Firefly luciferase intensity was normalized to renilla luciferase intensity to control for cell number and transfection efficiency.

Single Cell Microinjection

Cells were injected with Miridian mimics (GE Dharmacon) to miR-29, miR-24, miR-124, miR-128 or cel-miR-67 as a negative control (30 uM needle concentration) or miR-24/negative control LNAs (10uM needle concentration, Exiqon) as previously described (Kole et al., 2011). Briefly, mimics/LNAs were dissolved in sterile RNAse-free water and mixed with microinjection buffer containing 100 mM KCl and 10 mM KPi (pH 7.4) along with 8 mg/mL lysine-fixable Fluorescein Dextran (Invitrogen) to mark injected cells. For cyt *c* injections, 10 mg/mL of mammalian (bovine) or yeast cyt *c* were injected as

previously described (Potts et al., 2003) and cell survival was quantified by comparing the percentage of surviving cells immediately after injection and 24 hours post-injection.

Generation of miR-29 knockout neurons

To generate sympathetic neurons deficient for all three miR-29 family members, mice floxed at both genomic loci for miR-29 (miR-29a/b1 and miR-29b2/c)(kindly provided by Dr. He, Duke University) were crossed with ER-Cre (CAG-Cre/Esr1/ strain: 004453. Jackson Laboratories). Neurons were isolated from Cre-positive and Cre-negative littermates as previously described and treated with 500 nM 4-OH-tamoxifen (Sigma) for 10 days to induce recombination. Neurons were then matured until 28-35 DIV prior to experimental treatments. Cre-positive and negative littermates were genotyped for Cre expression using the following primers: Forward – gatggacatgttcagggatcgcc. Reverse: ctcccaccgtcagtacgtgagat. Knockout of miR-29 family expression was confirmed *via* RT-qPCR.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software version 5.0c. For comparisons between two means, unpaired student's T-Test was used. For comparisons between more than two groups, one-way ANOVA with Newman-Keuls multiple comparisons test was used. A p-value of less than 0.05 was considered significant.

Acknowledgements

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2.5 Figures and Figure Legends

Figure 2.1: Neuronal maturation is associated with progressive resistance to neuronal apoptosis at both pre and post-mitochondrial checkpoints. **A)** Representative images of identical fields of 5 DIV and 11 DIV neurons imaged at 24 hour intervals after NGF deprivation. **B)** Quantification of sympathetic neuronal survival in response to NGF deprivation for 48 hours after maturing in culture for the indicated amount of time. Data represent mean ± SEM of 3 independent experiments. **C)** Neurons isolated from neonatal $XIAP^{-/-}$ mice and cultured for the indicated time were injected with 10 mg/mL of either bovine cyt *c* to induce apoptosis or yeast cyt *c* as a negative control and survival was quantified 24 hours post-injection. Data are displayed as mean ± SEM of 3 independent experiments for 5, 10 and 15 DIV and 2 independent experiments for 20 and 25 DIV(**=P<0.01). **D)** Quantification of cyt *c* release from neurons cultured for the indicated time and deprived of NGF for 48 hours (**=P<.01, ***=P<.001). **E)** Representative images of cyt *c* and Tom20 staining in neurons matured for the indicated amount of time and maintained (+NGF) or deprived (-NGF) for 48 hours.

Figure 2.1

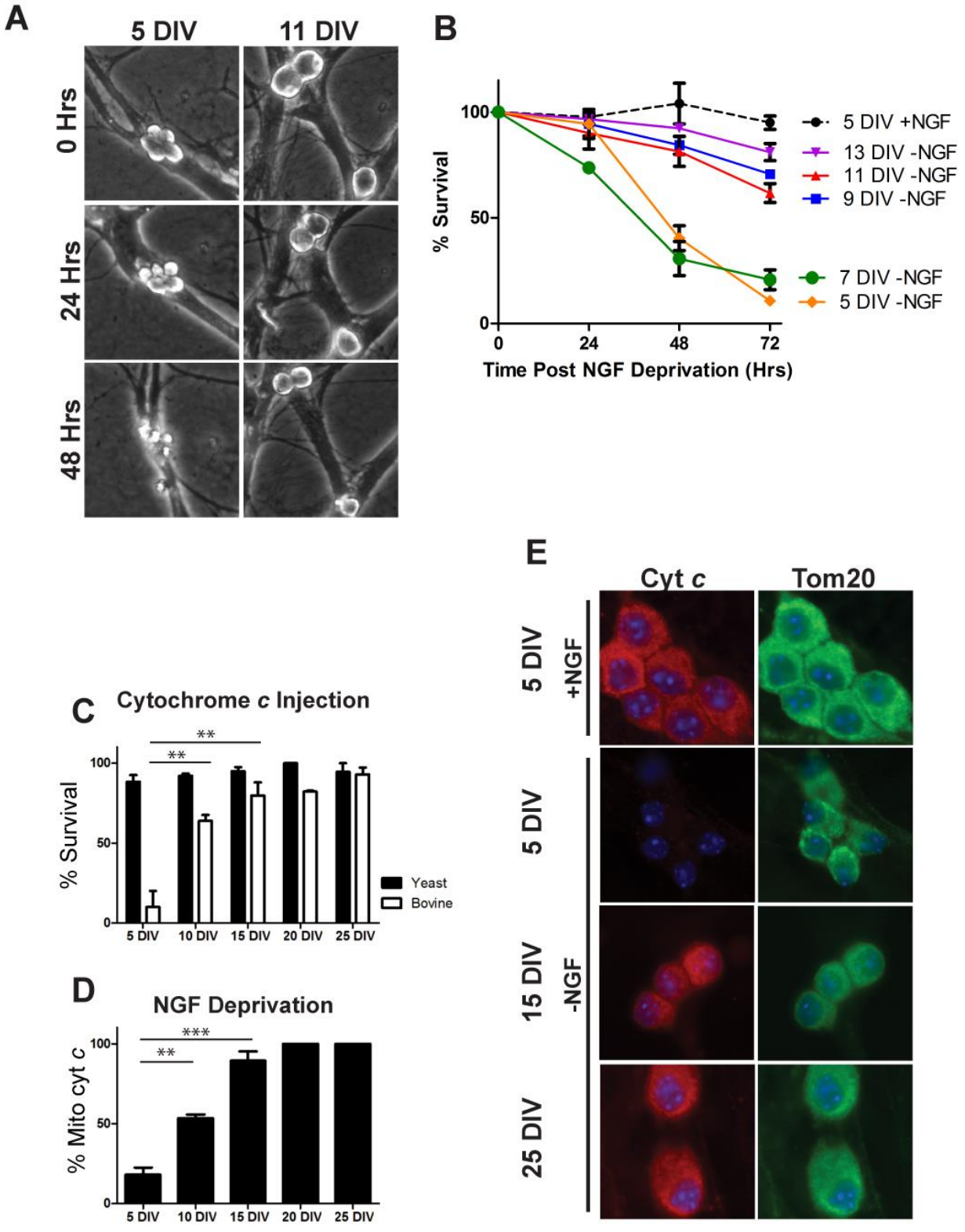

A
5 DIV
11 DIV
0 Hrs
24 Hrs
48 Hrs
B
% Survival
100
50
0
0
24
48
72
Time Post NGF Deprivation (Hrs)
5 DIV +NGF
13 DIV -NGF
11 DIV -NGF
9 DIV -NGF
7 DIV -NGF
5 DIV -NGF
C
Cytochrome c Injection
% Survival
**
**
100
50
0
5 DIV
10 DIV
15 DIV
20 DIV
25 DIV
Yeast
Bovine
D
NGF Deprivation
% Mito cyt c
***
**
100
50
0
5 DIV
10 DIV
15 DIV
20 DIV
25 DIV
E
Cyt c
Tom20
5 DIV
+NGF
5 DIV
15 DIV
25 DIV
-NGF

Figure 2.2: Mature miR-29 deficient neurons remain resistant to NGF-deprivation induced cyt *c* release. A) Timecourse of miR-29b induction in maturing neurons. Neurons were isolated from neonatal mice and cultured for the indicated amount of time and miR-29 levels were assessed by RT-qPCR. Values are expressed as mean fold change ± SEM relative to 5 DIV neurons from 3 independent experiments (**=p<0.01, ***=p<0.001, ns=not significant). **B)** Verification of miR-29 deficiency in mature mir-29 knockout neurons as assessed by RT-qPCR. Values are expressed as mean fold change ± SEM relative to WT 5 DIV neurons from 3 independent experiments. **C)** Representative images of cyt *c* staining in mature WT and miR-29 KO cells deprived of NGF for 48 hours. **D)** Quantification of cyt *c* release in young WT neurons and mature WT and mature miR-29 KO neurons deprived of NGF for 48 hours. Data represent the percentage of cells with mitochondrial cyt c and are presented as mean ± SEM of 3 independent experiments. **E)** RT-qPCR quantification of selected BH3-only genes in young, mature WT, and mature miR-29 KO neurons deprived of NGF for 48 hours. Fold changes were normalized to P5 +NGF neurons and represent mean ± SEM of 3 independent experiments.

Figure 2.2

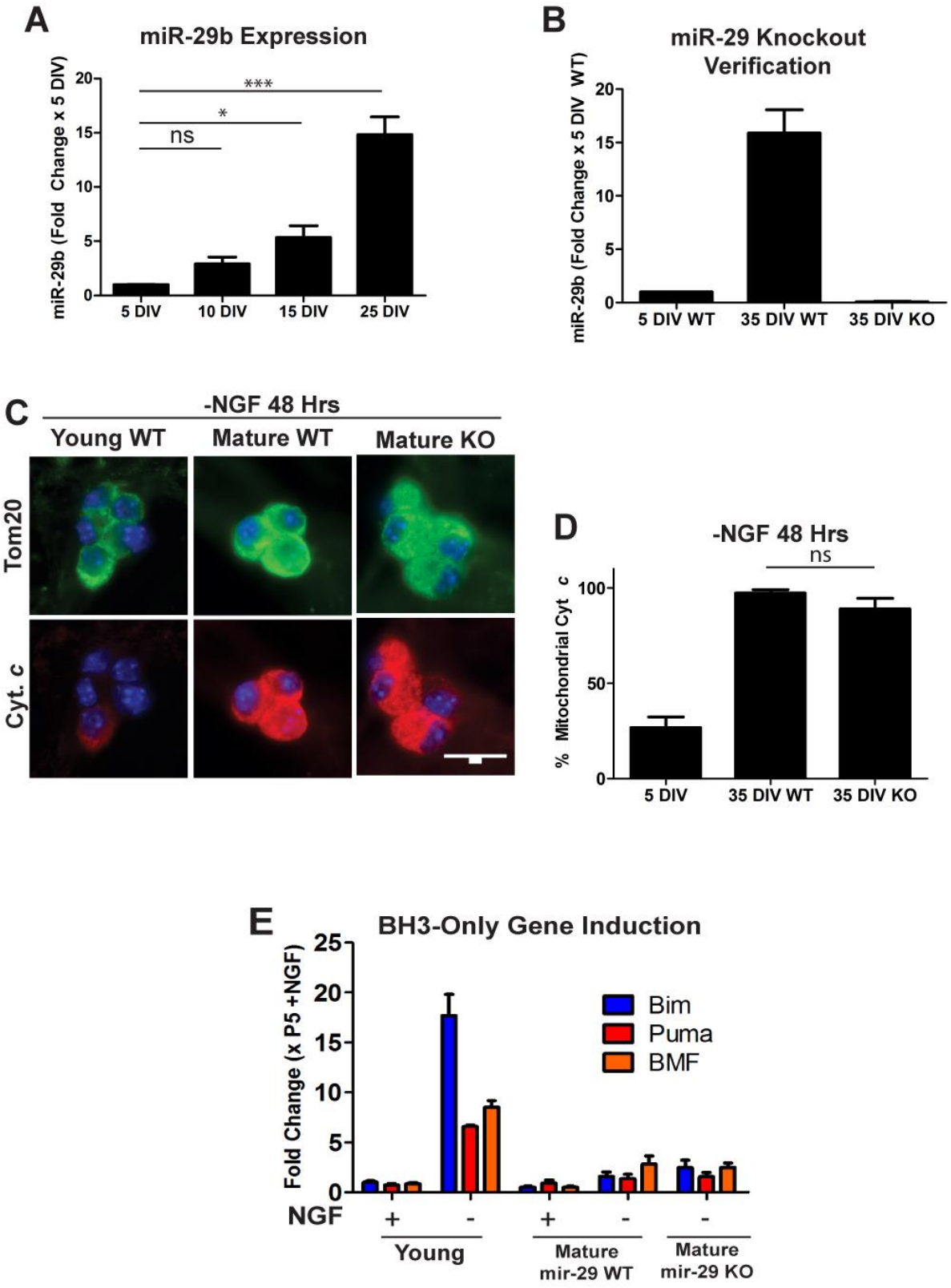
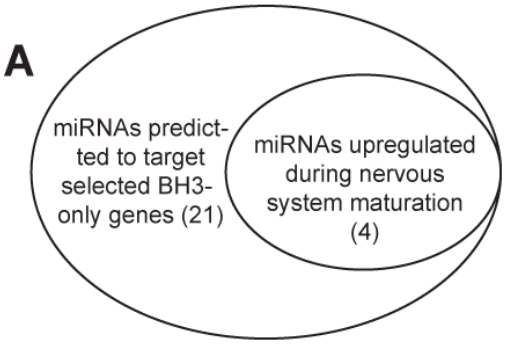

A
miR-29b Expression
miR-29b (Fold Change x 5 DIV)
***
*
ns
5 DIV
10 DIV
15 DIV
25 DIV
B
miR-29 Knockout Verification
miR-29b (Fold Change x 5 DIV WT)
5 DIV WT
35 DIV WT
35 DIV KO
C
-NGF 48 Hrs
Young WT
Mature WT
Mature KO
Tom20
Cyt. c
D
-NGF 48 Hrs
ns
% Mitochondrial Cyt c
5 DIV
35 DIV WT
35 DIV KO
E
BH3-Only Gene Induction
Fold Change (x P5 +NGF)
Bim
Puma
BMF
NGF
+
-
+
-
-
Young
Mature mir-29 WT
Mature mir-29 KO

Figure 2.3: Other miRNAs predicted to regulate the apoptotic pathway are also induced with neuronal maturation. **A)** Schematic showing candidate pools of miRNAs that may also regulate cell death in maturing neurons. **B)** Table of predicted targets for candidate miRNAs by TargetScan software. **C-E)** Relative expression levels of candidate miRNAs in maturing cerebellum. Values are expressed relative to young (P5) cerebellum and represent mean ± SEM of 3 independent experiments. **F-H)** Relative expression levels of candidate miRNAs in young (5 DIV) and mature (28 DIV) sympathetic neurons measured by RT-qPCR. Values are expressed as fold-change relative to expression in young neurons and represent mean ± SEM of 3 independent experiments (*=P<0.05 **=p<0.01 ***=P<0.001, ns=not significant).

Figure 2.3



B

MicroRNA	APAF-1	BIM	BMF	PUMA
miR-24		√	√	√
miR-124		√	√	
miR-128	√		√	√
miR-29 Family		√	√	√

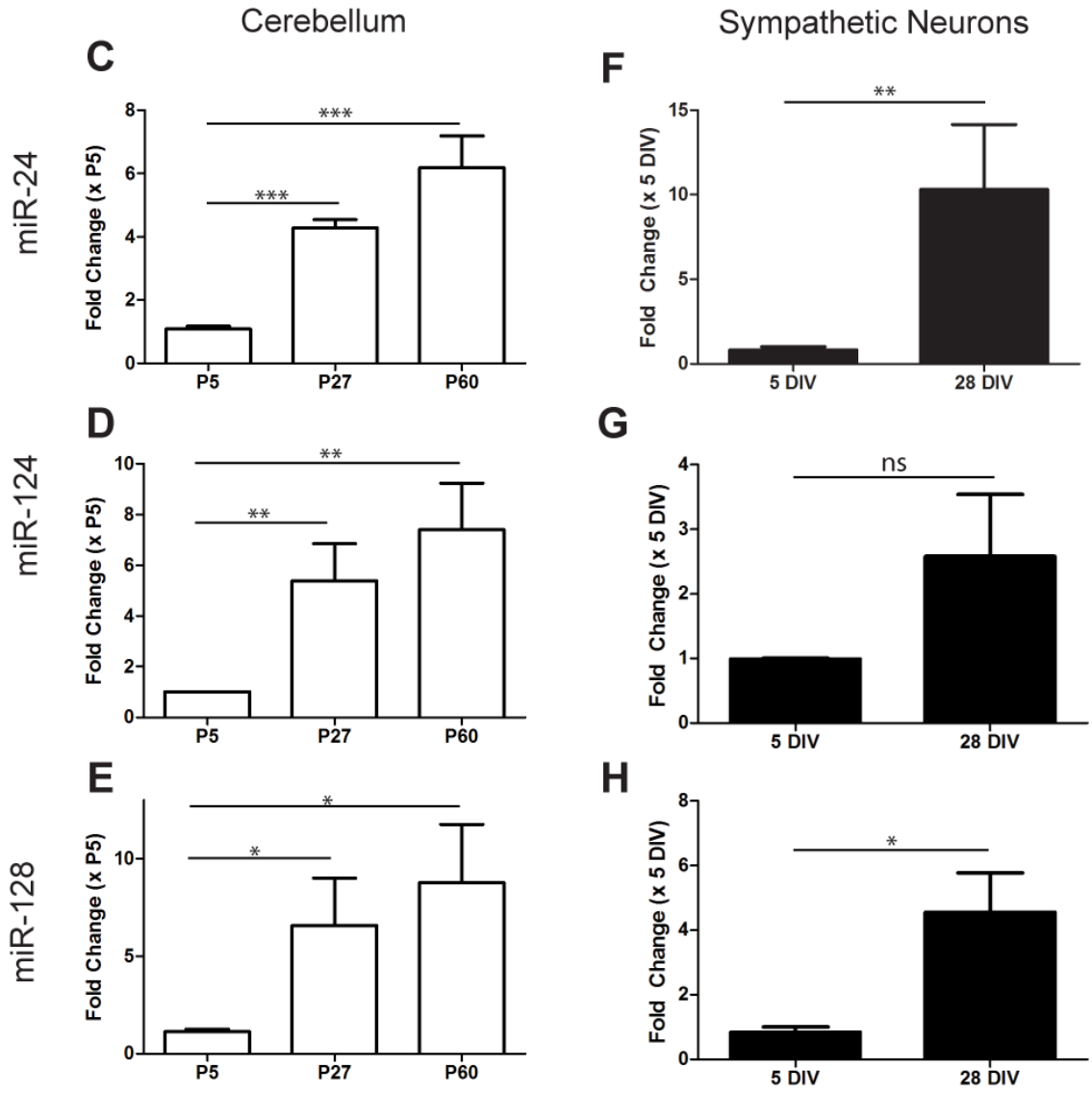


Figure 2.4: Overexpression of miR-29 or miR-24 is sufficient to inhibit cyt *c* release and cell death in young, NGF deprived neurons. A) Representative images of sympathetic neurons injected with mimics to candidate miRNAs or negative control mimic. Injected cells (arrows) are marked with FITC-Dextran (green) and cyt *c* release (red) was assessed after 48 hours of NGF deprivation. **B)** Quantification of cyt *c* release in injected cells after 48 hours of NGF deprivation. Data are represented as mean ± SEM of 3 independent experiments (*=P<0.05, ***=P<0.001). **C)** Quantification of neuronal survival in neurons injected with mimics to miR-29, miR-24, or a negative control (ncmiR) after 48 hours of NGF deprivation. Survival is expressed as the percentage of cells remaining compared to the number alive pre-deprivation. Data represent mean ± SEM of at least 3 independent experiments.

Figure 2.4

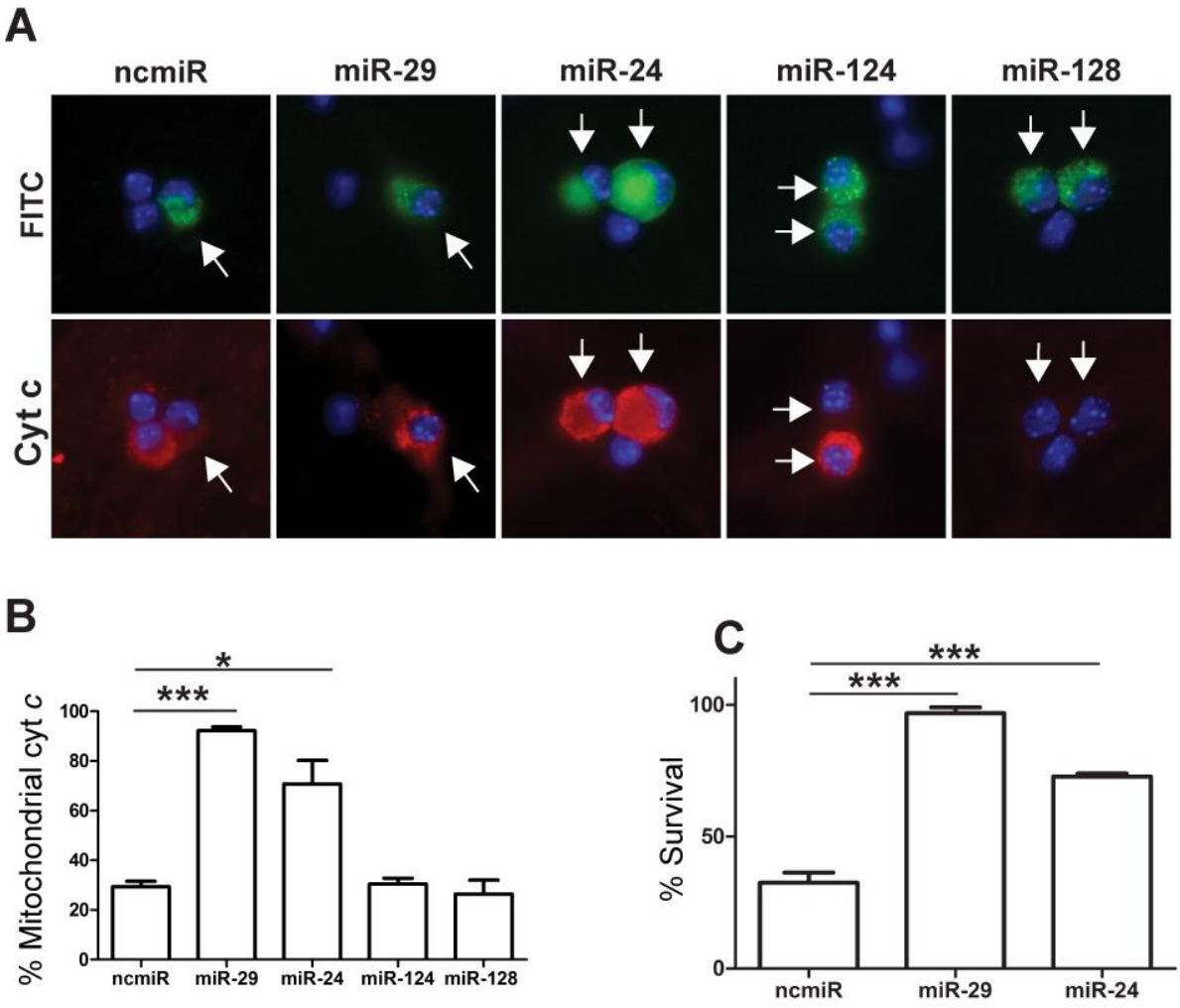

A
ncmiR
miR-29
miR-24
miR-124
miR-128
FITC
Cyt c
B
% Mitochondrial cyt c
*
***
100
80
60
40
20
0
ncmiR
miR-29
miR-24
miR-124
miR-128
C
***
***
% Survival
100
50
0
ncmiR
miR-29
miR-24

Figure 2.5: Overexpression of miR-29 or miR-24 is sufficient to inhibit the induction of Bim and Puma in young sympathetic neurons. A) Representative images of Bim staining in neurons. Neurons were injected at 3 DIV with mimics to miR-29, miR-24, or a negative control, along with FITC-Dextran (Green) to mark injected cells. At 5 DIV neurons were deprived of NGF and after 48 hours of NGF deprivation, neurons were fixed and stained for Bim (red). **B)** Quantification of normalized Bim staining intensity. Bim staining intensity was measured, and values for injected cells were normalized to NGF-deprived, mock injected neurons. Data presented as mean intensity ± SEM of 3 independent experiments (*=P>0.05). **C)** Sequence and alignment of miR-24 seed sequence with two putative miR-24 target sites in Puma 3'UTR. **D)** Luciferase activity was measured 48 hours after transfection in HEK293T cells transfected with reporter plasmids containing the Puma 3'UTR fused to a firefly luciferase gene. Plasmids were transfected either alone or with 100 nM mimics of miR-24 or negative control. Expression was normalized by measuring the ratio of firefly to renilla luciferase. Values are plotted relative to vector alone and represent mean ± SEM of 3 independent experiments (*=P<0.05). **E)** Representative images of cyt *c* staining in neurons injected with mimics to miR-29, miR-24, or negative control miR (ncmiR) and treated with 20 μM etoposide. Green indicates injected cells (arrows). **F)** Quantification of cyt *c* release in young neurons injected with mimics to miR-29, miR-24, or negative control after 48 hours of etoposide treatment. Data are plotted as mean ± SEM of 3 independent experiments (***=P<0.001).

Figure 2.5

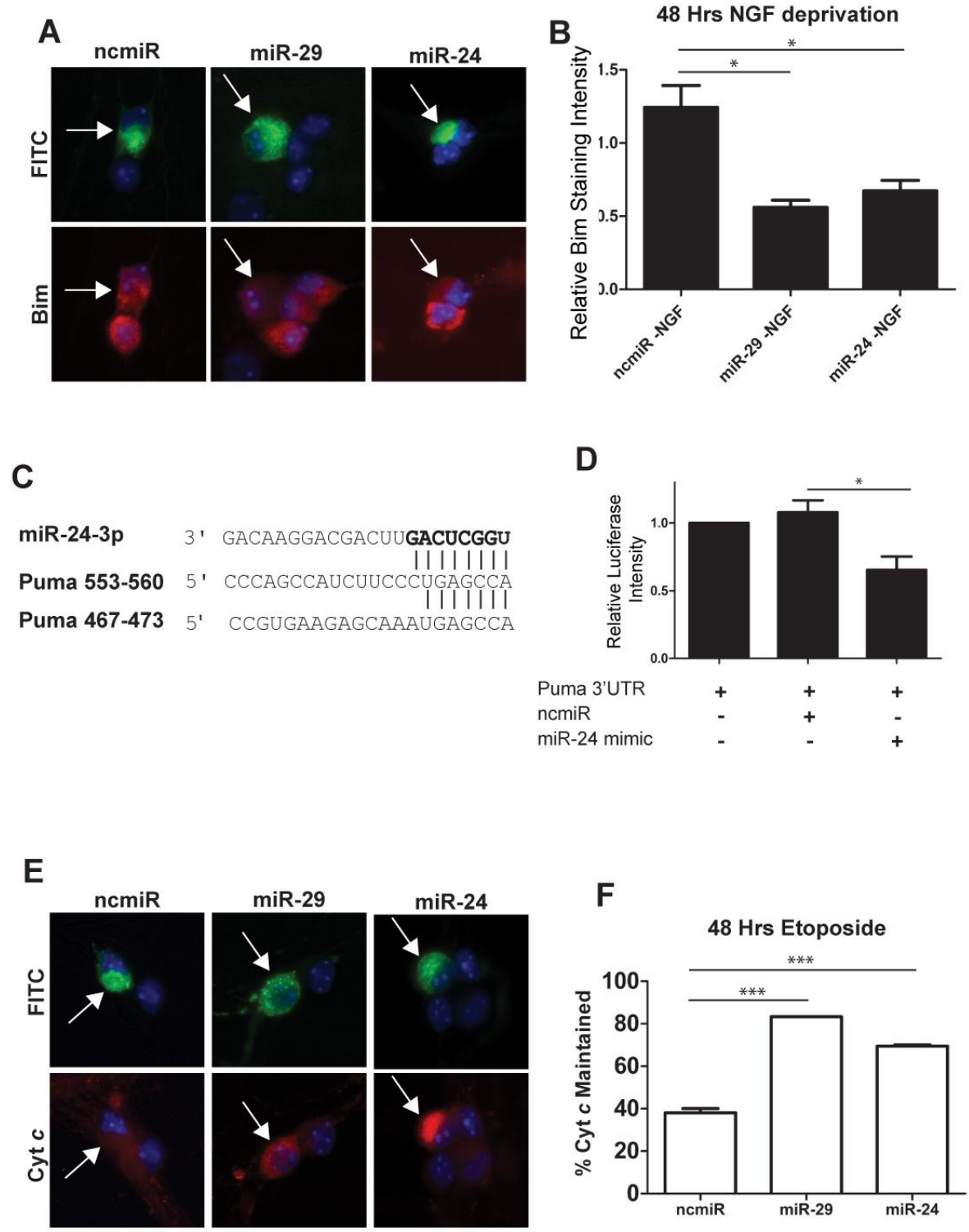

A
ncmiR
miR-29
miR-24
FITC
Bim
B
48 Hrs NGF deprivation
Relative Bim Staining Intensity
1.5
1.0
0.5
0.0
*
*
ncmiR -NGF
miR-29 -NGF
miR-24 -NGF
C
miR-24-3p 3' GACAAGGACGACUUGACUCGGU
Puma 553-560 5' CCCAGCCAUCUUCCCUGAGCCA
Puma 467-473 5' CCGUGAAGAGCAAAUGAGCCA
D
Relative Luciferase Intensity
1.0
0.5
0.0
*
Puma 3'UTR + + +
ncmiR - + -
miR-24 mimic - - +
E
ncmiR
miR-29
miR-24
FITC
Cyt c
F
48 Hrs Etoposide
% Cyt c Maintained
100
80
60
40
20
0
***
***
ncmiR
miR-29
miR-24

Figure 2.6: Inhibition of miR-24 Function in miR-29 Knockout Neurons Leads to Partial Restoration of Apoptotic Response A) Images of mature miR-29 knockout neurons microinjected with negative control LNA (ncLNA) or miR-24 LNA along with FITC dextran to mark injected cells. Neurons were deprived of NGF 48 hours post-injection and fixed for immunofluorescence 96 hours later. B) Quantification of the percentage of injected cells described in (A) with cyt *c* maintained at the mitochondria, presented as mean ±SEM of 3 independent experiments (*=P<.05). C) Representative images of young (5DIV) and mature (28DIV) neuronal cultures deprived of NGF for 48 hours and stained for phospo-c-jun (P-C-Jun). Dapi staining indicates nuclei. D) Quantification of the percentage of young (5 DIV) and mature (28 DIV) neurons expressing nuclear phospho-c-jun 48 hours post-NGF deprivation, presented as mean ±NGF of 2 independent experiments.

Figure 2.6

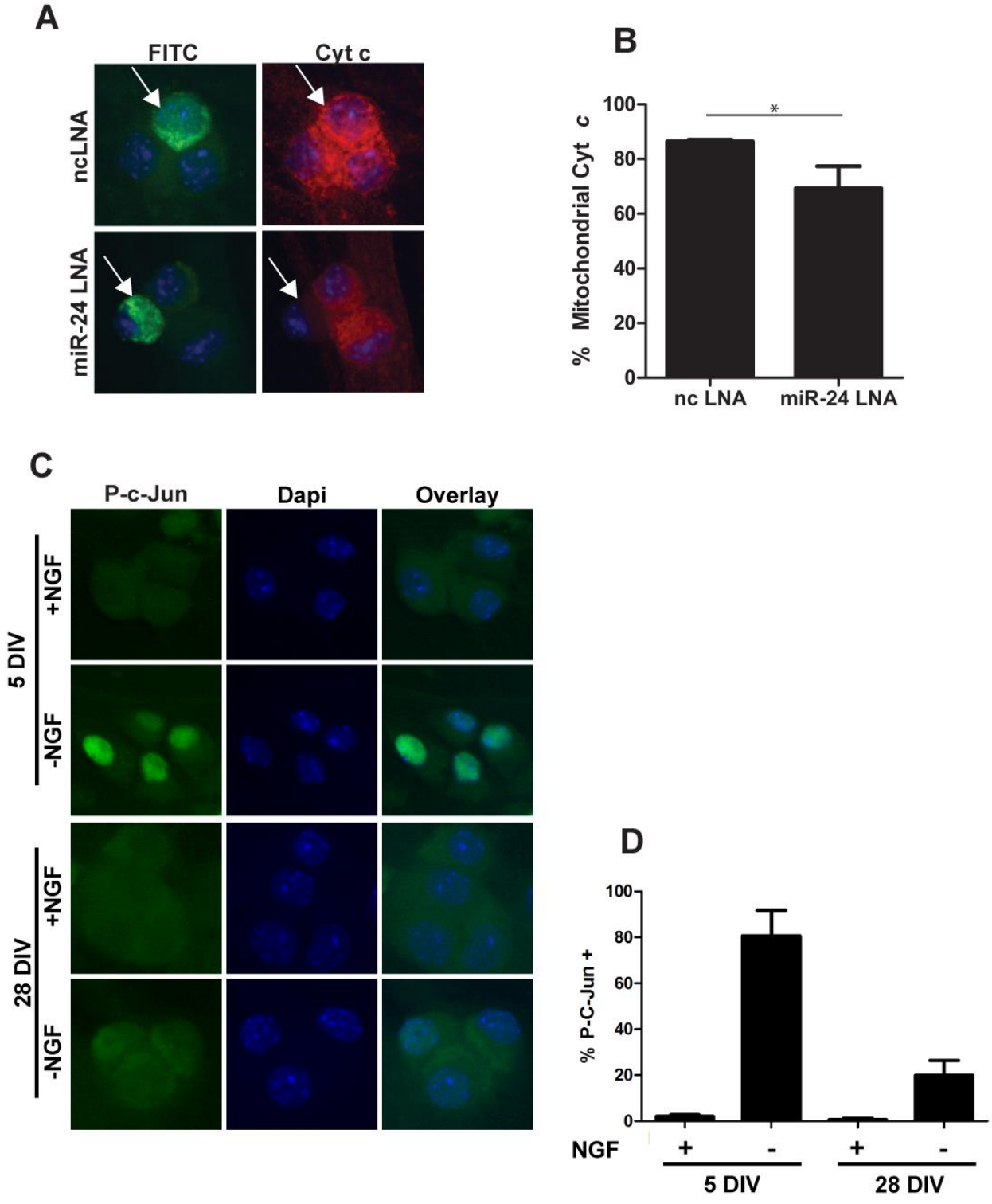

A
FITC
Cyt c
ncLNA
miR-24 LNA
B
% Mitochondrial Cyt c
100
80
60
40
20
0
*
nc LNA
miR-24 LNA
C
P-c-Jun
Dapi
Overlay
5 DIV
+NGF
-NGF
28 DIV
+NGF
-NGF
D
% P-C-Jun +
100
80
60
40
20
0
NGF
+
-
+
-
5 DIV
28 DIV

Table 2.1 Small-RNA-Seq Results from Maturing Cerebellum

Results of small-RNA-Seq on developing cerebellum. Data are shown as log(2) of the Fold-Change comparing P2 and P250 Cerebellum.

Table 2.1: Small-RNA-Seq Results from Maturing Cerebellum

miRNA	Log(2) Fold change
mmu-mir-29a-3p_-_1	9.23922243
mmu-mir-29b-1-3p	8.546476048
mmu-mir-29a-3p	8.418622901
mmu-mir-29b-2-3p	8.262343047
mmu-mir-29c-3p	6.112164983
mmu-mir-129-1-3p	4.249326455
mmu-mir-219-2-3p	4.192633704
mmu-mir-129-2-3p	3.914220479
mmu-mir-222-3p	3.442700429
mmu-mir-24-2-3p	3.377987339
mmu-mir-24-1-3p	3.377971596
mmu-mir-26a-2-5p_-_2	3.341914749
mmu-mir-128-2-3p_-_1	3.327847744
mmu-mir-137-3p	3.288597694
mmu-mir-1a-1-3p	3.047960954
mmu-mir-1a-2-3p	3.047960954
mmu-mir-128-1-3p_-_1	2.940839678
mmu-mir-128-1-3p	2.686971342
mmu-mir-128-2-3p	2.631897785
mmu-let-7i-5p_-_1	2.519435807
mmu-mir-153-3p	2.489955637
mmu-mir-22-3p	2.474592691
mmu-mir-124-3-3p_-_2	2.448247613
mmu-mir-124-2-3p_-_2	2.448247613
mmu-mir-124-1-3p_-_2	2.448247613
mmu-mir-34a-5p	2.282094191
mmu-mir-378-3p	2.12741106
mmu-mir-26a-2-5p_-_1	1.924286049
mmu-mir-218-2-5p	1.85367829
mmu-mir-137-3p_+_2	1.843588197

mmu-mir-218-1-5p	1.840203945
mmu-mir-143-3p	1.734142813
mmu-mir-127-3p	1.650469282
mmu-mir-136-5p	1.622172823
mmu-let-7g-5p_-_1	1.466853075
mmu-let-7g-5p_+_1	1.454552294
mmu-mir-137-3p_+_1	1.433874345
mmu-mir-23b-3p	1.430342439
mmu-let-7a-1-5p_-_1	1.366081503
mmu-mir-124-2-3p_-_1	1.314951681
mmu-mir-124-3-3p_-_1	1.314951681
mmu-mir-124-1-3p_-_1	1.311127769
mmu-mir-708-5p	1.30052708
mmu-mir-27b-3p	1.298943716
mmu-mir-411-5p_-_1	1.261353347
mmu-let-7b-5p_-_1	1.24969777
mmu-let-7c-2-5p_-_1	1.207648719
mmu-let-7f-2-5p_-_2	1.148975632
mmu-mir-26a-1-5p	1.124395481
mmu-mir-26a-2-5p	1.124300226
mmu-let-7g-5p	1.005429898
mmu-mir-26b-5p_-_1	0.976860419
mmu-mir-138-1-5p	0.896873914
mmu-mir-23a-3p	0.893842957
mmu-mir-124-3-3p_+_1	0.855112228
mmu-mir-124-1-3p_+_1	0.855112228
mmu-mir-124-2-3p_+_1	0.855112228
mmu-mir-191-5p	0.855015503
mmu-mir-124-2-3p	0.853413745
mmu-mir-124-3-3p	0.853308973
mmu-mir-124-1-3p	0.852844885
mmu-let-7d-5p_-_1	0.82206245
mmu-mir-101b-3p_-_1	0.817807326
mmu-mir-185-5p	0.816348799
mmu-let-7c-1-5p_-_1	0.755457368

mmu-mir-101a-3p_-_1	0.700314298
mmu-mir-26a-1-5p_-_1	0.616025135
mmu-mir-379-5p	0.55574293
mmu-mir-27a-3p	0.497080758
mmu-mir-7b-5p	0.458406739
mmu-mir-369-3p	0.425925906
mmu-mir-195-5p_-_1	0.408782549
mmu-mir-101b-3p	0.331923495
mmu-let-7a-1-5p_-_2	0.261049517
mmu-mir-335-5p_-_1	0.158111653
mmu-let-7f-2-5p_-_1	0.089385292
mmu-mir-30a-5p	0.052439317
mmu-mir-497-5p	0.041892357
mmu-mir-126-5p	0.028607886
mmu-mir-381-3p	0.018924586
mmu-mir-434-3p	-0.026370409
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mmu-mir-99a-5p_-_1	-0.161624895
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mmu-mir-425-5p	-0.24008956
mmu-let-7f-1-5p_-_2	-0.257280982
mmu-mir-411-3p	-0.264921938
mmu-let-7a-2-5p_-_1	-0.275268004
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mmu-mir-30d-5p	-0.524083907
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mmu-mir-181a-1-5p_-_1	-0.704041701
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mmu-let-7c-2-5p	-0.732192919
mmu-mir-99a-5p	-0.736326745
mmu-mir-335-5p	-0.773092289
mmu-mir-9-2-5p	-0.78268716
mmu-mir-9-3-5p	-0.783846295
mmu-mir-9-1-5p	-0.783867545
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mmu-let-7f-2-5p	-1.024257168
mmu-mir-543-3p	-1.029183671
mmu-let-7f-1-5p	-1.036474067
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mmu-mir-744-5p	-1.203349171
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mmu-mir-9-1-3p_+_2	-1.246505021
mmu-mir-7a-2-5p	-1.265238736
mmu-mir-7a-1-5p	-1.284369518
mmu-mir-125b-2-5p_-_1	-1.288522613
mmu-mir-125b-1-5p_-_1	-1.288522613
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mmu-mir-9-3-5p_+_1	-1.315373304
mmu-mir-9-2-5p_+_1	-1.316513399
mmu-mir-101a-3p	-1.318459897
mmu-mir-320-3p	-1.337016292
mmu-mir-9-2-3p_-_1	-1.337780279
mmu-mir-9-1-3p_-_1	-1.337780279
mmu-mir-9-3-3p_-_1	-1.352835029
mmu-mir-106b-5p_-_2	-1.359808157
mmu-mir-872-5p	-1.404430399
mmu-mir-107-3p	-1.419890057
mmu-mir-16-1-5p_-_1	-1.439861708
mmu-mir-411-5p	-1.441712221
mmu-mir-181b-2-5p	-1.447897426
mmu-mir-382-3p_-_2	-1.448765918
mmu-mir-16-2-5p_-_1	-1.468414749
mmu-mir-376c-3p	-1.525993473
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mmu-mir-9-2-3p	-1.566574733
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mmu-mir-186-5p	-1.646720015
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mmu-mir-16-1-5p	-1.885232943

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mmu-mir-92b-3p	-2.009621802
mmu-mir-34c-5p	-2.018246702
mmu-mir-152-3p	-2.059913093
mmu-mir-181c-5p	-2.073628414
mmu-mir-148b-3p	-2.133364514
mmu-mir-410-3p	-2.226426994
mmu-mir-99b-5p	-2.237806201
mmu-mir-487b-3p	-2.244155221
mmu-mir-342-3p	-2.351102694
mmu-mir-9-1-3p_+_1	-2.392679844
mmu-mir-9-2-3p_+_1	-2.392685836
mmu-mir-154-3p	-2.428520907
mmu-mir-652-3p	-2.439450885
mmu-mir-9-3-3p_+_1	-2.468227216
mmu-mir-127-5p_+_2	-2.470743612
mmu-mir-33-5p	-2.580869801
mmu-mir-421-3p	-2.58301589
mmu-mir-323-3p	-2.638004791
mmu-mir-181d-5p	-2.797523715
mmu-mir-135b-5p	-3.018604366
mmu-mir-15a-5p	-3.049165869
mmu-mir-340-5p_-_1	-3.201506566
mmu-mir-322-5p_-_1	-3.202972067
mmu-mir-204-5p	-3.291480082
mmu-mir-423-3p	-3.294485572
mmu-mir-340-5p	-3.396245583
mmu-mir-322-5p	-3.506050923
mmu-mir-350-3p	-3.569090388
mmu-let-7d-3p	-3.57574818
mmu-mir-135a-2-5p	-3.893240357
mmu-mir-135a-1-5p	-3.932005901
mmu-mir-106b-5p_-_1	-4.136345487
mmu-mir-484	-4.287377713
mmu-mir-25-3p	-4.471453512
mmu-mir-301a-3p	-4.689092404
mmu-mir-17-3p	-4.78722938
mmu-mir-106b-5p	-4.994931598
mmu-mir-93-5p	-5.069107244

mmu-mir-206-3p	-5.183435471
mmu-mir-92a-1-3p	-5.461448418
mmu-mir-449a-5p	-6.355656513
mmu-mir-15b-5p	-6.676286846
mmu-mir-18a-5p	-6.750281879
mmu-mir-130a-3p	-6.895129855
mmu-mir-17-5p	-7.038792003
mmu-mir-20a-5p	-7.066782902

CHAPTER 3: SPATIAL AND TEMPORAL REGULATION OF BAX ACTIVATION IN NEURONS

3.1 Introduction

The Bax/Bak mediated release of cyt. *c* from the mitochondria is the most important event in the execution of the intrinsic apoptotic pathway. Inhibition of apoptosis by Bax and Bak deletion in mice leads to perinatal lethality in ~90% of offspring. Mice that do survive past the perinatal period exhibit widespread defects, including persistant interdigital webs, imperforate vaginal canals in females, and excess cells present in both the nervous and hematopoietic systems (Lindsten et al., 2000).

As described in the introduction, the activation of Bax/Bak is governed by the BCL-2 family of proteins. The pro-apoptotic members of the BCL-2 family, known collectively as the BH3-only domain proteins, have been found to regulate Bax activation. Individual knockouts of various BH3-only genes in mice have revealed varying and often overlapping contributions to apoptosis that vary with tissue type and apoptotic stimulus. Among the BH3-only genes that play a role in neuronal cell death, systemic knockout of Bim leads to hyperplasia of lymphoid and myeloid cells, increased resistance of many cell types to cytokine deprivation, and slightly increased resistance of some cell types to DNA damage (Bouillet, Metcalf et al. 1999). Mice lacking Puma expression display widespread cellular resistance to DNA damage, underscoring the role that Puma plays in P53-dependent cell death (Villunger, Michalak et al. 2003).

Genetic deletion of HRK leads to only mild resistance of certain neuronal populations to apoptosis induced by nerve growth factor deprivation of axotomy (Imaizumi, Benito et al. 2004; Coultas, Terzano et al. 2007).

The reason for the varying phenotypes of BH3-only gene knockout mice is due in part to differences in gene expression (e.g. Hrk is thought to be a neuronal specific BH3-only gene), but also due to varying binding affinities for other members of the BCL-2 family, with whom BH3-only proteins interact extensively. The interactions of the BH3-only domain proteins with other members of the BCL-2 family has been intensively studied using yeast two-hybrid analyses, plasmon-resonance binding assays and cell free liposome/mitochondrial permeablization assays (Youle and Strasser 2008). This has led to the grouping of the BH3-only genes into two subgroups: activators, which include Bim, Bid, and probably Puma, are distinguished by their ability to directly interact with Bax and/or Bak (Letai, Bassik et al. 2002; Kuwana, Bouchier-Hayes et al. 2005; Kim, Tu et al. 2009). Sensitizers, which comprise the remaining members of the BH3-only domain proteins, fail to interact directly with Bax but interact with and inhibit pro-survival members of the BCL-2 family, preventing them from inhibiting the activation of Bax. This sub-grouping of pro-death proteins has coincided with the rise of two different, though not mutually exclusive, models of apoptosis: the indirect and direct activation models.

In the indirect activation model, a subpopulation of Bax is “primed” for apoptosis, or even constitutively active, and must be constantly restrained by pro-survival BCL-2 members (BCL-2, BCL-XL, BCL-W, and MCL-1). This model is supported by the finding that HEK cells lacking Bim and Bid and with Puma knocked down by siRNA were still

able to activate Bax and undergo apoptosis when pro-survival BCL-2 members were exogenously inhibited by drugs or overexpression of sensitizer proteins (Willis, Fletcher et al. 2007). Intriguingly, it was recently reported that a subset of Bax in healthy cells spontaneously activates and translocates to the mitochondria, and is continuously shuttled back to the cytoplasm in a BCL-XL dependent manner (Edlich, Banerjee et al. 2011). Under these circumstances, inhibition of BCL-XL may impair the retrotranslocation of active Bax from the mitochondria to the cytosol, leading to Bax accumulation and cyt *c* release. This hypothesis is also supported by experiments with Bax mutants that abolish its interaction with pro-survival BCL-2 family members, which lead to constitutive apoptosis (Fletcher, Meusburger et al. 2008; Czabotar, Lessene et al. 2014).

In the direct activation model, Bax activation and cyt *c* release are dependent on the interaction with the direct activator BH3-only proteins Bid, Bim, and/or Puma. While many lines of evidence for this model exist, the most definitive evidence was provided by the creation of Bid/Bim/Puma triple-deficient mice. These mice, which lack expression of all known direct activators, but maintain expression of sensitizer BH3-only proteins, were found to recapitulate many of the phenotypes of the Bax/Bak double knockout mice. While Bid/Bim/Puma triple-knockout did not have as profound an effect on perinatal survival as double deletion of Bax and Bak, surviving Bid/Bim/Puma knockouts displayed many of the same developmental abnormalities, including persistant interdigital webbing, imperforate vaginal canals, and accumulation of hematopoietic cells. Bid/Bim/Puma triple-knockout cerebellar granule neurons also

displayed marked resistance to ionizing radiation and potassium deprivation (Ren et al., 2010).

These two models of cell death are not mutually exclusive and can be difficult to distinguish from one another. It is likely that different cell types have different activation thresholds for apoptosis. In some cell types, Bax may be “primed” in such a way that simply removing the anti-apoptotic brakes allows apoptosis to proceed, while in others, apoptosis may be restricted to such an extent that activation of both activators and sensitizers is required for proper execution of apoptosis.

A method that has emerged recently for measuring the threshold for apoptosis in different cell types is known as BH3-profiling. BH3 profiling is performed by subjecting either isolated mitochondria or permeablized cells from different cell lines or patients to treatment with purified BH3-only domain peptides and measuring the degree of mitochondrial permeablization and/or cyt *c* release that results (Del Gaizo Moore and Letai 2012).

Other tools that can be used to explore the apoptotic threshold in different cell types are small-molecule mimetics of BH3-only genes. The first of these, ABT-737, was discovered in 2005 and remains widely used (Oltersdorf, Elmore et al. 2005). An orally bioavailable version of ABT-737, ABT-263, is currently in clinical trials as a chemotherapeutic agent under the trade name Navitoclax, along with a related compound, Obatoclax. ABT-737 is known to act by inhibiting the anti-apoptotic BCL-2 family members BCL-2, BCL-XL, and BCL-W. In cancer cells that overexpress one of

these proteins, treatment with ABT-737 can sensitize the cells to chemotherapeutic drugs.

While extensive studies have been conducted using these tools in cancer cells and immortalized cell lines, there are few studies that have made use of them to study the regulation of apoptosis in primary cells like neurons. Neurons make for an especially interesting system in which to utilize these tools for two reasons: first, as has already been described in this dissertation, neurons dynamically regulate apoptosis as they mature; and second, neurons are the most highly spatially specialized cells in the body.

When studying the temporal regulation of apoptosis in neurons, we have focused on downregulation of certain pro-apoptotic genes, such as Apaf-1, and restriction of BH3-only gene induction by microRNAs. However, other possibilities for the regulation of apoptosis exist. Namely, while the expression of Bax does not change in maturing sympathetic neurons, it is not clear whether there may be modifications of Bax that change its threshold for activation. BH3-profiling and treatment with BH3-mimetic drugs provide tools that allow more direct assessment of Bax activation in neurons, and how its activation threshold may be modulated in maturing neurons independent of miRNAs or BH3-transcription.

Spatial Regulation of Apoptosis in Neurons

The spatial localization of apoptosis in neurons is a field of study that has exploded in the past 8 years, beginning with the publication of the finding that neurons subjected to local deprivation of growth factors employ a specialized pathway to prune

or destroy axons while sparing the cell body. Unlike whole-neuron apoptosis, axon pruning is dependent on activation of Caspase 6, which is not required for whole-neuron apoptosis (Nikolaev, McLaughlin et al. 2009; Cusack, Swahari et al. 2013). In order to locally deprive axons while leaving somas in the presence of growth factor, compartmented culture systems such as microfluidic or campenot chambers are employed. These chambers place barriers between the soma and axon compartments, allowing independent treatment of each subcellular compartment.

Since the first study implicating Caspase 6, many aspects of this dedicated axon pruning pathway have become clearer. Loss of TRKA signaling in the axons leads to a DLK-dependent retrograde signal being transmitted to the soma (Simon, Pitts et al. 2016). Activation of DLK leads to local phosphorylation of JNKs, which are required for axon pruning to take place (Ghosh, Wang et al. 2011). Similar to whole-cell apoptosis, c-jun is phosphorylated and translocates to the nucleus when axons are locally deprived of NGF, although it is unclear whether c-jun activation is required for the execution of the axon degeneration program (Mok, Lund et al. 2009; Ghosh, Wang et al. 2011). Axon pruning has also been found to require the presence of Bax, Caspase-9, and Caspase-3 (Nikolaev, McLaughlin et al. 2009; Schoenmann, Assa-Kunik et al. 2010; Cusack, Swahari et al. 2013). Knockout of any of those three genes renders axons resistant to local-deprivation induced pruning, although interestingly, knockout of Apaf-1, a gene which is required for the execution of whole cell apoptosis, was not found to inhibit local-deprivation induced axon pruning. Consistent with this, mature neurons, which are known to downregulate Apaf-1 to restrict the apoptotic pathway, maintain their ability to locally activate apoptosis for the purpose of axon pruning (Cusack et al., 2013).

These findings, that neurons can locally activate the apoptotic machinery for the purposes of axon pruning, and that mature neurons maintain this ability despite restricting apoptosis in the context of whole-cell growth factor deprivation, raise many interesting questions. Chief among these questions is how neurons maintain a permissive environment for apoptosis in axons while restricting it in somas. To examine this further, we have examined the effects of small molecule BH3-mimetics as well as purified BH3-peptides on the neuronal apoptotic pathway.

3.2: Materials and Methods

Cell Culture and Drug Treatments

Neurons were isolated from neonatal (P0/P1) sympathetic neurons as previously described (Potts et al., 2003). ABT-737 (Selleckchem) was used at a final concentration of 10µM. NGF deprivation was conducted as described in Chapter 2. For mature neuron experiments, neurons were maintained in culture until P28-equivalent prior to treatments. For cell permeablization experiments, Digitonin (Promega) was added to cells for the indicated time period at the indicated concentrations.

Immunofluorescence

Immunofluorescence for cyt *c* and Tom20 was conducted as described in Chapter 2. Propidium Iodide (Sigma) was applied to cells at 2.5ug/mL.

Image Acqusition

Images were acquired and processed as described in Chapter 2.

Western Blot

Western blots were performed as described in Potts et al., 2003. Primary antibodies used for western blot were anti-cytochrome *c* (BD Pharmingen 556433, 1:500 Dilution), anti-Bim (Cell Signaling, 2819, 1:1000 Dilution), anti-alpha-tubulin (Sigma, 1:10000 Dilution)

Single-Cell Microinjection

Microinjection solutions were made as described in Chapter 2. For BH3 peptide injections, purified BH3-only domain peptides for Bim (Anaspec), BMF (Genscript) and Puma (Genscript) were dissolved in 33% DMSO and injected into sympathetic neurons as described above at a concentration of .6mg/mL (Bim) or 12mg/mL (BMF, Puma). Neurons were fixed 24 hours post-injection and the status of cyt *c* was assessed using immunofluorescence.

3.3 Results

Treatment with ABT-737 Causes Selective Degeneration of Axons in Young Neurons

To explore the apoptotic "priming" status of neurons, we treated young neurons with the small-molecule BH3-mimetic drug ABT-737. ABT-737 is a small molecule designed to mimic the effects of the BH3-protein Bad, and binds to the pro-survival BCL-2 family members BCL-2, BCL-XL, and BCL-W, inhibiting their activity. We find that upon treatment with ABT-737 for 48 hours, sympathetic neurons exhibit extensive axon beading that resembles the axon degeneration observed in sympathetic neurons locally deprived of NGF (Figure 3.1 B,D, F). Interestingly, the cell bodies of these neurons fail to exhibit the characteristic condensation and degeneration associated with apoptosis (Figure 3.1 A, C, E).

ABT-737 Induces Global Cytochrome c Release in Young Sympathetic Neurons

Immunostaining experiments revealed that treatment with ABT-737 in young neurons induced the release of cyt *c* from the mitochondria in both the somas and axons (Figure 3.2 A). This observation is not inconsistent with the previous observation that axons of young ABT-737-treated neurons degenerate while Somas do not. It has previously been found that the proper execution of neuronal apoptosis requires the development of so-called "competence to die". In experiments that simulate the release of cyt *c* from mitochondria by injecting purified exogenous cyt *c* directly into the cytosol, it was found that neurons were remarkably resistant to death induced by cyt *c* injection as long as they were maintained in NGF. If neurons were deprived of NGF but saved by

the addition of the protein synthesis inhibitor cycloheximide, they maintained their own cyt *c* at the mitochondria, but underwent robust apoptosis in response to cyt *c* injection (Deshmukh and Johnson 1998). The development of competence to die was later found to involve the degradation of the inhibitor of apoptosis XIAP. Neurons cultured from XIAP -/- animals undergo apoptosis in response to cyt. *c* injection in the presence of NGF (Potts et al., 2003). Thus, the failure of neuronal somas to degenerate after ABT-737 induced cyt *c* release may reflect inhibition of active caspases by XIAP. Interestingly, XIAP was recently found to be present in axons, and loss of XIAP expression lead to accelerated axon degeneration (Unsain, Higgins et al. 2013). Axons treated with ABT-737 may degenerate because XIAP is differently regulated/enriched or more easily overwhelmed in axons compared with somas. This result was also surprising because it suggested that neurons were more primed than expected to release cyt *c*, since inhibition of apoptotic brakes with ABT-737 was sufficient to induce the release of cyt *c* on its own.

ABT-737 Induces Axon-Specific Cytochrome c Release in Mature Neurons

Since mature neurons are well known to be more resistant to cyt *c* release and apoptosis than young neurons, we next tested the response of mature neurons to treatment with ABT-737. Consistent with our previous findings that mature neurons are more resistant to apoptosis than young neurons, mature neurons failed to release cyt *c* from mitochondria in their somas in response to ABT-737. In contrast, mature neuron axons still exhibited robust cyt *c* release in response to ABT-737 treatment (Figure 3.2

B). This result is consistent with the previously published finding from our lab that mature neurons subjected to local deprivation of NGF were still able to degenerate their axons, while mature neurons globally deprived of NGF did not degenerate either axons or somas (Cusack et al., 2013). This also suggests that apoptotic brakes are differentially regulated within mature neurons, and that mature neurons maintain a permissive environment for apoptosis in their axons while restricting the apoptotic pathway in their somas. This finding may be partially explained by recently published findings from another lab that explored the subcellular distribution of apoptotic brakes. It was found that BCL-W -/- mice exhibited a progressive sensory neuropathy that exhibited manifested as decreased axonal innervation of the epidermis, but that cell-bodies of the innervating neurons were unaffected, suggesting that loss of BCL-W led to an axon-specific degeneration phenotype. BCL-W was found to be enriched in axons compared to BCL-2 and BCL-xl, and BCL-W -/- neurons exhibited defects in axonal mitochondrial morphology and function (Courchesne, Karch et al. 2011). Degeneration of mature neuron axons in response to ABT-737 may therefore be due to inhibition of axonal BCL-W. However, the concentration of ABT-737 employed should be sufficient to overwhelm all present apoptotic brakes, so it would be surprising if differential distribution of BCL-W alone explained the observed degeneration.

NGF Deprivation Sensitizes Mature Neuron Somas to ABT-737-Induced Cytochrome c Release

As discussed above, sensitivity of neurons to cytosolic cyt *c* injection is governed by the development of competence to die, where NGF deprivation sensitizes neurons to death induced by purified cytochrome *c*. It has not yet been tested whether a similar phenomenon exists in neurons pre-mitochondrially. To examine this question, we paired ABT-737 stimulation with NGF deprivation. Neurons were deprived of NGF for 24 hours prior to the addition of ABT-737, and maintained in the absence of NGF during treatment. To our surprise, we found that deprivation of NGF was able to sensitize mature neurons to ABT-737, resulting in robust cyt *c* release (Figure 3.2 C). We quantified the release of cyt *c* from the mitochondria of young and mature neurons subjected to the treatments described above using immunofluorescence (Figure 3.2 D), and further confirmed these findings by comparing the levels of cyt *c* in these neurons using Western Blot (Figure 3.2 E). Loss of the neuronal cyt *c* signal in immunofluorescence or western blot experiments was interpreted in these experiments as being indicative of cyt *c* release, as neurons have been found to degrade the majority of released cyt *c* in response to apoptotic stimuli (Gama et al., 2014).

To determine if the sensitization of mature neurons to NGF-deprivation requires new protein transcription or translation, possibly due to limited induction of BH3-only domain proteins, we added the drugs actinomycin D (actin D) or cyclohexmide (CHX) during the 24-hour NGF deprivation pre-treatment (Figure 3.2 F). We find that CHX and actin D have no effect on the sensitization of mature neurons to ABT-737, suggesting

that this sensitization is perhaps due to loss of a pro-survival signal from NGF, rather than the induction of a positive pro-death signal.

ABT-737 Treatment Results in Elevated Bim Levels in Young, but not Mature Neurons

Due to the unexpected finding that ABT-737 can induce robust cyt *c* release in young neurons, suggesting that the apoptotic pathway is more primed in young neurons than anticipated, we ran experiments to ensure that ABT-737 was acting as intended. If ABT-737 was acting solely by inhibiting apoptotic brakes, simultaneous addition of a translation inhibitor would be expected to have no effect on the ability of ABT-737 to induce cyt *c* release. However, when we treated sympathetic neurons with a combination of ABT-737 and CHX simultaneously, we found that cyt *c* release was substantially decreased in the ABT-737/CHX treated cells when compared to the cells treated with ABT-737 alone (Figure 3.3 A). Western blot analysis of ABT-737 treated neuron lysates showed that ABT-737 unexpectedly led to an increase in the levels of the pro-apoptotic protein Bim, which acts as a direct activator of Bax, confounding the results of the ABT-737 experiments (Figure 3.3 B). Interestingly, however, preliminary experiments suggest that treatment with CHX does not prevent the axon degeneration caused by ABT-737 (Figure 3.3 C). It is also noteworthy that ABT-737 is able to sensitize mature neurons to release cyt *c* in the presence of NGF, in spite of the fact that no elevation of Bim was detected in mature neurons in the +ABT/-NGF condition.

Although ABT-737 is a widely used drug in the field of apoptosis research, the possibility for off-target effects of ABT-737 treatment has rarely been explored. One

study in 2012 reported that application of ABT-737 to HeLa cells resulted in increased Bim levels, and that this increase in Bim occurred in a JNK-dependent manner. While this may further enhance the efficacy of ABT-737 as a cancer therapeutic, it negatively impacts our ability to use this compound as a tool to explore the regulation of the apoptotic pathway, since the entire reason to use it was to remove the anti-apoptotic proteins without affecting the pro-apoptotic genes. That said, there are several findings from these experiments that remain interesting and merit further explanation in spite of the confounds introduced by ABT-737-induced elevation of Bim levels. The fact that CHX cotreatment with ABT-737 did not prevent axon degeneration suggests that the threshold for Bax activation may indeed be different in somas and axons. The case for differential regulation of Bax in axons is further strengthened by the observation that axons in mature neurons still degenerate in response to ABT-737 treatment, and that ABT-737 does not induce Bim accumulation in mature neurons. Furthermore, the sensitization of mature neurons to ABT-737-induced cyt *c* release does not require protein synthesis, and neurons sensitized by NGF deprivation do not display increased Bim levels. In order to test these hypotheses properly, we have decided to explore alternative approaches that do not require the use of small-molecule BCL-2 family inhibitors.

Direct Stimulation of Bax with BH3-Peptides as an Alternative Approach to Explore Bax Activation in Neurons

Due to the unforeseen complications arising from off-target effects of ABT-737 treatment, an alternative approach is required to properly assess the spatial and

developmental regulation of Bax activation in neurons. To this end, we have pursued methods for directly stimulating Bax activation with purified BH3 peptides.

Direct stimulation of the apoptotic pathway with purified BH3 peptides is an approach that has become increasingly popular in the study of cancer cell apoptosis in the past decade (Letai, Bassik et al. 2002; Del Gaizo Moore and Letai 2012). BH3 peptides are used instead of full-length purified proteins because BH3 proteins have intrinsically disordered and transmembrane domains that complicate protein purification efforts. Purified proteins also allow more precise control over dosage levels and can circumvent issues with transfection of different cells when compared to plasmid based overexpression studies.

To validate the activity of BH3 peptides in neurons, we injected young and mature neurons with a pool of peptides comprising the BH3 domains of Bim, Puma, and BMF, which are three of the four BH3-only family members that have been found to be upregulated in neurons undergoing apoptosis (Kristiansen et al., 2011). Notably, while HRK-BH3 was not available at the time these experiments were initially conducted, it has become available recently and will be included in future experiments. Injection of pooled Bim, BMF, and Puma was sufficient to induce cyt *c* release (assessed by immunofluorescence) in the majority of injected young sympathetic neurons (Figure 3.6 A, top panels). Interestingly, mature sympathetic neurons injected with the same combination of peptides at the same concentration displayed markedly less cyt *c* release compared to young neurons (Figure 3.6 A, bottom panels, Figure 3.6 B). These results suggest that there is indeed another brake in mature neurons at the level of Bax mediated cyt *c* release. The precise nature of this brake is currently unknown, but Bax

activation has been found to be regulated by post-translational modifications such as phosphorylation, and more recently, by the formation of autoinhibitory Bax dimers (Gardai, Hildeman et al. 2004; Kim, Ryu et al. 2006; Quast, Berger et al. 2013; Garner, Reyna et al. 2016). It is possible that differences in Bax posttranslational modification or dimerization between young and mature neurons lead to differences in Bax sensitivity between young and mature neurons.

A limitation of this microinjection-based approach is that the effect of soma-injected peptides on axons is difficult to properly discern. Peptides are introduced directly in the soma of the neurons, and it is unclear if the peptides can get into the axons in sufficient concentrations to have an effect by passive diffusion. Additionally, the status of cyt *c* in the axons of individual injected neurons is difficult to determine because of tendency of sympathetic axons in culture to form large fasciculated bundles, which makes it difficult to discern one axon from another within a given bundle.

Recent advances in BH3 profiling provide a potential solution to the limitations of the single-cell microinjection based approach. Early experiments in BH3 profiling were performed on purified mitochondria isolated from cell lines. These experiments are difficult to perform in primary neurons due to difficulties in obtaining sufficient material from cultured neurons, and obtaining axonal mitochondria specifically to explore the spatial aspects of apoptosis regulation would be even more difficult. However, techniques have recently been developed to perform BH3 profiling on adherent cells without having to purify intact mitochondria. Using low concentrations of the detergent digitonin, the cellular membrane can be permeablized while leaving the mitochondrial membrane intact. This allows the bath application of BH3-peptides in adherent cells,

using either cyt *c* status or mitochondrial membrane potential as a read out of Bax activity (Ryan and Letai 2013).

BH3-profiling on adherent cells is an attractive complement to single-cell microinjection experiments, because it would allow for the stimulation of Bax activation in larger populations of cells and permit analysis of differential Bax activation in neuronal somas and axons. Since existing BH3-profiling studies using adherent cells have been conducted mostly in cancer cells and immortalized cell lines, we performed initial experiments to determine the feasibility of this technique for use in primary neurons. While the injection experiments described above validated the ability of BH3 peptides to promote cyt *c* release in primary sympathetic neurons, the key requirement for performing BH3-profiling on adherent cells is the ability to selectively permeablize the cell membrane while leaving the outer mitochondrial membrane intact.

To determine the feasibility of this approach in sympathetic neurons, we incubated 5 DIV neurons with increasing concentrations of digitonin for 15 or 60 minutes. Initial digitonin concentrations were determined using existing literature as guidelines (Ryan and Letai, 2014). As a reporter for membrane permeablization, we employed the DNA intercalating agent Propidium Iodide (PI). PI is unable to cross the membrane of healthy, unpermablized cells, but intercalates into DNA and fluoresces upon entering the cell, allowing cellular membrane permeability to be assessed by the presence or absence of nuclear PI staining in live cells. After the initial assessment of cellular membrane permeability, the integrity of the mitochondrial membrane was assessed by fixing the cells and evaluating the status of cyt *c* in the cells by immunofluorescence, using Tom20 staining as a mitochondrial marker. The goal of this

experiment was to find a concentration of digitonin that permeablized the cell membrane, resulting in PI-positive nuclei, but did not permeablize the mitochondrial membrane, as determined by the presence of mitochondrial cyt *c*. As a positive control for cellular permeablization, cells were incubated with a high concentration of the detergent Triton-X-100, which would be expected to completely permeablize both cell and mitochondrial membranes.

We find that incubation of digitonin at concentrations of less than .005% is insufficient to permeablize the cell membrane of sympathetic neurons, even when cells are incubated for up to 60 minutes. .005% digitonin is insufficient to permeablize the cell membrane after 15 minutes, but succeeds in permablizing the membrane after 60 minutes (Figure 3.7A). Immunofluorescence revealed that in these cells treated with .005% digitonin for 60 minutes, cyt *c* was maintained at the mitochondria, indicating that the mitochondrial membrane had not been permeablized. As expected, incubation with Triton-X-100 resulted in complete permeablization of both cellular and mitochondrial membranes within 15 minutes, resulting in PI-positive neurons with no detectable cyt *c* at their mitochondria (Figure 3.7B). Taken together, these results demonstrate the feasibility of digitonin permeablization as a means to study sympathetic neuron apoptosis.

In order to build on the promising preliminary results obtained thus far, I propose the following experiments:

1) Combinatorial studies utilizing single-cell injection of BH3 peptides in varying combinations to determine their relative ability to induce cyt *c* release in young

neurons. It will be interesting to determine if the injection of BMF-BH3, which is known to act only as a sensitizer, is sufficient to promote the release of cyt *c* in neurons, or if it requires the presence of activator BH3-peptides as well. The ability of BMF-BH3 to accelerate cyt c release in response to NGF deprivation would serve as a useful control for ensuring that the peptide is biologically active. It has also been reported that the transmembrane domain of Puma is important for its ability to bind Bax, resulting in purified Puma-BH3 acting more like a sensitizer while full-length Puma can act as an activator. The results of experiments expressing all four BH3 genes (Bim, Puma, BMF, and HRK) would likely be difficult to interpret, as there is no way to determine if a given microinjected neuron is expressing all four plasmids to the same level, or indeed if one or more plasmids is not being expressed at all. However, the overexpression of individual full-length BH3 genes by plasmid microinjection should be more straightforward and would serve as a useful complement to BH3 peptide injections. Different results between peptide injections and plasmid based overexpression studies could provide useful insight into the relative importance of the different domains of BH3 proteins in neuronal apoptosis.

2) Further experiments to determine the mechanistic reasons for the differences observed in cyt *c* release in young and mature neurons in response to BH3-peptide injections. BH3-peptide injection bypasses the known brakes on cyt *c* release engaged in mature neurons; namely the miRNAs miR-29 and miR-24. Thus, at least one additional brake likely exists at the level of Bax activation or

integration into the mitochondrial membrane. Bax activation could be inhibited in mature neurons by post-translational modification or interaction with a binding partner. There is precedence in the literature for both of these events; Bax can be phosphorylated by AKT at Serine 184, which inhibits its activation (Gardai et al., 2004). Bax activity can also be inhibited by protein binding partners (Su, Wang et al. 2006; Garner, Reyna et al. 2016). Given the range of known ways that Bax activation can be inhibited, as well as the potential for novel regulatory mechanisms, this is perhaps best examined by mass-spectrometric comparison of Bax immunoprecipitated from young and mature neurons.

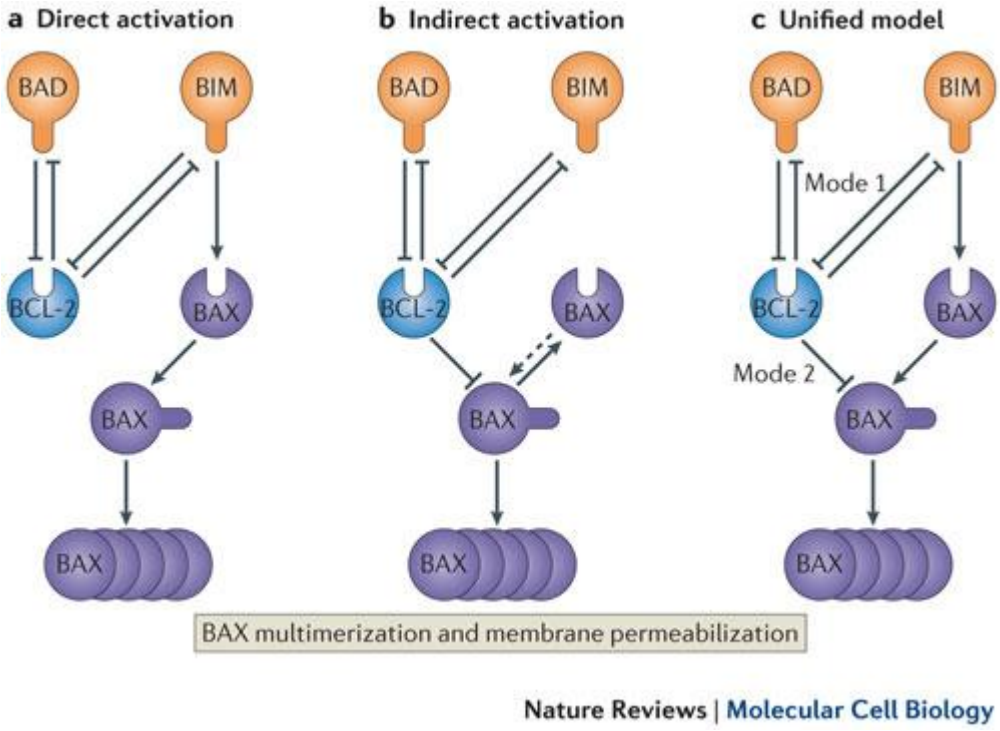
3) Following further optimization of the cellular permeablization protocol, BH3-profling experiments on adherent sympathetic neurons can be carried out. Permeablization of a large population of cells would allow evaluation of cyt *c* release in response to BH3 peptide stimulation by both immunofluorescence and western blot, providing an advantage over single-cell microinjection experiments. More importantly, these experiments provide the opportunity to examine differences in cyt *c* release induced by these peptides in different cellular compartments, namely somas and axons. The effects of varying combinations of BH3 peptides can be evaluated by assaying the release of cyt *c* by immunofluorescence and/or western blot in young and mature neurons.

3.4: Figures and Figure Legends

Figure 3.1: Indirect vs Direct Activation Models for Bax Activation

In the direct activation model for Bax activation (A), direct activator BH3 proteins, such as Bim, are bound and inhibited by the anti-apoptotic members of the BCL-2 family, such as BCL-2 or BCL-xl. Sensitizer BH3 proteins, such as Bad, sequester the anti-apoptotic BCL-2 family proteins, freeing the activator BH3 proteins to bind and activate Bax. In the indirect activation model (B), Bax is constitutively active to some degree, and is held in check by the anti-apoptotic BCL-2 family members. In this model, inhibition of this interaction between anti-apoptotic BCL-2 family members by BH3-only family members is sufficient to promote Bax activation and cell death. The unified model (C) proposes that both direct and indirect activation can occur, depending on cellular context and relative protein levels of each family member.

Figure 3.1



Adapted from Czabotar et al., 2014

Figure 3.2: Treatment of Young Sympathetic Neurons with ABT-737 Induces Axon Degeneration

Neurons isolated from neonatal mice were cultured for 5 days and then either maintained in NGF (A, B), treated with ABT-737 (C, D) or deprived of NGF (E, F). Images of somas and axons were captured from the same plate. ABT-737 treated neurons exhibit beaded axons similar to NGF-deprived neurons, but maintain phase bright somas similar to NGF-maintained neurons.

Figure 3.2

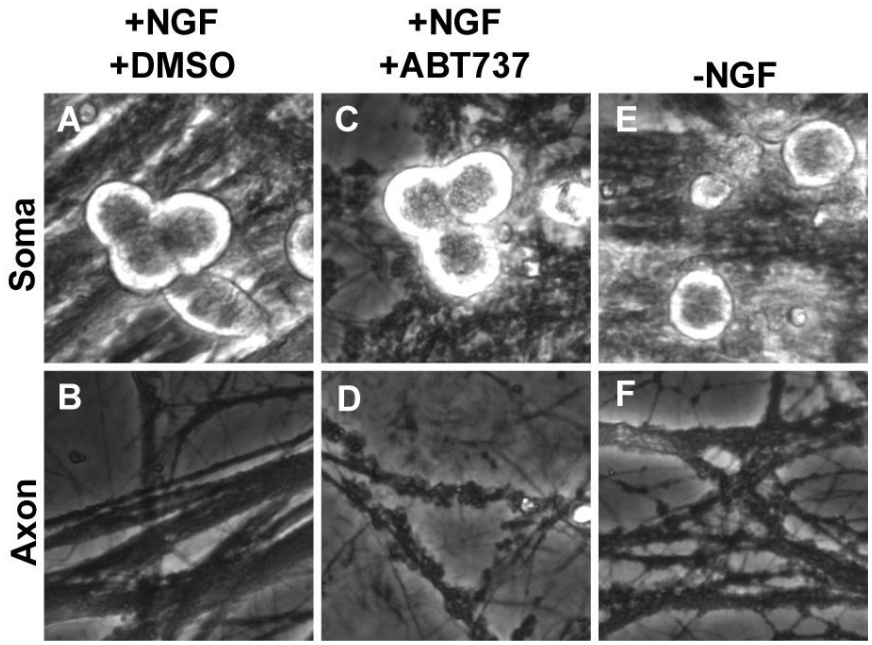


Figure 3.3: Differing Effects of ABT-737 on cyt *c* Release in Young and Mature Neurons

Neurons isolated from neonatal mice and cultured for either 5 days (P5) or 28 days (P28), subjected to treatment with ABT-737 for 48 hours, then fixed and stained for cyt c or the mitochondrial marker Tom20. A) Treatment of young neurons with ABT-737 leads to widespread cyt *c* release in both somas and axons. B) Treatment of mature neurons with ABT-737 leads to cyt c release mainly in the axons, while somas largely maintain cyt *c* at the mitochondria. C) Mature neurons subjected to NGF deprivation for 24 hours prior to ABT treatment release cyt *c* release cyt *c* in both axons and somas, similar to young neurons. D) Quantification of the percentage of cells with mitochondrial (maintained) cyt *c* in each of the indicated condition. Data presented as mean ± SEM of at least 2 independent experiments. E) Western blot of cyt *c* levels in neurons subjected to the indicated treatments. Loss of cyt *c* expression or staining reflects degradation of cyt *c* after its release from the mitochondria. F) Percentage of cells displaying mitochondrial cyt *c* in mature neurons deprived of NGF in the presence or absence of Actin D or CHX for 24 hours prior to treatment with ABT-737, compared with NGF deprived neurons and NGF maintained, vehicle treated neurons, presented as mean ±SEM from two independent experiments.

Figure 3.3

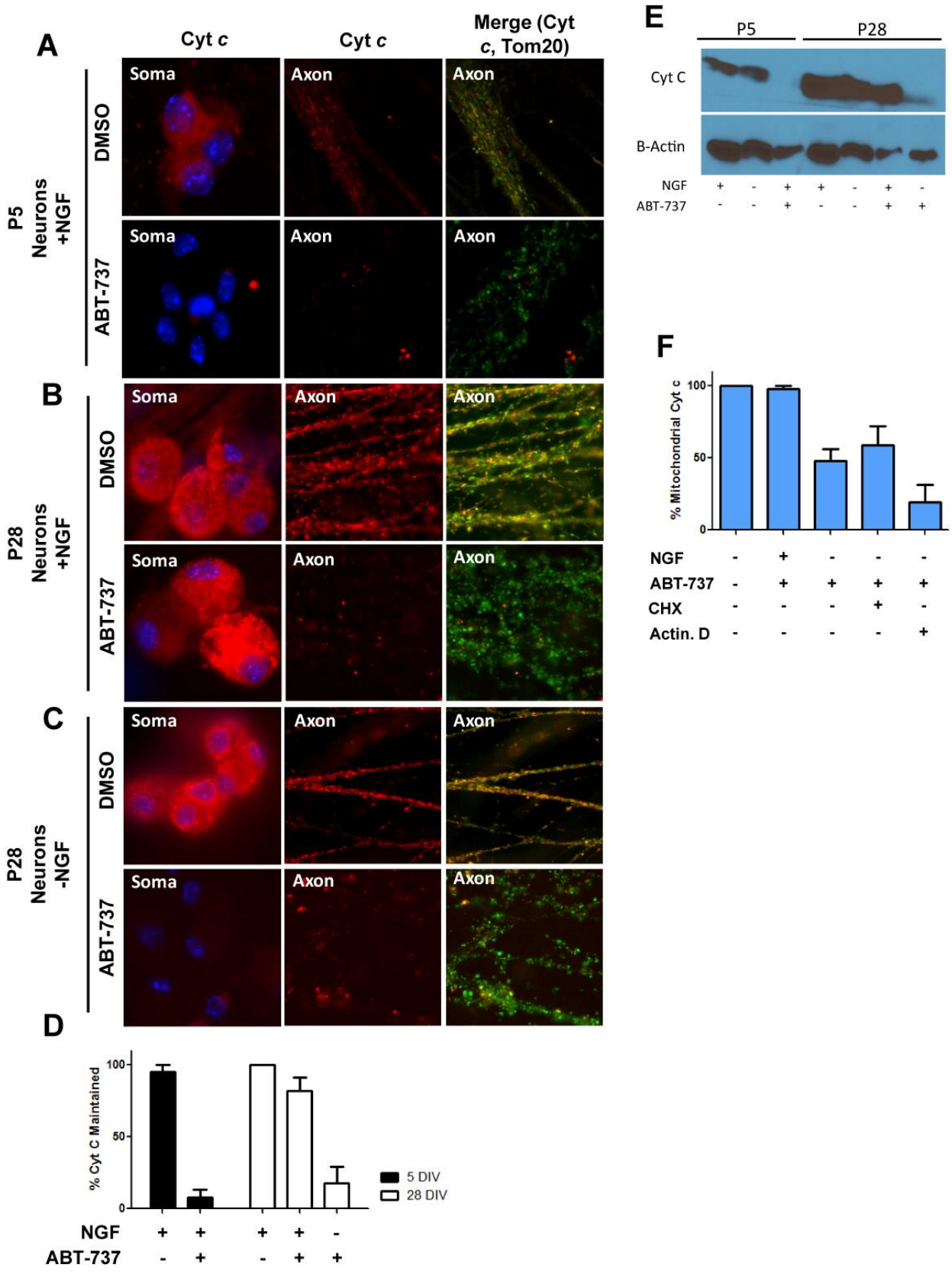

A
Cyt c
Cyt c
Merge (Cyt c, Tom20)
P5 Neurons +NGF
DMSO
ABT-737
Soma
Axon
B
P28 Neurons +NGF
C
P28 Neurons -NGF
D
% Cyt C Maintained
100
50
0
5 DIV
28 DIV
NGF + + + + -
ABT-737 - + - + +
E
P5
P28
Cyt C
B-Actin
NGF + - + + - + -
ABT-737 - - + - - + +
F
% Mitochondrial Cyt c
100
50
0
NGF - + - - -
ABT-737 - + + + +
CHX - - - + -
Actin. D - - - - +

Figure 3.4: ABT-737 Upregulates Bim Expression in Sympathetic Neurons

A) Quantification of neurons exhibiting mitochondrial cyt *c* in sympathetic neurons treated with ABT-737 in the presence or absence of CHX for 48 hours. –NGF +CHX condition is shown as a control for CHX efficacy. B) Western blot analysis of young (P5) and mature (P28) sympathetic neuron lysates probed with anti-Bim antibody. Tubulin is present as a loading control. C) Axon morphology of P5 neurons treated with vehicle, ABT-737 or ABT-737 +CHX for 72 hours.

Figure 3.4

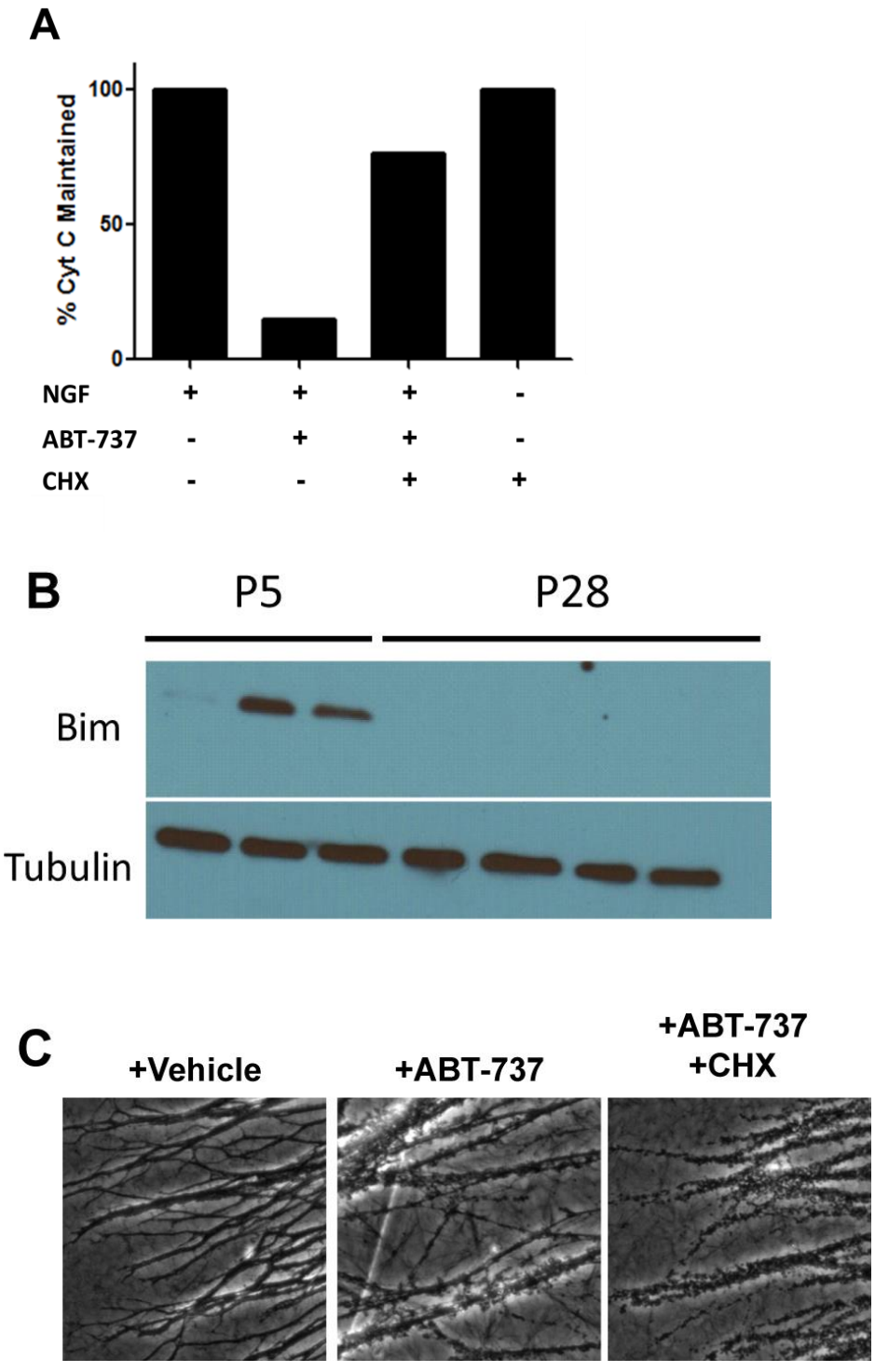

A
% Cyt C Maintained
100
50
0
NGF + + + -
ABT-737 - + + -
CHX - - + +
B
P5
P28
Bim
Tubulin
C
+Vehicle
+ABT-737
+ABT-737
+CHX

Figure 3.5 Direct Injection of BH3-only Domain Peptides Is Sufficient to Cause Cytochrome *c* Release in Young, but not Mature SCGs

A) Representative images of Neurons injected with a pooled combination of peptides comprising the purified BH3-domains of Bim, BMF, and Puma (BBP) or vehicle control (DMSO) were fixed and stained with anti-cytochrome *c* antibody. Injected cells are marked with FITC-Dextran. B) Quantification of the number of injected cells with mitochondrial cyt *c*, displayed as mean ± SEM of 3 independent experiments.

Figure 3.5

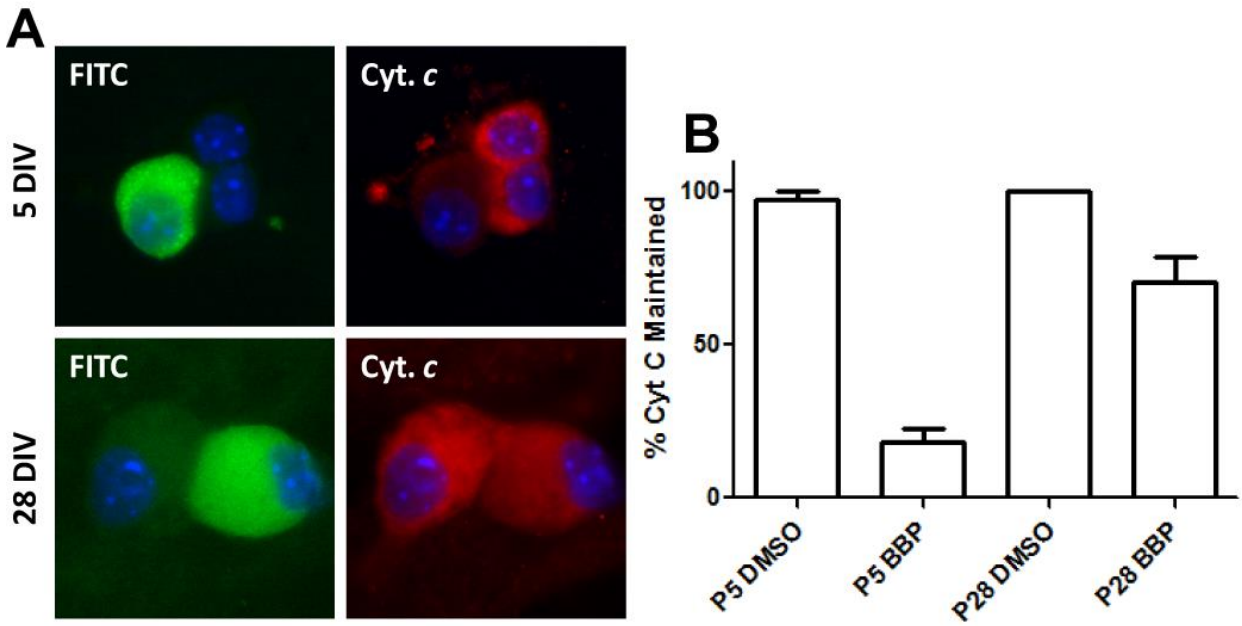
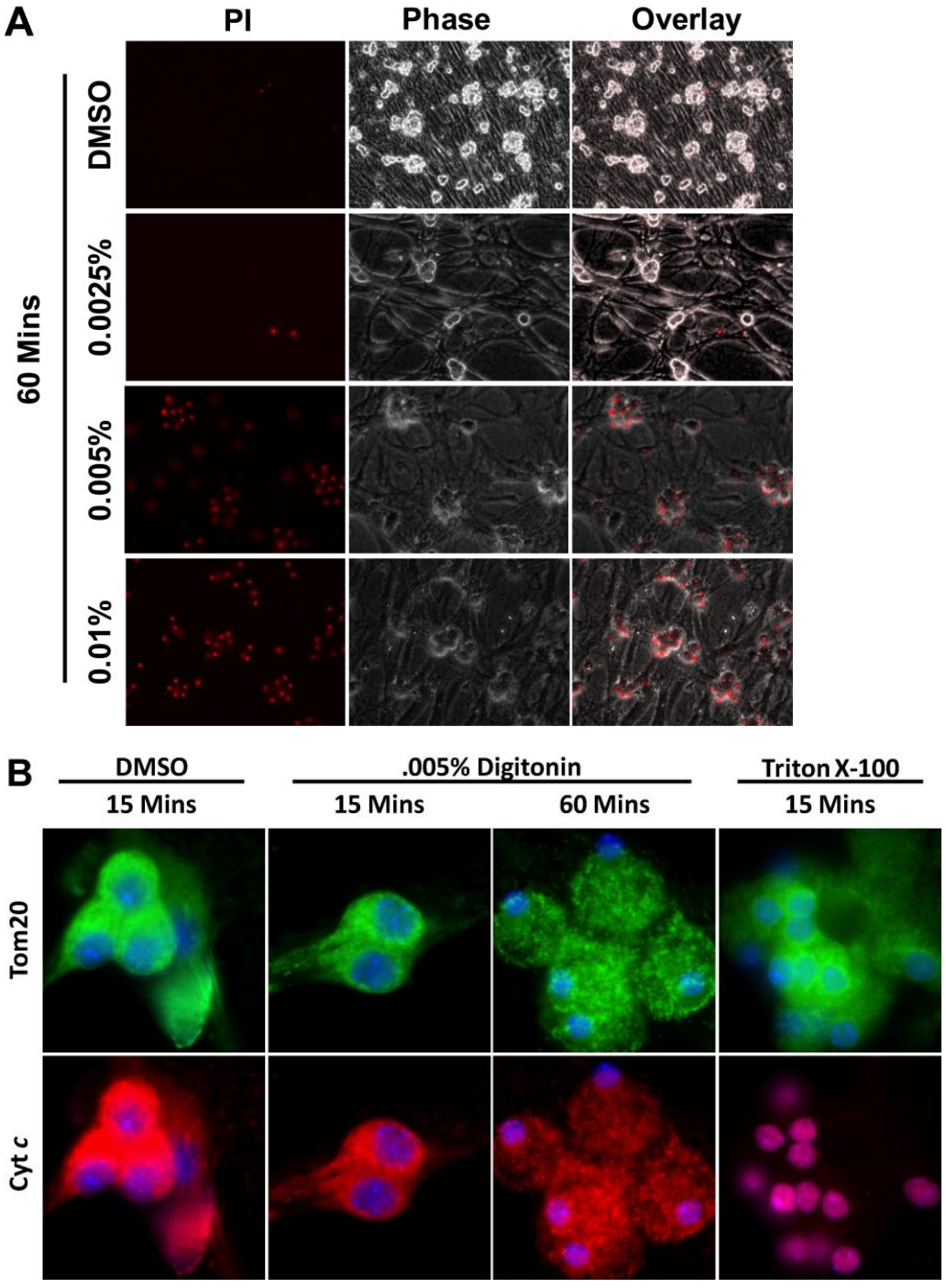

A
FITC
Cyt. c
5 DIV
FITC
Cyt. c
28 DIV
B
% Cyt C Maintained
100
50
0
P5 DMSO
P5 BBP
P28 DMSO
P28 BBP

Figure 3.6 Optimization of Digitonin Concentration for Permeablizing Adherent Sympathetic Neurons

A) Representative images of neuron cultures permeablized with digitonin. DMSO indicates vehicle control. Percentages represent digitonin concentration. PI staining is used as an indicator of cell membrane permeablization. B) Immunofluorescence staining of neurons incubated with the optimum concentration of digitonin (.005%) as determined in (A). Neurons were stained with anti-cyt *c* antibody as a readout for mitochondrial membrane permeablization and Tom20 as a mitochondrial marker. Triton X-100 was used as a positive control for mitochondrial membrane permeablization.

Figure 3.6


A
PI
Phase
Overlay
60 Mins
DMSO
0.0025%
0.005%
0.01%
B
DMSO
.005% Digitonin
Triton X-100
15 Mins
15 Mins
60 Mins
15 Mins
Tom20
Cyt c

CHAPTER 4: DISCUSSION

4.1: Summary of Findings

Creating a properly wired nervous system in which the appropriate neurons match up with the correct targets in the correct numbers is an intricately regulated process that requires a combination of neuronal differentiation, migration, axon outgrowth, and regulated degeneration, or apoptosis. Apoptosis serves a key role in development by allowing for the controlled removal of neurons that fail to differentiate, migrate, or innervate their targets properly. During the early stages of development, neurons voluntarily initiate their own destruction in a well regulated, orderly manner that depends on new protein synthesis. However, after the formation of the nervous system, the apoptotic pathway becomes restricted in neurons, likely to prevent aberrant activation of the apoptotic pathway which would be deleterious as neurons, with few exceptions, are not easily replaced if lost. This work provides important insight into the molecular mechanisms utilized by neurons to restrict the apoptotic pathway as they mature.

While it is clear that mature neurons restrict the apoptotic pathway by multiple redundant mechanisms, recent findings in the fields of axon pruning and synaptic plasticity have revealed that regulation of the apoptotic pathway can be controlled with far greater spatial precision than previously appreciated, even in mature neurons that have restricted the apoptotic pathway. This precise spatial regulation

allows neurons to engage the apoptotic pathway in a limited fashion in dendrites or axons to permit the controlled destruction of processes or even individual synaptic sites, such as dendritic spines, to facilitate nervous system plasticity. Here, we have investigated both the temporal regulation of the apoptotic pathway in developing neurons, and the spatial regulation of the apoptotic pathway that differs between somas and axons.

Mature Neurons Dynamically Regulate Apoptosis Via Redundant Pre-Mitochondrial Brakes

1) Sympathetic neurons acquire resistance to apoptosis by activating pre and post-mitochondrial apoptotic brakes as early as 10-15 days in culture.
2) Knockout of all three miR-29 family members in maturing sympathetic neurons fails to resensitize these neurons to apoptosis in response to NGF deprivation, indicating the presence of other brakes on the apoptotic pathway upstream of cyt *c* release.
3) Other miRNAs with predicted targets in the apoptotic pathway, miR-24, miR-124, and miR-128, are upregulated in maturing neurons.
4) Overexpression of miR-29 or miR-24, but not miR-124 or miR-128, is sufficient to inhibit cyt *c* release in response to NGF deprivation in sympathetic neurons.
5) miR-29 and miR-24 can repress the induction of endogenous Bim in sympathetic neurons.
6) miR-24 can target the Puma 3’ UTR and inhibit DNA-damage-induced apoptosis in sympathetic neurons.

7) Inhibition of miR-24 activity in miR-29 deficient neurons does not completely resensitize mature neurons to apoptosis, possibly due to the presence of an additional brake on the apoptotic pathway at the level of c-jun phosphorylation.

Spatial and Temporal Regulation of Bax Activation in Neurons

1) ABT-737 treatment induces degeneration of axons, but not somas, in young sympathetic neurons.
2) ABT-737 induces cyt *c* release in both somas and axons of young sympathetic neurons, but only in axons of mature sympathetic neurons.
3) Pre-treatment with NGF-deprivation sensitizes mature neurons to ABT-737 induced cyt *c* release in a protein-synthesis-independent manner.
4) Treatment with ABT-737 unexpectedly leads to the accumulation of Bim in young, but not mature sympathetic neurons.
5) Inhibition of protein synthesis at the time of CHX treatment in young neurons inhibits the cyt *c* release induced by ABT-737 treatment in neuronal somas, but axons still appear to degenerate.
6) Cytosolic injection of BH3-only domain peptides is sufficient to induce cyt *c* release in young sympathetic neurons, but mature neurons are markedly more resistant.
7) Partial permeablization of adherent sympathetic neurons with digitonin appears to be a feasible model for investigating the regulation of Bax activation by BH3-only domain peptides.

4.2: Clinical Relevance

Overexpression of miRNAs for Therapeutic Purposes

As discussed in the introduction, a number of miRNAs are dysregulated in cases of neurodegenerative disease. Additionally, overexpression of miR-29 has been found to be neuroprotective in *in vivo* models of stroke and spinal cord injury (Wang et al., 2015; Khanna et al., 2013; Ouyang et al., 2013; Liu et al., 2015). miR-29 has also been found to target Beta-Amyloid Cleaving Enzyme (BACE1), which is an enzyme that cleaves Amyloid Precursor Protein (APP) to produce toxic Amyloid-β proteins in Alzheimer's Disease (Hebert, Horre et al. 2008; Roshan, Ghosh et al. 2012; Lei, Lei et al. 2015). These findings, coupled with our own findings that miR-29 and miR-24 overexpression can restrict the apoptotic pathway, suggest that overexpression of pro-survival miRNAs such as miR-29 and/or miR-24 could have therapeutic potential for human pathologies.

Development of miRNAs as therapeutic entities is still in the early stages. The ability of miRNAs to simultaneously regulate multiple molecular pathways makes them attractive from a therapeutic perspective for disorders like cancer and neurodegeneration, where multiple pathways are known to be perturbed. Therapeutic strategies being pursued in clinical trials include both interfering with miRNA activity, as in the case of miR-122 and Hepatitis C Virus (Janssen, Reesink et al. 2013), and in replacing/overexpressing miRNAs, as with miR-34a and cancer (Mirna Therapeutics Clinical Trial NCT01829971).

Treatment of diseases by miRNA overexpression or replacement has two chief obstacles to overcome: first, RNA degradation machinery, such as Ribonucleases, in the mammalian bloodstream can inhibit the stability of the miRNA duplex, preventing it from reaching its target tissue. Second, miRNAs do not readily cross cellular membranes, and miRNAs can only have a therapeutic effect from inside the cell. A number of different approaches are currently being explored to overcome these obstacles.

To enhance miRNA stability in the body, miRNA duplex mimics can be chemically modified; the addition of 2'F can protect against exonuclease activity, while conjugation to a molecule like cholesterol can enhance cellular uptake. Additional chemical modifications can be made to the sense strand of the miRNA duplex to prevent RISC loading and stop the sense strand from acting as an antimiR, which would diminish therapeutic efficacy. miRNA duplexes can also be loaded into liposome nanoparticles to aid their *in vivo* delivery. Indeed, the first miRNA restoration study to enter clinical trials makes use of liposomal nanoparticles to facilitate miRNA mimic delivery. miRNAs are also good candidates for gene-therapy mediated overexpression using lentiviral or adenoviral vectors (Zhang, Wang et al. 2013; Chen, Gao et al. 2015).

Delivery of miR-29 or miR-24 to the brain poses additional challenges compared to delivery of miRNAs to other tissues or tumors. Any treatment targeting the brain must cross the blood-brain barrier to achieve its effects. Animal studies have used direct injection of mimics into the brain as a straightforward means of overcoming this barrier, but this approach has obvious drawbacks for therapeutic use in humans. An approach that has been successfully used to deliver siRNAs and LNAs to the brain is the

conjugation of the RNA to a neurotropic peptide derived from the Rabies virus, known as RVG, which leads to neuronal uptake of RNAs (Kumar et al., 2007; Roshan et al., 2014). This approach has allowed the brain-specific knockdown of target genes through systemic injection of the siRNA/RVG complex. This approach has also recently been adapted to work with miRNA mimics (Kumar, Wu et al. 2007; Hwang, Son et al. 2011).

Targeted delivery of miR-29 mimics in particular is important because overexpression of miR-29 in many mitotic cells has been found to induce apoptosis and cellular senescence, in contrast to its survival-promoting effects found in neurons (Mott et al., 2007; Martinez et al., 2011; Hu et al., 2014). Interestingly, a recent study from the pharmaceutical company Miragen explored the use of miR-29 mimics for the treatment of pulmonary fibrosis. miR-29 is known to target multiple extracellular matrix components, and it is thought that overexpression of miR-29 can inhibit the formation of fibrotic tissue and scars (He, Huang et al. 2013). In the Miragen preclinical study, a miR-29 mimetic molecule with modifications to promote its stability and cellular uptake was able to inhibit disease progression in a mouse model of pulmonary fibrosis (Montgomery, Yu et al. 2014). Another group has also recently reported the successful delivery of a recombinant pre-miR-29 mimic into N2a695 cells, resulting in the downregulation of BACE1 expression and amyloid-beta protein levels (Pereira, Tomas et al. 2016).

It remains to be seen how well miR-29, miR-24, or indeed miRNAs in general, will ultimately work astherapeutic agents. Treating neurological diseases with miRNA will likely require further advances in enhancing miRNA delivery and efficacy. However, early clinical results manipulating other miRNAs appear promising (Janssen et al.,

2013), and preclinical data from our lab and others certainly makes the case that miR-29 and miR-24 merit further investigation as a therapeutic, both because of their ability to inhibit apoptosis as well as the ability of miR-29 in particular to target disease specific proteins such as BACE1 in Alzheimer's Disease.

Cell Death in Neurodegeneration

The work presented here invites an obvious question: if mature neurons are more resistant to cell death, how do neurons die in cases of neurodegeneration, which predominantly affect older individuals whose neurons should be mature? A definitive answer to this question is not yet known, and likely varies from one disease to another. There is evidence that some apoptotic brakes, such as miR-29 induction, are partially reversed in some neurodegenerative disorders, such as Alzheimer's Disease or Huntington's Disease (Hebert et al., 2008; Shioya et al., 2010). However, as discussed in the introduction, inhibition of apoptosis has not proved to be a "magic bullet" for stopping neurodegenerative disease. Indeed, evidence for the involvement of apoptosis in neurodegenerative disease has been controversial at best. The most noteworthy success for inhibiting a neurodegenerative disorder is likely the finding that Bax/Bak double deficiency delays cell death and disease progression in a mouse model of ALS (Reyes et al., 2010). However, even in this case, complete inhibition of the mitochondrial apoptotic pathway only had a modest effect on disease progression, neuronal survival, and animal lifespan, suggesting that neurons in ALS that would normally die of apoptosis die by other means when the apoptotic pathway is blocked.

When surveying the literature on neurodegeneration and developmental apoptosis, one feature that is common to both is the activation of the cell cycle machinery in neurons. Apoptosis in young neurons is well established as requiring activation of cell-cycle proteins (Freeman et al., 1994). Inhibition of cell-cycle progression by pharmacological or genetic means protects neurons from apoptosis. Interestingly, aberrant activation of cell cycle machinery is also known to occur in the brains of Alzheimer's Disease patients, as determined by the presence of elevated cell-cycle related proteins and even multinucleate neurons in AD patient samples. This aberrant cell cycle reentery is thought to be a cause, rather than a consequence, of neurodegeneration (Vincent, Pae et al. 2003; Currais, Hortobagyi et al. 2009; Bonda, Lee et al. 2010). Enforced activation of the cell cycle in the brain by overexpression of Simian-Virus-40 (SV40) Large T Antigen has been found to induce neurodegeneration in mice, and this neurodegeneration exhibits two hallmarks of Alzheimer's Disease: neurofibrillary tangles and Amyloid-β deposits (Park, Hallows et al. 2007). Induction of neuronal cell-cycle reentry by overexpression of c-myc in forebrain neurons also leads to neurodegeneration and cognitive decline (Lee, Casadesus et al. 2009).

A limitation of many studies of neuronal cell death is that they make use of young, often embryonically derived cultured neurons. Stimulation of apoptosis in these neurons leads to cell-cycle reentry and relatively rapid death. However, in mature neurons where the apoptotic pathway is arrested, as described in this dissertation, sustained exposure to an apoptotic or pathological stimulus that lead to apoptosis in young neurons could lead to the partial induction of the apoptotic pathway in mature neurons, resulting in cell-cycle reentry without progressing to the point of caspase

activation and cell death. This theory is consistent with the “two-hit” hypothesis of Alzheimer’s Disease, which posits that neuron death in Alzheimer’s is a consequence of a combination of aberrant cell-cycle reentry and oxidative stress. Aberrant cell-cycle reentry is thought to place neurons in a weakened state, which renders them more vulnerable to a second “hit”, which this hypothesis proposes to be oxidative stress (Zhu, Lee et al. 2007)(Zhu et al., 2007). This second hit overwhelms the already stressed neurons, ultimately leading to the death of the cells. Interestingly, aberrant cell-cycle activation is not limited to Alzheimer’s Disease, and has also been observed in human cases and animal models of ALS and Huntington’s Disease (Ranganathan and Bowser 2003; Pelegri, Duran-Vilaregut et al. 2008). It is unclear at this point whether this cell-cycle reentry does in fact represent the aborted activation of the apoptotic pathway, or indeed what the precise stimulus is that causes cell-cycle reentry at all; however the fact that many of the toxic protein species found in the mature brain during neurodegenerative disorders are capable of inducing apoptosis in young neurons suggests that further investigation is warranted in this area.

4.3: Non-Apoptotic Roles for Apoptotic Proteins

Another question raised by our findings that neurons become more resistant to apoptosis with maturation is: why would neurons continue expressing any apoptotic proteins at all? If the goal is to shut down the pathway, why not shut the pathway all the way down? The likely answer to this is that apoptotic proteins have jobs in the cell independent of their role in apoptosis. Indeed, a large number of recent studies have demonstrated that apoptotic pathway genes have roles outside of their “day jobs” as the

executioners of the cell death pathway. While the classic example of this phenomenon is cytochrome *c*, which was well established as an electron-transport-chain component prior to its key role in the apoptotic pathway being identified, recent evidence demonstrates non-apoptotic roles for other members of the apoptotic pathway as well.

Bax and Caspases in Synaptic Plasticity and Synapse Remodeling

The first major finding indicating that the apoptotic pathway might be involved in synaptic plasticity in the mammalian brain was made in the lab of Dr. Morgan Sheng in 2010 (Li et al., 2010). Their study found that in hippocampal neurons, stimulation of NMDA receptors can lead to a low level of Caspase 3 activation, and that Caspase activity was required for the onset of Long-Term Depression (LTD) in these neurons by a mechanism that involves the regulation of AMPA receptor trafficking. LTD could be prevented by pharmacological caspase inhibitors, overexpression of the endogenous caspase inhibitor XIAP, or genetic knockout of Caspase 3. Interestingly, they also noted that overexpression of BCL-XL, which acts upstream of Caspases, could also inhibit LTD, suggesting the involvement of Bax. Sure enough, a paper published the very next year (Jiao and Li 2011) reported the finding that Bax and the BH3-only protein Bad are also required for the development of NMDAR-Dependent LTD in hippocampal neurons. Interestingly, this paper was unable to detect increased active Bax in the mitochondrial fraction obtained from NMDA-stimulated cells by immunoprecipitation. This raises the possibility that Bax could activate Caspase 3 in a manner that does not involve mitochondria under these circumstances, but it could also be that Bax permeablizes only a small number of mitochondria, or does so in a limited fashion in these cells that is difficult to detect by Western blotting on mass cultures. Indeed, so-called “minority

MOMP", where a limited population of mitochondria in within a given cell are permeablized, has recently been described in cancer cells (Ichim, Lopez et al. 2015).

Another mechanism through which limited Caspase activation is believed to regulate nervous system plasticity is through the elimination of synapses themselves. Synaptic spine density has been found to be increased in the brains of Caspase 3 knockout mice, and local activation of Caspase 3 by optogenetic means has been found to result in the loss of synaptic spines in cultured hippocampal neurons (Ertürk, Wang et al. 2014). Caspase 3 was also found to regulate post-synaptic structures at the neuromuscular junction by cleavage of the protein Dishevelled (Wang, Chen et al. 2014). The cell death pathway was also found to regulate synapse elimination in the nematode worm *C*. *elegans* (Meng, Mulcahy et al. 2015).

Sublethal activation of caspases has also been implicated in other neuronal processes, such as axon pathfinding (Unsain and Barker, 2015). The chemotrophic response of neuronal axons in the retina to netrin or lysophosphatidic acid has been found to depend on the activity of caspase 3 and 9 (Campbell and Holt 2003; Campbell and Okamoto 2013). In olfactory neurons, Caspase-9 and Apaf-1 knockout animals were found to display defects in axon projection and synapse formation (Ohsawa, Hamada et al. 2010). Inhibition of Caspase 3 activity has also been found to cause defects in the regeneration of injured neurons in the mouse Dorsal Root Ganglia (DRG) and in injured sensory and motor axons in *C. elegans* (Verma, Chierzi et al. 2005; Pinan-Lucarre, Gabel et al. 2012; Ozturk, Cengiz et al. 2013).

Neuronal plasticity is important for learning and memory as organisms adapt to new environments. Combined with the results from our lab and others that demonstrate inhibition of the apoptotic pathway in mature neurons, these findings suggest that neurons possess a previously unappreciated ability to precisely control both the level and location of the activation of the apoptotic pathway. The precise mechanisms through which cells achieve this measure of control are still being studied, but it is likely that the increasing restriction of the apoptotic pathway in mature neurons that we have described helps prevent limited activation of apoptosis for the purposes of synaptic plasticity from compromising the survival of the cell.

A more complete understanding of how local or low-level activation of the apoptotic machinery is regulated is crucial, as there is accumulating evidence that aberrant caspase activation in different subcellular compartments may play a role in neurodegeneration. In Huntington's Disease, the toxic protein Huntingtin is known to be a Caspase substrate, and its cleavage by Caspases may play a role in Huntingtin clearance from the brain (Gafni, Papanikolaou et al. 2012). In Alzheimer's Disease, patients are known to exhibit decreases in synaptic density and changes in synaptic function (Ferrer and Gullotta 1990; Ingelsson, Fukumoto et al. 2004). Animal models investigating the effects of Aβ overexpression have found similar defects in synaptic function, and these defects have been found to be caspase dependent (Kim, Anwyl et al. 2001; Shankar, Li et al. 2008; D'Amelio, Cavallucci et al. 2011).

Concluding Remarks

The results described here provide important insights into how neurons restrict the apoptotic pathway during development at the level of miRNAs and byond. They highlight how upregulation of certain miRNAs can make neurons more resistant to stressful stimuli like trophic factor deprivation or DNA damage, which could have therapeutic implications in neurological diseases. Interesting future directions to explore include optimizing neuronal delivery of miRNAs to the brain *in vivo* for the treatment of neurological disorders, as well as further mechanistic exploration of how certain neurons in the adult brain are able to engage the apoptotic pathway in a limited fashion to help facilitate neuronal plasticity.

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