THE MOLECULAR INTERSECTION BETWEEN AXON-SPECIFIC PRUNING AND NEURONAL APOPTOSIS

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ABSTRACT

COREY LEIGH CUSACK: The Molecular Intersection between Axon-Specific Pruning and Neuronal Apoptosis
(Under the direction of Dr. Mohanish Deshmukh)

Neurons can activate pathways to either destroy the whole cell via apoptosis or specifically degenerate only the axon. Axon-specific degeneration, also known as pruning, is required to establish and refine neuronal connections during development and to permit plasticity in the adult nervous system. Aberrant axon degeneration is also observed in several neurodegenerative diseases and can long precede cell body death in neurons. However, despite its biological importance and clinical relevance, the exact mechanism underlying axon degeneration remains unclear.

Apoptosis and axon degeneration pathways were widely recognized to be distinct based on the prevalent axotomy-induced model of axon removal called Wallerian degeneration. Five years ago, however, it was discovered that developmental axon pruning induced by axon-specific trophic factor deprivation required the key apoptotic protein Bax as well as a caspase, caspase-6. Several studies, including work described here, also demonstrate the requirement of the classically apoptotic caspases-9 and -3 in axon pruning. This dissertation examines several critical questions raised by these unanticipated findings: Are other components of apoptosis also involved in axon degeneration? How can neurons activate Bax to selectively degenerate axons without simultaneously triggering

apoptosis? What is the exact mechanism for activating caspase-6 during axon degeneration?

By applying the lab's expertise in neuronal apoptosis and adapting microfluidic technology to study axon-specific degeneration, I have uncovered several novel aspects about the molecular points of overlap and distinction between neuronal apoptosis (where both axons and somata degenerate) and axon pruning (where only axons degenerate). This work is the first to distinguish that caspase-6 is essential for axon degeneration that occurs during pruning but not apoptosis. Second, while apoptosis requires both Apaf-1 and caspase-9, axon pruning requires caspase-9 but not Apaf-1. Third, I found that neurons utilize both the potent endogenous caspase inhibitor XIAP and the proteasome to compartmentalize axonal caspase activation and protect the soma. Fourth, this work demonstrates that mature neurons are exquisitely capable of selectively restricting apoptosis while permitting axon-specific degeneration. Lastly, I have also focused on how transcription, translation, and the localization of BH3-only proteins and the microRNA, miR-29, may facilitate axon-specific Bax activation.

To my parents

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"Do not follow where the path may lead,
go instead where there is no path and leave a trail."
- Ralph Waldo Emerson

PREFACE

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AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

Apaf-1: Apoptosis protease activating factor 1

APP: Amyloid precursor protein

ATP: Adenosine triphosphate

BACE1: β-site APP-cleaving enzyme 1

BH: Bcl-2 homology

BIR: Bacculoviral IAP repeat

CARD: Caspase activation and recruitment domain

Casp: Caspase

C. elegans: Caenorhabditis elegans

CNS: Central nervous system

dATP: Deoxyadenosine triphosphate

DD: Death domain

DED: Death effector domain

DIABLO: Direct IAP binding protein with low pI

DIV: Days *in vitro*

DNA: Deoxyribonucleic acid

DR: Death receptor

DRIE: Deep reactive ion etching

E: Embryonic day

ER: Endoplasmic reticulum

FADD: FAS-associated protein with Death Domain

GAPDH: Glyceraldehyde phosphate deyhydrogenase

HD: Huntington's disease

HSP27/70/90: Heat shock proteins

IAPs: Inhibitor of apoptosis proteins

ICE: Interleukin-1β converting enzyme

JNK: c-Jun N-terminal kinase

miR-29: microRNA-29

miRNA: microRNA

MLK: Mixed lineage kinase

MOM: Mitochondrial outer membrane

MOMP: Mitochondrial outer membrane permeabilization

mRNA: Messenger RNA

NB-ARC: Nucleotide-binding adaptor shared by Apaf-1, R proteins and ced-4

NGF: Nerve growth factor

NOD: Nucleotide-binding oligomerization domain

P: Post-natal day

PD: Parkinson's disease

PIDD: p53-induced death domain protein

PNS: Peripheral nervous system

qRT-PCR: quantitative RT-PCR

RAIDD: RIP associated Ich-1/CED homologous protein with death domain

RING: Really interesting new gene domain

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

ROS: Reactive oxygen species

Ser: Serine

SMAC: Second mitochondrial activator of caspases

TRAIL: TNF-related apoptosis-inducing ligand

TRAMP: Trf4/Air2/Mtr4p polyadenylation complex

WD40: 40 amino acid repeat ending in tryptophan-aspartic acid (W-D)

XIAP: X-linked inhibitor of apoptosis protein

CHAPTER ONE: INTRODUCTION

1. Overview of Apoptosis

"If you don't know how to die, don't worry; Nature will tell you what to do on the spot, fully and adequately. She will do this job perfectly for you; don't bother your head about it."
-Montaigne (1533-1592), French philosopher and essayist

A historical perspective:

Death is a fundamental and universal part of life; every living cell, vibrant organism, and breathing creature has an end. Cells can die in a variety of ways, yet the most understood energetic, genetically conserved death process is programmed cell death, or apoptosis. First observed by Carl Vogt as early as 1842, apoptosis was coined much later in 1972 by Drs. J.F.R. Kerr, A.H. Wyllie, and A.C. Currie (Clarke, Sphyris et al. 1996) when they recognized that the phenotype of spontaneously dying cells in organisms greatly differed from cells that died following trauma or injury. The latter swell and violently burst, spilling their contents into surrounding tissue, while the former simply shrink, condense their chromatin, and quickly become engulfed by debris-clearing cells. Meaning "dropping or falling off" in Latin, as in leaves falling from a tree (Kerr, Wyllie et al. 1972), apoptosis has since become a widely studied phenomenon. As the topic of now more than 260,000 publications, apoptosis is highly valued as an essential mechanism of normal development across all organisms as well as a key contributor to human disease states ranging from

cancer to neurodegeneration.

Apoptosis is well appreciated as a genetically programmed and tightly regulated pathway required for proper development and tissue homeostasis (Danial and Korsmeyer 2004), yet this has not always been the case. Studies from Dr. Bob Horvitz's lab during the mid-1980s laid the foundation for our understanding of the genetic and biochemical processes that control programmed cell death. The Horvitz lab mapped the fate of every single cell in the nematode *C. elegans* and found that exactly 131 cells die during normal development, always resulting in a worm with precisely 959 cells (Sulston and Horvitz 1977; Sulston, Schierenberg et al. 1983). A forward genetic screen then revealed two genes, *ced-3* and *ced-4*, whose function is absolutely required for the death of those 131 cells (Ellis and Horvitz 1986; Yuan and Horvitz 1990). The Horvitz lab also identified *ced-9*, the first anti-apoptotic gene; mutations in *ced-9* resulted in excessive and unnecessary cell death in the nematode (Hengartner, Ellis et al. 1992). These insightful discoveries about the genetic underpinnings of apoptosis earned Dr. Horvitz the Nobel Prize and pioneered the search for conserved cell death machinery beyond the nematode and into mammalian cells.

Apoptosis is essential for proper development:

Cell death occurs at astonishingly high levels during normal mammalian development. Apoptosis is required to generate properly sculpted tissues and organs, to eliminate unnecessary or misplaced cells after development, and to remove cells that may harm the organism (Jacobson, Weil et al. 1997; Danial and Korsmeyer 2004). One favorite example of apoptosis is the precise death of skin cells between digits *in utero* that gives rise to the individual digits of each mouse paw (Figure 1.1)(Garcia-Martinez, Macias et al. 1993;

Wood, Turmaine et al. 2000). Apoptosis is also essential for proper nervous system development for the following three known functions:

1.) to achieve balance between the number of innervating neurons and their target cells (Oppenheim 1991);
2.) to limit the number of proliferating neuronal precursors in germinal zones of the CNS and PNS (Voyvodic 1996; Kuan, Roth et al. 2000), therefore secondarily affecting the final number of neurons in the nervous system;
3.) to correct any errors, most notably by removing neurons that have migration deficits or misprojecting axon outgrowth (Kim and Sun 2011).

Apoptosis is required for adult homeostasis:

Apoptosis is critical beyond development and throughout adulthood. Steady cell populations are achieved in adult tissues by complementing the removal of older cells by apoptosis with the generation of new cells by mitosis (Guo and Hay 1999). For example, bone marrow and intestinal cells normally undergo apoptosis at rapid rates to balance out the continuous addition of new cells in these organs (Alberts 2002). Apoptosis is also critical for maintaining healthy cell populations in the face of damage caused by insults such as toxins, carcinogens, ultraviolet radiation and reactive oxygen species; apoptosis ensures the rapid and efficient removal of damaged cells following such toxic insults (Carson and Ribeiro 1993; Mates and Sanchez-Jimenez 2000; Norbury and Hickson 2001; Truong-Tran, Grosser et al. 2003; Van Laethem, Claerhout et al. 2005). For cell populations with high mitotic rates, in particular, it is healthier for the organism to kill off damaged

cells rather than risk passing genetic damage down to daughter cells (Green and Evan 2002). Notably, in contrast to cells that are replaced throughout life, post-mitotic cells with little or no proliferative capacity (such as neurons, cardiomyocytes and myotubes) must engage pathways that strictly inhibit apoptosis (Wright and Deshmukh 2006).

Apoptosis in disease:

Too little or too much apoptosis can cause developmental disease, cancer, autoimmunity, and neurodegeneration. The importance of developmental cell death is highlighted by the disrupted tissue formation observed in genetically altered mice that cannot execute apoptosis (Kuida, Haydar et al. 1998). In the immune system, apoptosis is critical for proper development of immune B and T cells to ensure the execution of any cells that recognize self-antigens (Marsden and Strasser 2003); failure to complete this process results in the survival of cells that recognize and respond to self-antigens (O'Reilly and Strasser 1999), leading to autoimmune disease. Lastly, the ability of cancer cells to evade apoptosis promotes their vigorous survival and uncontrolled growth (Vaux, Cory et al. 1988; Hanahan and Weinberg 2000).

In contrast to situations involving too little apoptosis, disease also occurs in cases of over-active apoptosis. One highly relevant example is neurodegeneration, which results when unwanted, excessive apoptosis occurs in the mature nervous system. While many diverse events contribute to the pathologies and neuron death observed in neurodegenerative situations (described in more detail later in the Introduction), apoptosis can be triggered by toxic insults associated with these diseases (Figure 1.2)(e.g. reactive oxygen species, misfolded proteins, aggregate formations, etc.)(Mattson 2000; Yuan and

Yankner 2000). Apoptosis has also been implicated in cell death following stroke or myocardial infarctions (Choi 1996; Schulz, Weller et al. 1999; Rodriguez, Lucchesi et al. 2002; Li, Zheng et al. 2005). Ideally, achieving a more complete understanding of the molecular mechanisms that enhance or suppress apoptosis will lead to or improve the development of therapeutics for a variety of diseases.

1.2 Apoptosis in developing neurons

Nerve growth factor and the neurotrophic hypothesis:

Seminal studies by Viktor Hamburger and Rita Levi-Montalcini ultimately led to the discovery of the first neurotrophic factor (NGF, nerve growth factor)(Cohen, Levi-Montalcini et al. 1954; Levi-Montalcini and Cohen 1956; Levi-Montalcini and Booker 1960; Aloe 2004) and the neurotrophic hypothesis, which provided insight into the basic mechanism that initiates cell death in developing neurons. Simply stated, the neurotrophic hypothesis posits that an abundance of neurons are generated during development that must compete for limited amounts of survival-promoting growth factors secreted by target cells; the fates of developing neurons are ultimately regulated by their targets (Northcutt 1989; Yuan and Yankner 2000; Dekkers and Barde 2013; Dekkers, Nikoletopoulou et al. 2013). Neurons that reach and properly innervate their target will survive and incorporate into the developed nervous system, while those without adequate trophic factor support are eliminated by apoptosis. As many as 50% of neurons undergo apoptosis during this developmental period, elegantly matching the number of target cells to a precise population of neurons.

Molecular modulators of neuronal apoptosis induced by NGF deprivation:

Sympathetic neurons of the superior cervical ganglia provide an excellent model to study developmental apoptosis. Indeed, we have discovered much of what we know about the mechanisms and regulation of neuronal apoptosis by examining sympathetic mouse neurons deprived of NGF *in vitro* (Levi-Montalcini and Booker 1960; Gorin and Johnson

1979; Crowley, Spencer et al. 1994; Smeyne, Klein et al. 1994; Deshmukh and Johnson 1997; Putcha, Deshmukh et al. 2000).

In the presence of NGF, a neuron promotes its own survival through the tyrosine kinase receptor (known as TrkA), which signals through the PI3-kinase/Akt and MEK/MAPK pathway (Kaplan and Miller 2000). One long-held and reasonable assumption posited that neurons died following NGF deprivation because NGF signaling promoted neuron survival. Simply stated, the loss of trophic factor support meant the loss of pro-survival signals and a passive death. We now know, however, that neurons initiate an active death process following NGF deprivation that requires signal transduction, transcription and translation; strikingly, treating neurons with transcription or translation inhibitors during NGF deprivation protects them from death (Martin, Schmidt et al. 1988).

Although new factors involved in neuronal death continue to be discovered, many of the essential events and modulators of the apoptosis pathway induced by NGF deprivation are well known (Figure 1.3). When an NGF-dependent neuron is deprived of trophic factor, the following initial changes occur: glucose uptake rapidly decreases, protein synthesis is globally reduced, levels of reactive oxygen species (ROS) spike, and the Rho GTPase Cdc42 becomes activated (Greenlund, Deckwerth et al. 1995; Bazenet, Mota et al. 1998). Precisely how each of these events is triggered remains unknown, yet their downstream effects are well characterized; following these events, a kinase cascade is initiated that involves the mixed lineage kinases (MLKs) and c-Jun N-terminal kinase (JNK), leading to the phosphorylation of the transcription factor c-Jun (Mota, Reeder et al. 2001; Xu, Maroney et al. 2001). Phosphorylated c-Jun translocates from the cytosol to the nucleus where it activates the transcription of (at least) the following four BH3-only proteins: Bim, Hrk/DP5,

Puma, and Bmf (Estus et al., 1994; Ham et al., 1995; Imaizumi et al., 1997; Eilers et al., 1998; Xu et al., 2001; Mota et al., 2001; Harris and Johnson, 2001; Whitfield et al., 2001; Kristiansen et al., 2011). While phosphorylated c-Jun activity is absolutely required for NGF-deprivation-induced death in sympathetic neurons (Estus, Zaks et al. 1994; Ham, Babij et al. 1995; Eilers, Whitfield et al. 1998), its targets for transcriptional upregulation appear to act redundantly as the loss of any one BH3-only protein's expression only modestly protects neurons from death (Putcha, Moulder et al. 2001; Imaizumi, Benito et al. 2004).

Following Bax activation by the BH3-only proteins, cytochrome *c* is released from mitochondria, which leads to apoptosome formation, caspase-9 activation and, lastly, caspase-3 activation (Deshmukh, Kuida et al. 2000; Wright, Vaughn et al. 2007). Similarly, the intrinsic apoptotic pathway involving BH3-only proteins, Bax, apoptosome formation and caspase activation is also activated in neurons faced with stimuli other than NGF withdrawal (e.g. DNA damage and endoplasmic reticulum stress) (Besirli, Deckwerth et al. 2003; Smith and Deshmukh 2007; Vaughn and Deshmukh 2007).

1.3 Intrinsic Apoptosis: The Key Players

"After all, to the well-organized mind, death is but the next great adventure."
–Albus Dumbledore in Harry Potter and the Sorcerer's Stone *by J.K. Rowling*

Bcl-2 family of proteins: Key initiators of cell death:

The translocation of cytochrome *c* from the mitochondria into the cytosol is one major point of apoptosis regulation, and this step is primarily controlled by the Bcl-2 family of proteins. The discovery of *ced-9* in *C. elegans* led to the critical identification of its mammalian homolog, Bcl-2 (B-cell lymphoma 2) protein, as both proteins potently prevent apoptosis (Hockenbery, Nunez et al. 1990; Garcia, Martinou et al. 1992; Vaux, Weissman et al. 1992; Chipuk, Moldoveanu et al. 2010). Since its initial discovery in the 1990s, the Bcl-2 family of proteins has grown extensively and is divided into the following three distinct classes based on structure and function:

1.) the effector proteins that oligomerize and permeabilize the mitochondrial outer membrane (MOM), thereby inducing the release of intermembrane components (such as cytochrome *c* and SMAC/DIABLO) that lead to downstream caspase activation;
2.) the BH3-only proteins that sense cellular stress and become activated, either directly or indirectly;
3.) the anti-apoptotic members that inhibit both of the groups described above (Giam, Huang et al. 2008; Youle and Strasser 2008).

More specifically, each Bcl-2 protein is classified by its pro- or anti-apoptotic role and its number of Bcl-2 homology (BH) domains (Figure 1.4). The first group of pro-apoptotic members contains multi-domain proteins that have BH1, BH2 and BH3 domains; this group comprises two proteins, Bax and Bak. In healthy cells, Bax and Bak are normally expressed but remain inactive. During an apoptotic stimulus, however, both proteins can insert into the MOM and oligomerize to form a pore-like structure, resulting in mitochondrial outer membrane permeabilization (called MOMP)(Chipuk, Bouchier-Hayes et al. 2006). MOMP permits the release of cytochrome *c* into the cytoplasm where, as described in the previous section, it associates with Apaf-1 and promotes caspase activation. Interestingly, in neurons, only Bax is capable of triggering MOMP as Bax-deficient neurons are highly resistant to intrinsic apoptotic stimuli (Deckwerth, Elliott et al. 1996). Bak is present in neurons but exists in a uniquely spliced isoform called N-Bak that cannot directly permeabilize mitochondria (Sun, Yu et al. 2001; Uo, Kinoshita et al. 2005). Rather, the domain structure of N-Bak indicates this Bcl-2 protein may function like a BH3-only protein (described below) in neurons (Ham, Towers et al. 2005). While little is known about the function of N-Bak, the redundancy between Bax and Bak is clearly lost in neurons, which may permit tighter apoptosis regulation and prevent the release of cytochrome *c* until absolutely necessary.

Members of the second group of pro-apoptotic Bcl-2 proteins contain only one BH3 domain and are aptly named the BH3-only proteins (Giam, Huang et al. 2008; Lomonosova and Chinnadurai 2008). This group included the following proteins: Bid, Bim, Bik, Bad, Bmf, Hrk/DP5, Puma, and Noxa. As the key responders to apoptotic stimuli, the BH3-only proteins can become activated through transcriptional upregulation, cleavage,

phosphorylation, and altered localization. For example, in the presence of trophic factor, Bad is phosphorylated, bound to the protein 14-3-3, and safely held in the cytoplasm. Upon trophic factor deprivation, however, Bad becomes dephosphorylated and re-localizes to the mitochondria to inhibit the anti-apoptotic protein Bcl-X_L (Zha, Harada et al. 1996; Puthalakath and Strasser 2002). Inactive Bim, in turn, is normally secured to the microtubule-associated dynein motor complex but can be freed and translocate to the mitochondria to block anti-apoptotic Bcl-2 (Puthalakath, Huang et al. 1999). Additionally, full length Bid is cleaved into truncated Bid (tBid) in response to stimulation of the extrinsic apoptotic pathway, resulting in Bax activation and cytochrome *c* release from the mitochondria (Esposti 2002). Transcriptional upregulation of Bim, DP5/HRK, Puma, and Noxa, respectively, has also been observed in various apoptotic situations (Dijkers, Medema et al. 2000; Harris and Johnson 2001; Nakano and Vousden 2001; Puthalakath and Strasser 2002; Freeman, Burch et al. 2004). Activated members trigger apoptosis either indirectly by blocking anti-apoptotic Bcl-2 protein activity or by directly binding Bax and Bak. Only Bim, Bid, and Puma are classified as direct activators of apoptosis due to their ability to directly bind Bax and Bak (Ren, Tu et al. 2010). The rest of the BH3-only proteins are classified as sensitizers because of their ability to indirectly inhibit apoptosis by blocking the anti-apoptotic Bcl-2 proteins (Letai, Bassik et al. 2002). With the activation of BH3-only proteins ultimately resulting in MOMP and cytochrome *c* release, these Bcl-2 family members are accepted as some of the most significant cell death initiators.

The third group within the Bcl-2 family consists of anti-apoptotic proteins that contain four BH domains (BH1-BH4). Members include Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1, all of which are usually localized to the MOM and associate with Bax and Bak to prevent their

oligomerization and activation (Cory and Adams 2002). These anti-apoptotic Bcl-2 proteins must be relieved by BH3-only sensitizers or bypassed in order for apoptosis to proceed.

The apoptosome: Apaf-1, caspase-9, dATP and cytochrome c:

The effort to identify proteins capable of cleaving and activating caspase-3 resulted in the discovery of the mammalian homolog of *ced-4*, called apoptotic protease-activating factor (Apaf-1)(Liu, Kim et al. 1996; Zou, Henzel et al. 1997). Apaf-1 contains an N-terminal CARD domain, a nucleotide binding and oligomerization domain (NOD, or NB-ARC), and several repeats of the regulatory WD40 domain at its C-terminus (Riedl and Salvesen 2007). More complex than its nematode precursor, mammalian Apaf-1 contains a WD40 repeat motif that keeps Apaf-1 in an auto-inhibited conformation and prevents caspase-3 activation in the absence of the cytosolic Apaf-1 binding partner, cytochrome *c* (Hu, Ding et al. 1998); identified concomitantly with Apaf-1, cytochrome *c* is a well-studied, critical component of the mitochondrial respiratory chain (Liu, Kim et al. 1996). Securely sequestered away from Apaf-1 and destructive caspases in the mitochondrial intermembrane of healthy cells, cytochrome *c* must translocate from the intermembrane space to the cytoplasm in order to induce Apaf-1 activation and downstream caspase activation during apoptosis (Wang, Zhou et al. 2001).

Apaf-1 normally exists in a monomeric, inactive state in healthy cells because the WD40 repeats mask the NB-ARC and CARD domains to form an auto-inhibited conformation (Hu, Ding et al. 1998). Apaf-1 becomes active when cytochrome *c* binds to the WD40 domain, which alters Apaf-1's conformation to expose its CARD and NB-ARC domains (Figure 1.5). Apaf-1 is then able to bind dATP (at the NB-ARC) and caspase-9 (via

CARD-CARD interactions) to form the caspase-activating scaffolding complex called the apoptosome (Liu, Kim et al. 1996; Hu, Benedict et al. 1998; Acehan, Jiang et al. 2002; Yu, Acehan et al. 2005). The main function of the apoptosome is to initiate enzymatic activity of caspase-9. With functional Apaf-1 in place at the central 'hub of the wheel', caspase-9 is recruited around Apaf-1 via its CARD domains and becomes active after dimerization on the apoptosome (Boatright, Renatus et al. 2003; Boatright and Salvesen 2003); active initiator caspase-9 then cleaves and activates effector caspase-3 to cause the ultimate destruction of the cell.

The powerful association between Apaf-1 and caspase-9 on the apoptosome enhances processed caspase-9 activity more than 1000-fold (Rodriguez and Lazebnik 1999), yet this model of activation (termed the "induced proximity model") does not entirely explain caspase-9 activation. For example, constitutively dimeric caspase-9 is far less catalytically active than its apoptosome-associated counterparts (Chao, Shiozaki et al. 2005), and non-Apaf-1-mediated mechanisms of caspase-9 activation have been described (explored in more detail in the Discussion).

Unlike other initiator caspases, neither prodomain removal nor cleavage is required to achieve catalytic activation of caspase-9 (Riedl and Salvesen 2007). Whether the apoptosome induces conformational changes in caspase-9 that enhance its activity following dimerization remains an intensive area of investigation (Bratton and Salvesen 2010). Recent studies suggest that the zymogen form of caspase-9 possesses higher affinity for the apoptosome compared to the cleaved form, thereby supporting a continuous cycle of procaspase-9 recruitment, activation, processing and release from the apoptosome. Analogously, others have proposed that the apoptosome functions as a proteolytic-based

'molecular timer' of apoptosis - the overall length of the timer is set by the intracellular concentration of procaspase-9, procaspase-9 autoprocessing starts the timer, and the rate of active caspase-9 dissociation from the complex (resulting in its inability to activate caspase-3) determines the speed at which the timer 'ticks' (Malladi, Challa-Malladi et al. 2009). Overall, this line of thinking maintains that, rather than merely activating caspase-9, the chief purpose of procaspase-9 autoprocessing is to initiate a molecular timer that regulates the duration of apoptosome activity (Bratton and Salvesen 2010). Although the precise events mediating caspase-9 activation continue to be explored, the requirement for the apoptosome in activating caspase-9 during apoptosis remains undeniably clear as the phenotypes of caspase-9-/- and Apaf-1-/- animals are very similar - the deletion of either apoptosome-associated protein potently inhibits neuron death during development (Kuida, Zheng et al. 1996; Cecconi, Alvarez et al. 1998; Hakem, Hakem et al. 1998).

Regulators of apoptosome function:

Our lab and others have shown that fully differentiated postmitotic cells lose their ability to undergo cytochrome *c*-mediated apoptosis, and this heightened resistance to apoptosis enables these cells to survive for the lifetime of the organism. For postmitotic cells such as neurons, myotubes, and cardiomyocytes, the decreased sensitivity to cytochrome *c* is partly due to the substantial transcriptional downregulation of Apaf-1 (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005). Such low levels of Apaf-1 render the apoptosome incapable of activating caspases at adequate levels required to overcome the cell's endogenous levels of caspases inhibitors, such as XIAP (X-linked inhibitor of apoptosis; discussed in greater detail below). Alternative splicing of Apaf-1 can also

regulate apoptosome function as splice variants encoding fewer WD40 domains have reduced activity (Benedict, Hu et al. 2000).

Activity of cytochrome *c* can also be regulated at transcriptional, translational and redox levels. For example, cytochrome *c* mRNA and protein levels are induced in response to an apoptotic stimulus (Sanchez-Alcazar, Ault et al. 2000; Chandra, Liu et al. 2002). Post-translationally, addition or removal of a heme group can confer or remove cytochrome *c*'s pro-apoptotic facilities, respectively (Yang, Liu et al. 1997; Martin and Fearnhead 2002). Tri-methylation (at lysine 72) and the redox state of cytochrome *c* have also been shown to regulate this molecule's ability to promote apoptosis (Pan, Voehringer et al. 1999; Hancock, Desikan et al. 2001; Suto, Sato et al. 2005; Vaughn and Deshmukh 2008). More specifically, oxidized cytochrome *c* is a more potent caspase activator than the reduced form. While healthy neurons maintain a highly reducing environment that restricts cytochrome *c*-mediated caspase activation (even after cytochrome *c* is released into the cytosol), cytochrome *c* potently activates caspases when the environment becomes more oxidizing following insults such as NGF withdrawal (Vaughn and Deshmukh 2008). It is interesting to note that, unlike most cells, neurons have the capability to survive and even recover after the point of cytochrome *c* release. If caspase activation is prevented during NGF withdrawal, sympathetic neurons can maintain their mitochondrial membrane potential for a short period after cytochrome *c* is released into the cytosol; this 'safety window' provides neurons with an opportunity to reverse apoptotic processes before mitochondrial membrane potential is lost (Martinou, Desagher et al. 1999; Deshmukh, Kuida et al. 2000).

Given the crucial role of the apoptosome in cell death, it is unsurprising that numerous binding partners have been reported to regulate its function (Schafer and

Kornbluth 2006). First, cytochrome *c* can be negatively modulated by Heat Shock Protein 27 (HSP27), which binds and sequesters cytochrome *c* away from Apaf-1 (Bruey, Ducasse et al. 2000). In turn, Apaf-1 binding by Heat Shock Protein 90 (HSP90) inhibits apoptosome assembly (Pandey, Saleh et al. 2000), and Heat Shock Protein 70 (HSP70) has also been shown to bind Apaf-1 and block caspase-9 processing (Beere, Wolf et al. 2000; Saleh, Srinivasula et al. 2000; Beere 2004). Apaf-1 can be inhibited by Rsk-mediated phosphorylation, which promotes the recruitment of 14-3-3 to effectively block cytochrome *c* from binding to Apaf-1 (Kim, Parrish et al. 2012). Two proteins, Aven and ProT, can effectively block apoptosome assembly (Chau, Cheng et al. 2000; Jiang, Kim et al. 2003), and TUCAN, APIP, JNK, and various truncated forms of caspase-9 are all able to inhibit caspase-9 recruitment to and activation on the apoptosome (Srinivasula, Ahmad et al. 1999; Angelastro, Moon et al. 2001; Pathan, Marusawa et al. 2001; Cao, Xiao et al. 2004; Tran, Andreka et al. 2007). Even non-protein interactors (including nitric oxide donors, high potassium and calcium levels, and intracellular nucleotides) have been shown to negatively regulate Apaf-1 oligomerization by binding to cytochrome *c* or inhibiting nucleotide exchange (Purring-Koch and McLendon 2000; Cain, Langlais et al. 2001; Bao and Shi 2006; Chandra, Bratton et al. 2006; Mei, Yong et al. 2010). Lastly, several kinases have been shown to modulate caspase-9 activity. Specifically, ERK2, DYRK1A, CDK1-cyclin B1 and p38α phosphorylate caspase-9 at Thr125 to inhibit cleavage and, conversely, c-Abl inactivates caspase-9 via phosphorylation at Tyr153 (Cardone, Roy et al. 1998; Allan, Morrice et al. 2003; Brady, Allan et al. 2005; Martin, Allan et al. 2005; Raina, Pandey et al. 2005; Allan and Clarke 2007; Martin, Allan et al. 2008; Seifert, Allan et al. 2008; Seifert and

Clarke 2009). In all of these cases, however, the physiological importance of caspase-9 modification remains unclear.

When viewed in the context of the growing evidence for non-apoptotic caspase activation, the potential role of the apoptosome (as well as its many modulators) in this process is particularly exciting and puzzling. If the apoptosome is involved in non-apoptotic caspase activation, how does it function without killing the cell? Chapter Three of this dissertation examines the role of the apoptosome in one situation – developmental axon pruning – that involves caspase activation without death.

Caspase proteases execute cell death:

One of the principal families of apoptotic proteins was discovered during the search for the mammalian homolog to the *ced-3* gene: the cysteine-dependent aspartate-directed proteases called caspases (Yuan, Shaham et al. 1993; Hengartner and Horvitz 1994). Over the past twenty years, our knowledge about mammalian caspases has been steadily growing with regard to their physiological functions, distinct mechanisms of activation and regulation, and the diverse signal transduction pathways that trigger their activation and consequential involvement in disease pathogenesis.

Caspases are synthesized as relatively inactive zymogens containing a prodomain, a p20 large subunit and a p10 small subunit (Figure 1.6). Proteolytic cleavages that separate the large and small subunits and remove the prodomain lead to caspase activation, as can scaffold-mediated transactivation. Each active caspase originates from the processing and self-association of two procaspase (inactive) zymogens. The tetrameric enzymes, described as homodimers of heterodimers, contain two active sites at either end of the molecule.

Within each heterodimer the large and small subunits interdigitate to form a core that is unique to proteases. For some caspases (such as caspase-1 and -3), the maturation of caspase precursors depends on the p10 subunit from one zymogen forming a complex with the p20 subunit of a second zymogen. For others, however, maturation can occur via self-activation (caspase-6) or transactivation with the aid of scaffolding proteins (caspase-9). Notably, caspase-9 is the only caspase reported to possess catalytic properties in the absence of any cleavage; mutating all active sites within caspase-9 does not block its downstream apoptotic effects, suggesting that caspases can mature in different fashions (Stennicke, Deveraux et al. 1999).

Active caspases cleave diverse intracellular polypeptides, such as major nuclear and cytoskeletal structures, to cause the characteristically deliberate and neat disassembly of the cell during apoptosis. Caspases recognize at least four contiguous amino acids in their designated substrates, P4-P3-P2-P1, and cleave after P1, which must always be an aspartate residue. Of the thirteen identified mammalian caspases (eleven are in humans and ten are in mice), caspase-3 and caspase-9 are essential for developmental apoptosis; deletion of either caspase in C57/Black6 mouse background prevents the substantial apoptosis that occurs during nervous system development, resulting in hydrocephaly (enlarged brain) and embryonic lethality (Kuida, Haydar et al. 1998).

Caspases are classified into two groups based on the length of their prodomains, which corresponds to their positions in the apoptotic cascade: the initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) and the effector caspases (caspase-3, -6, -7, -14). Initiator caspases possess a long prodomain that exists in two forms:

a. the death effector domain (DED), which is present in caspase-8 and -10 and mediates interactions with signaling adapter proteins such as FADD;
b. the caspase recruitment domain (CARD), found in caspase-1, -2, -4, and -9, promotes the ability of caspases to interact with each other as well as a wide range of other regulatory and adapter proteins.

Adapter proteins recruit specific caspases via interactions with the long prodomain. The following four specific adapter protein complexes that support caspase activation have been identified in mammalian cells:

a. the apoptosome, which mediates caspase-9 activation by the adaptor Apaf-1 bound to cytosolic cytochrome *c* in the intrinsic pathway;
b. the death-inducing signaling complex (DISC), which activates caspase-8 and -10 by interactions with the adaptor FADD in the extrinsic pathway;
c. the inflammasome, which activates caspase-1 and -5 by interactions with the adaptors ASC (apoptosis-associated speck-like protein containing a CARD) or the NLR family during inflammatory responses;
d. the PIDDosome, which mediates caspase-2 activation during the DNA damage-induced apoptosis pathway by interacting with the adaptors RAIDD (receptor-interacting protein (RIP)-associated ICH-1 CED-3 homologous protein with a death domain) and PIDD (p53-induced protein with a death domain).

Allocating separate caspase activation complexes to distinct insults allows cells to fine-tune and carefully control apoptosis, ensuring that specific caspases are activated appropriately (Hyman and Yuan 2012).

The effector caspases with short prodomains cleave various cellular substrates to perform the downstream execution steps of apoptosis, and these proteases are typically processed and activated by the upstream initiator caspases. To date, almost 400 distinct substrates have been reported for mammalian caspases (Luthi and Martin 2007).

It is important to note that not all caspases are created equal. Initiator caspases are much more specific and cleave only their own precursors and downstream caspases, whereas effector caspases are far more promiscuous during the demolition phases of apoptosis. Additionally, the upstream-downstream relationship between caspases is likely a transient and death-specific one; increasing evidence suggests important roles of caspases in non-apoptotic processes (detailed in the Discussion), and understanding the non-apoptotic substrates of caspases continues to be an important ongoing area of study.

XIAP and the regulation of apoptosis:

The ability to carefully regulate caspase activation to prevent unwanted death and destruction is critical for a cell, particularly for postmitotic cells such as neurons that must last a lifetime. While caspase activity can be regulated by direct caspase modifications such as phosphorylation or nitrosylation (Kim, Ju et al. 2002; Allan, Morrice et al. 2003; Brady, Allan et al. 2005), the most potent and well understood endogenous caspase regulators are members of the Inhibitor of Apoptosis Protein (IAP) family.

Mammalian IAPs include the following: XIAP, cIAP1, cIAP2, ML-IAP, NAIP, Survivin, and Apollon/Bruce (Salvesen and Duckett 2002). All IAPs contain a specific motif called the baculovirus IAP repeat (BIR) domain, so called because this motif was originally isolated from baculovirus during a screen to identify regulators of host-cell viability during viral infection. Overexpression of most IAP family members can suppress apoptosis (Duckett, Nava et al. 1996; Liston, Fong et al. 2003), yet XIAP reigns as the most potent inhibitor of caspases (Salvesen and Duckett 2002; Scott, Denault et al. 2005; Eckelman and Salvesen 2006; Eckelman, Salvesen et al. 2006).

XIAP binds directly to cleaved caspases to block their proteolytic activity. XIAP contains three BIR domains, the third of which binds to cleaved and activated caspase-9 to prevent its interaction with other substrates (Figure 1.7)(Sun, Cai et al. 2000; Srinivasula, Hegde et al. 2001). The small linker region on the N-terminal side of the BIR2 domain forms a reversible, steric occlusion of substrates from caspase-3 and caspase-7 (Figure 1.7)(Chai, Shiozaki et al. 2001; Huang, Park et al. 2001; Riedl, Renatus et al. 2001). XIAP also contains a C-terminal RING finger domain that possesses E3 ubiquitin ligase activity (Yang, Fang et al. 2000). Despite evidence demonstrating XIAP's ability to ubiquitinate several proteins in the apoptotic pathway (Silke, Ekert et al. 2001; Suzuki, Nakabayashi et al. 2001; MacFarlane, Merrison et al. 2002; Sun 2003; Morizane, Honda et al. 2005), how the RING finger regulates apoptosis in intact cells remains unclear.

Our lab has shown that XIAP is a primary safety brake on caspase activation induced by the release of mitochondrial cytochrome *c*. Even though XIAP-/- mice show no overt phenotype, XIAP-deficient neurons and cardiomyocytes are more susceptible to cytochrome *c*-mediated apoptosis (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005).

Similarly, neurons in XIAP-deficient mice are more vulnerable to stroke injury *in vivo* (West, Stump et al. 2009). In contrast to mice, the deletion of the XIAP homolog DIAP1 in Drosophila causes massive apoptosis and fly lethality, and it was discovered that four proteins (Reaper, Grim, HID, and Sickle) bind and inhibit DIAP1 in the cytosol (Martin 2002). The search for mammalian XIAP binding partners uncovered two proteins, SMAC/DIABLO and Omi/HtrA2, that localize to the mitochondria in healthy cells (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000; Suzuki, Imai et al. 2001; Hegde, Srinivasula et al. 2002; Martin 2002; van Loo, van Gurp et al. 2002; Verhagen and Vaux 2002). Upon apoptotic stimulation, SMAC and HtrA2 are released into the cytosol and bind to XIAP at the same regions as caspase-9, -3, and -7 (Sun, Cai et al. 2000; Srinivasula, Hegde et al. 2001; Salvesen and Duckett 2002; Shi 2002; Verhagen, Silke et al. 2002). At sufficiently elevated ratios in the cytoplasm, these pro-apoptotic mitochondria-released proteins can exclude caspase-9 and disrupt caspase-3/7 binding with XIAP, resulting in freed active caspases and cell destruction. Curiously, endogenous SMAC does not inhibit XIAP in neurons undergoing cytochrome *c*-mediated apoptosis (Vaughn and Deshmukh 2007); only ectopic expression of SMAC by microinjection allows cytochrome *c* to induce death in sympathetic neurons (Potts, Singh et al. 2003; Potts, Vaughn et al. 2005).

Importantly, XIAP activity in neurons can be regulated by SMAC-independent mechanisms. XIAP is degraded in neurons undergoing apoptosis in response to NGF-deprivation (Potts, Singh et al. 2003), although the mechanism that mediates this degradation remains elusive; it is possible that the RING finger domain of XIAP functions as an E3 ligase to target itself for ubiquitylation (Yang, Fang et al. 2000). Apaf-1 levels and the apoptosome also indirectly regulate XIAP activity. In healthy cells, minimal caspase

activation maintains XIAP as an extremely effective apoptosis inhibitor. However, a high level of Apaf-1 (and hence apoptosome activity) causes maximal levels of caspase activation that overpower XIAP's ability to prevent apoptosis (Wright, Linhoff et al. 2004; Vaughn and Deshmukh 2007). Our lab has shown that differentiating neurons and cardiomyocytes, as well as neurons undergoing DNA damage, regulate XIAP activity via Apaf-1 levels (Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005; Vaughn and Deshmukh 2007).

1.4 Extrinsic Pathway

The extrinsic cell death pathway is induced by ligand binding to death receptors (DRs) of the tumor necrosis factor receptor (TNFR) family that contain the death domain (DD). This receptor family includes the following members: CD95, TNF receptor (TNF-R1), DR3/APO-3/TRAMP, TRAIL receptor 1/DR4, TRAIL-2/DR5, and DR6 (Peter, Scaffidi et al. 1999). Following ligand binding (by ligands such as Fas and TRAIL), the death receptors undergo conformational changes that permit the recruitment of multiple proteins that form the death-inducing signaling complex (DISC). The ligand-bound death receptors then recruit a DD-containing adapter protein called FADD (Fas-associated DD). FADD contains the critical death-effector domain (DED) that allows FADD to recruit initiator caspases-8 and -10. FADD-bound caspase-8/10 then undergoes autoproteolytic cleavage on the DISC and is released into the cytoplasm where it can cleave and activate effector caspase-3 to fully engage the apoptotic caspase cascade (Figure 1.8). In neurons, caspase-8 connects the intrinsic and extrinsic pathways by cleaving the BH3-only protein Bid into its Bax-activating form, tBid (Ricci, Kim et al. 2007; Dekkers and Barde 2013). The extrinsic pathway can be regulated by IAPs (Vaux and Silke 2005) and cFLIP, which inhibits caspase-8 and blocks formation of the DISC at the plasma membrane (Wang, Wang et al. 2005).

Dependence receptors:

Classically, transmembrane receptors are activated when bound by their respective ligands and remain relatively inactive in their unbound state. Consequently, for many years the conventional model of neuronal apoptosis assumed that any cell death resulting from the lack of receptor activation (e.g. the absence of trophic factor from Trk receptors) was

simply due to the loss of positive survival signals. Extensive work over the past two decades, however, supports a novel form of signal transduction that is mediated by unique receptors called dependence receptors. Dependence receptors support cell survival signaling when bound to their respective ligands (just as seen with other transmembrane receptors), yet they can fully switch to activating cell death in the absence of ligand (Bredesen, Mehlen et al. 2005; Tauszig-Delamasure, Yu et al. 2007). Dependence receptors create a highly tuned state of cellular dependence on their ligands. Fifteen dependence receptors had been identified as of 2010, although more likely exist that have yet to be discovered (Eilers, Whitfield et al. 1998).

A. DCC

Among the first dependence receptors to be characterized is the protein Deleted in Colorectal Carcinoma (DCC). DCC was discovered to promote apoptosis in the absence of its ligand, netrin, while inhibiting apoptosis when bound to netrin (Llambi, Causeret et al. 2001; Bredesen, Mehlen et al. 2005; Vanderhaeghen and Cheng 2010). Curiously, the death induced by netrin is believed to be dependent on caspase-3 and caspase-9 but independent of Apaf-1 or cytochrome *c* (Forcet, Ye et al. 2001). Although, as its name implies, DCC was first discovered due to its association with cancer, it also plays an important role in neurons. Netrin-DCC signaling is crucial for axon guidance during nervous system development, and subsequent studies have found that it also plays a role in promoting the survival of some populations of neurons (Mehlen and Mazelin 2003; Mehlen and Bredesen 2011).

B. Trks and p75NTR:

It was only recently shown that TrkA, the receptor for NGF in sympathetic neurons, is a dependence receptor: TrkA causes neuronal cell death by merely being expressed, and its death-inducing activity is blocked by the presence of NGF. Correspondingly, sympathetic neurons deficient for TrkA survive following NGF deprivation (Tauszig-Delamasure, Yu et al. 2007; Nikoletopoulou, Lickert et al. 2010). Closely related to TrkA, TrkC also acts as a dependence receptor. Exactly how TrkA and TrkC trigger cell death remains unclear. Proteolysis of the receptors themselves may be involved (Tauszig-Delamasure, Yu et al. 2007) or the proteolysis of the TrkA/C-associated receptor p75NTR could be responsible (Nikoletopoulou, Lickert et al. 2010). p75NTR colocalizes with TrkA and TrkC in lipid rafts in the plasma membrane, which is significant because the localization of certain pro-apoptotic proteins is known to be important for their death-inducing function. For example, during the extrinsic apoptotic pathway, activated death receptors must translocate to lipid rafts in the membrane in order to assemble the DISC, and cFLIP prevents this translocation to mitigate cell death (Davis, Lotocki et al. 2007; Song, Tse et al. 2007). Likewise, DCC's localization in lipid rafts is required for its death-inducing function in the absence of netrin (Furne, Corset et al. 2006). Notably, despite its overall structural similarity to TrkA and TrkC, TrkB does not function as a dependence receptor, most likely due to differences in both its membrane localization (TrkB does not colocalize with p75NTR in lipid rafts) and transmembrane domain (Bibel, Hoppe et al. 1999; Dekkers and Barde 2013).

1.5 Resistance of mature neurons to apoptosis

With the exception of neurons located in the olfactory bulb and dentate gyrus, neurons that survive the massive cull during nervous system development must subsequently last the lifetime of the organism (which, for the average human, means over seven decades). As neurons are post-mitotic and have extremely limited potential for regeneration, these cells must strictly control the apoptotic pathway as they mature to prevent unwanted neuron loss (and risk neurodegeneration). Growing evidence supports the idea that mature neurons are far more resistant to apoptosis than young neurons (Figure 1.9). Although the exact changes that occur during neuronal maturation continue to be identified, several distinct mechanisms have been discovered that allow neurons to switch from a state of apoptotic susceptibility to one in which apoptosis is nearly completely shut off.

Mechanisms that restrict apoptosis in mature neurons:

Mature neurons engage several mechanisms that act at multiple points to restrict the apoptotic pathway, and this redundancy is necessary to safeguard neuronal survival in the event that one safety mechanism fails. The diverse ways in which a neuron becomes resistant to NGF deprivation-induced apoptosis are detailed below.

1.) *Extended TrkA phosphorylation*: Maturing neurons alter the timeline of events following NGF binding to its receptor, the tyrosine kinase receptor (TrkA). In developing neurons exposed to ample NGF, NGF binding to TrkA induces TrkA phosphorylation and the retrograde transport of both the bound receptor and

its ligand to the cell body where it promotes neuronal survival. When NGF is withdrawn from young NGF-dependent neurons, TrkA is dephosphorylated within one hour. However, in mature neurons deprived of NGF, TrkA remains phosphorylated for many hours and likely promotes the robust resistance of these cells against the loss of trophic support (Tsui-Pierchala and Ginty 1999).

2.) *Failed translocation of Bax and cytochrome c release*: Instead of translocating from the cytoplasm to mitochondria to induce cytochrome *c* release following NGF deprivation (as seen in young neurons), Bax remains cytoplasmic in mature neurons (Putcha, Deshmukh et al. 2000) and cytochrome *c* maintains its mitochondrial localization (Easton, Deckwerth et al. 1997).

3.) *Transcriptional restriction of Apaf*-1: Our lab has shown that mature neurons rearrange their chromatin to form a more condensed state at the Apaf-1 locus, resulting in a block on Apaf-1 expression (Wright, Smith et al. 2007). The addition of exogenous cytochrome *c* fails to induce apoptosis in both mature wildtype (essentially Apaf-1-deficient) and XIAP-deficient neurons, indicating that high levels of XIAP expression do not underlie mature neuron survival after this post-mitochondrial insult.

4.) *MicroRNA 29b (miR-29b) inhibits multiple BH3-only mRNAs:* Recent work from our lab shows that while the vast majority of miRNAs are downregulated during neuronal maturation, the expression of miR-29 is markedly induced. Remarkably, miR-29b targets the 3'UTR of multiple BH3-only mRNAs to repress BH3-only protein induction and block apoptosis (Kole, Swahari et al. 2011).

While the mechanisms utilized by maturing neurons to restrict apoptosis have been best characterized in sympathetic neurons, studies performed on other types of neurons indicate that they, too, restrict apoptosis with maturation. Cerebellar, cortical, and photoreceptor neurons have been found to greatly downregulate Apaf-1 expression as they mature (Northcutt 1989; Kuan, Roth et al. 2000; Yuan and Yankner 2000; Aloe 2004). In contrast to sympathetic neurons, however, many neurons also downregulate caspase-3 expression (Northcutt 1989; Kuan, Roth et al. 2000; Yuan and Yankner 2000; Aloe 2004; Liu, Siesjo et al. 2004). Even the IAPs have been shown to increase their reach in maturing neurons - motor neurons downregulate the endogenous XIAP inhibitor, XAF, to allow XIAP to more potently inhibit caspases (Kim and Sun 2011).

Neuronal cell death in the mature nervous system:

Despite the protective mechanisms described above, neurons in the adult nervous system are still vulnerable to death, particularly in neurodegenerative situations (Orike, Middleton et al. 2001; Walsh, Orike et al. 2004). Why do the multiple brakes developed over the course of neuronal maturation become ineffective in neurodegenerative states? Are these mechanisms somehow reversed or disengaged during processes of degeneration and injury? As my research career was born from a deep personal interest in neurodegeneration and a hope to positively impact those suffering from neurodegenerative disease, I have dedicated the following sections to providing a brief overview of apoptosis in several neurodegenerative situations.

Neuronal death is a fundamental characteristic of multiple neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease,

amyotrophic lateral sclerosis, and stroke, among others. The extremely limited regenerative capacity of the central nervous system makes neurodegenerative diseases particularly devastating – dead neurons cannot be replaced, resulting in worsening symptoms and a declining quality of life as neurodegeneration progresses over time. Thus, understanding how to limit or prevent the damage caused by various destructive mechanisms will enable the development of more effective and preventative treatments across the spectrum of neurodegeneration.

Neurodegenerative disorders are caused by multi-factorial stimuli that may include genetic and environmental factors, metabolic dysregulation, excitotoxicity, neuroinflammation, cellular stressors such as oxidative stress and the overproduction of free radicals, disrupted calcium regulation, mitochondrial dysfunction, interrupted cell transport, sustained activation of microglia (the brain's immune cells), and the accumulation of misfolded or toxic proteins (Figure 1.2). Delineating the causes of neuronal cell death in degeneration and injury is complicated by the fact that one stimulus can trigger multiple death pathways depending on its degree, duration, cell type, brain region, and the affected neuron's bioenergetic state.

Several different pathways of neuronal cell death are implicated in neurodegeneration, including apoptosis, necrosis, glial injury, excitotoxicity, and toxicity resulting from damaging reactive oxygen species. Indeed, it is not unusual to observe more than one type of cell death pathway occurring in neurodegenerative states; necrotic and apoptotic markers have even been observed in the same neuron following ischemia, indicating that more than one cell death mechanism can be concomitantly active in a cell

(Unal-Cevik, Kilinc et al. 2004). A brief overview of neuronal cell death across several of the most prevalent neurodegenerative diseases and stroke is described here.

The following sections on neurodegeneration and stroke were previously published in Cusack CL, Annis RP, Kole AJ, and Deshmukh M. Neuronal Death Mechanisms in Development and Disease. In: Wu H, ed. *Cell Death: Mechanism and Disease*. New York, NY: Springer Science and Business Media; 2014: 167-188.

A. Alzheimer's disease:

Alzheimer's disease (AD) is the most common form of neurodegeneration worldwide, and the vast majority of AD cases are sporadic. This disease is characterized by the loss of neurons and synapses in the cerebral cortex and several subcortical regions, resulting in massive brain atrophy of these areas (Figure 1.10). AD is most often diagnosed in people over 65 years of age, although the less prevalent early-onset form occurs much earlier. AD is progressive, meaning that the disease pathologies and symptoms become more severe over time. As neurodegeneration spreads throughout the brain, bodily functions are eventually lost, ultimately leading to death.

There is currently no known cure for AD, and understanding the causes and progression of AD is an area of intense investigation. The clinical hallmarks of AD include extracellular senile plaques, which are mostly composed of amyloid-β (Aβ) peptides, and neurofibrillary tangles composed of intracellular, hyperphosphorylated microtubule-associated tau protein. Aberrant build-up of these proteins interferes with proper neuronal processes, such as intracellular transport, calcium ion homeostasis, and mitochondria

dynamics, and these aggregates also induce inflammation; all of these processes can trigger death cascades.

The direct relationship between aggregate lesions, their toxic soluble monomers, and neuronal loss in AD is still unclear, but evidence of caspase activation in AD may provide a link. Initiating and executioner caspases are both upregulated in the brains of patients with AD (Matsui, Ramasamy et al. 2006), and caspases mediate the cleavage of multiple targets associated with AD pathology (LeBlanc, Liu et al. 1999; Guo, Albrecht et al. 2004; Albrecht, Bogdanovic et al. 2009; Halawani, Tessier et al. 2010; D'Amelio, Cavallucci et al. 2011; Hyman 2011). In particular, Caspase-6 has been shown to cleave both Aβ and tau, and caspase activation plays a role in the synaptic loss associated with Aβ toxicity (LeBlanc, Liu et al. 1999; Guo, Albrecht et al. 2004; Albrecht, Bogdanovic et al. 2009; D'Amelio, Cavallucci et al. 2011). There is now evidence that neurons can tolerate chronic low levels of caspase activation without dying in a mouse model of AD(de Calignon, Spires-Jones et al. 2010), and caspase-mediated cleavage of Aβ and tau produces toxic protein aggregates (Graham, Ehrnhoefer et al. 2011; Reddy 2011; Zhang, Thompson et al. 2011). These observations may help to explain how caspase activation – a normally acute, rapid event during apoptosis – can occur at sub-apoptotic levels for long periods of time, thus long-preceding but culminating in the progressive neurodegeneration of AD.

B. Parkinson's disease:

Parkinson's disease (PD) is a degenerative movement disorder caused by the death of dopaminergic neurons in the substantia nigra, a region of the midbrain that plays an important role in motor planning. Early symptoms of PD are movement-related and include

shaking, rigidity, slowness, and unstable posture. More advanced stages of the disease involve dementia as well as cognitive, sleep-related, and behavioral issues. PD symptoms are considered to be the direct result of the progressive dysfunction and loss of dopaminergic neurons, yet how and why these particular neurons are targeted for neurodegeneration remains unclear.

The pathology of PD is strongly linked to the progressive accumulation of alpha-synuclein into inclusions called Lewy bodies in neurons. The formation of Lewy bodies is a leading theory as the development and spread of these inclusions appears to correspond with disease progression and region-specific degeneration (Schulz-Schaeffer 2010); however, in some cases alpha-synuclein has been shown to be protective (Alves da Costa, Paitel et al. 2002; Li and Lee 2005). Alpha-synuclein can be secreted by neurons, partake in cell-to-cell transmission, induce neurotoxicity, make neurons more sensitive to apoptosis, and trigger inflammatory glial responses (Tanaka, Engelender et al. 2001; Schulz-Schaeffer 2010; Arduino, Esteves et al. 2011). Inflammation and dysfunction of the proteasomal and lysosomal systems also contribute to PD pathologies, and defects in mitochondrial quality control processes such as mitochondrial fission, fusion, and autophagy have also been linked to substantia nigral neuron degeneration (Obeso, Rodriguez-Oroz et al. 2010; Youle and van der Bliek 2012).

Caspase-dependent and independent death pathways contribute to neurodegeneration in PD (Tanaka, Engelender et al. 2001; Venderova and Park 2012). Positive labeling for numerous apoptotic markers in postmortem human tissue samples confirm the presence of apoptotic nigrostriatal dopaminergic neurons, and similar observations have been replicated in multiple mouse models of PD. Bax suppression,

overexpression of Bcl-2 family members, and overexpression of XIAP, respectively, protect nigrostriatal neurons in certain PD mouse models. Additionally, inhibiting the opening of the mitochondrial permeability transition pore or overexpressing the E3 ubiquitin ligase Parkin to block cytochrome *c* release prevents apoptosis in dopaminergic neurons.

There is also evidence of paraptosis, a caspase-independent death pathway, in PD. Paraptosis depends on the activation of poly(ADP-ribose)polymerase (PARP-1), a DNA repair enzyme that, along with p53, is induced by genotoxic stress and DNA damage. PARP-1 activation leads to the translocation of apoptosis inducing factor (AIF) from the inner mitochondrial membrane to the nucleus where it participates in chromatin condensation and large-scale DNA fragmentation (Yu, Wang et al. 2002). Indeed, nuclear AIF is observed in postmortem PD patient brain tissue (Burguillos, Hajji et al. 2011). Additionally, PARP-1 inhibition blocks alpha-synuclein cytotoxicity as well as cell death in toxic (MPTP/MPP+) models of PD (Venderova and Park 2012). With multiple signaling pathways regulating several types of neuronal cell death in PD (and in other neurodegenerative diseases), basic research on neurodegeneration will provide much needed insight into the pathogenesis and regulation of cell death mechanisms in neurons.

C. Amyotrophic lateral sclerosis:

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive loss of motor neurons throughout the brain, brainstem, and spinal cord. Onset of this disease normally occurs during one's fifties, and the average life expectancy after clinical onset is 4 years. Unfortunately, the causes of almost all ALS cases remain unknown. Several of the mutant genes found in familial forms of ALS are involved in mitochondrial maintenance,

and mitochondrial damage and dysfunction are widely observed in patients with ALS and in mouse models of the disease (i.e. transgenic animals that overexpress the gene for human mutant superoxide dismutase (SOD1), mutant TAR DNA-binding protein 43 (TDP-43), or mutant fused in liposarcoma (FUS)). Disrupted axonal transport, impaired mitochondrial fusion, decreased mitochondrial size and density, and defective mitochondrial membrane potential are also present in degenerating motor neurons of ALS mouse models (Johri and Beal 2012).

Excitotoxicity also contributes to ALS pathology. Glutamate-mediated excitotoxicity caused by repetitive neuronal firing and/or elevation of intracellular calcium levels by calcium-permeable glutamate receptors is known to cause neuronal death. In normal physiological conditions, glutamate is the primary excitatory neurotransmitter in the nervous system. The release of glutamate into the synaptic cleft activates glutamate receptors, which leads to the influx of calcium and sodium into the post-synaptic neuron to trigger its depolarization. The removal of synaptic glutamate by glutamate transporters is required to prevent repetitive firing, and the glial glutamate transporter EAAT2 is responsible for approximately 90% of glutamate clearance in motor neurons (Rothstein, Dykes-Hoberg et al. 1996; Tanaka, Watase et al. 1997). Elevated levels of glutamate are found in 40% of sporadic ALS patients (Spreux-Varoquaux, Bensimon et al. 2002), and loss of EAAT2 protein causes significantly reduced glutamate transport in brain regions affected in ALS (Rothstein, Van Kammen et al. 1995). The abundance of data indicating dysfunctional astroglia in both sporadic and familial forms of ALS suggests that restoring astrocyte health and glutamate transport capabilities may be a very effective therapy for this neurodegenerative disease (Cleveland and Rothstein 2001).

Despite the combined contributions of oxidative damage, axonal stress, and toxicity to motor neuron dysfunction in ALS, it is well established that Caspase-3 activation is the final event in the death cascade of these neurons (Cleveland and Rothstein 2001). Other evidence for the role of apoptosis in ALS includes the observation that administration of a pan-caspase inhibitor to ALS mice is neuroprotective and significantly extends survival, indicating a role for caspase-1 and caspase-3 in ALS (Li, Ona et al. 2000). As ALS mice age, there is a progressive transcriptional upregulation of Caspase-1 and Caspase-3 messenger RNA (mRNA)(Li, Ona et al. 2000; Vukosavic, Stefanis et al. 2000). These findings support that clinical relevance of mouse models of ALS as both Caspase-1 and Caspase-3 activation have been found in spinal cord samples from human ALS patients. In addition, other central markers of apoptosis – including Caspase-9 activation, the release of cytochrome *c* from mitochondria, and pro-apoptotic changes in the Bcl-2 family - have also been found in the spinal cords of ALS mice, and ALS mice that overexpress the anti-apoptotic Bcl-2 gene survive longer than other ALS mice (Sathasivam and Shaw 2005).

D. Huntington's Disease:

Huntington's Disease (HD) is a universally fatal autosomal dominant neurodegenerative disease in which neostriatal medium spiny neurons and eventually certain regions of the cortex progressively waste away. Onset typically occurs during the mid-thirties and forties, and persons with HD usually die within 15 to 20 years. Symptoms include abnormal involuntary movements, cognitive decline, and psychiatric problems. The disease is caused by a genetic mutation encoding for the aberrant expansion of CAG-encoded polyglutamine repeat regions in a protein called Huntingtin (Htt), resulting in

mutant huntingtin protein (mHtt). Post-translational modifications of mHtt result in cleavage of the protein that leaves behind toxic, shorter fragments that form damaging protein aggregates instead of folding properly into functional proteins. These aggregates, called inclusion bodies, accumulate over time and ultimately interfere with proper neuronal functions. There is also extensive evidence for deficient energy metabolism, mitochondrial dysfunction, and glutamate-mediated excitotoxicity in HD (Estrada Sanchez, Mejia-Toiber et al. 2008; Johri and Beal 2012). With regard to key apoptotic proteins, in a mouse model of HD, cytochrome *c* changes its localization from a mitochondrial to cytosolic pool as the animal ages and the disease progresses (Mangiarini, Sathasivam et al. 1996; Kiechle, Dedeoglu et al. 2002). Caspases, particularly caspase-6, have also been strongly implicated in HD (Graham, Deng et al. 2006; Majumder, Chattopadhyay et al. 2006; Leyva, Degiacomo et al. 2010). Caspase-mediated cleavage of Htt appears to be critical for initiating striatal degeneration, and inhibiting such cleavage blocks Htt-mediated toxicity (Graham, Deng et al. 2006).

E. Stroke:

Stroke is an acute, rapid form of neurodegeneration that is caused by ischemia (lack of blood supply) or hemorrhage. Both the severity and duration of the insult induce complex metabolic changes that then determine the type and extent of cell death in stroke (Sims and Muyderman 2010). Irreversible neuron loss is caused by necrosis in the core tissue of the stroke lesion. The region surrounding the necrotic core, called the penumbra, is damaged by apoptotic processes but retains enough structural integrity to support functional restoration following therapeutic interventions.

Cell death mechanisms induced by ischemic injury occur on a dynamic time-scale. At early time points, neurons in the necrotic core display many features of apoptosis, such as nuclear condensation and evidence of caspase-1 and caspase-8 activation (Benchoua, Guegan et al. 2001). As the penumbral region develops in later time points, the intrinsic apoptotic pathway is activated in the infarct core but appears to be aborted due to severe energy depletions and mitochondrial failure. Reperfusion of blood to the penumbral region permits energy-dependent caspase activation and complete apoptosis (Hyman and Yuan 2012). Bcl-2 protein family members play a significant role in neuronal death following ischemia as the overexpression of anti-apoptotic Bcl-2 protein (Martinou, Dubois-Dauphin et al. 1994) and the loss of Bid (Plesnila, Zinkel et al. 2002), a BH3-only protein, respectively protect against ischemic injury by reducing mitochondrial damage. Caspases are also critical mediators of stroke-induced neuronal death as treatment with caspase inhibitors following stroke improve functional outcomes in ischemic mouse models (Akpan, Serrano-Saiz et al. 2011; Al-Jamal, Gherardini et al. 2011).

Neuronal death following stroke is largely due to the massive disruption of glutamate homeostasis. Ischemia greatly compromises cellular respiration, resulting in a marked drop in ATP levels that impairs ATP-dependent glutamate transporters. The unregulated accumulation of glutamate in the synaptic space overactivates glutamate receptors and causes a large influx of calcium, the production of reactive oxygen and nitrogen species, and mitochondrial damage, all of which lead to neuron death. Secondary neuronal death then occurs due to the inflammation caused by ROS and cytokines released by dying neurons that activate surrounding microglia, which secrete their own ROS and cytokines that only exacerbate the damage (Vila, Castillo et al. 2003; Chapman, Dale et al.

2009; Yenari and Han 2012). Future therapies for stroke may therefore involve an appropriate combination of treatments that prevent apoptosis, antagonize glutamate, and reduce oxidative stress.

In summary, key apoptotic molecules have been implicated across the span of neurodegenerative disease and stroke. While mature neurons maintain several brakes on the apoptotic pathway (likely with others yet to be discovered), the loss of these protective mechanisms contributes to the pathogenesis of neurodegeneration in the adult nervous system.

1.6 Axon Pruning

"The fidelity with which adult neurons are connected to their postsynaptic partners belies a time of far less discretion during their younger days."
-David B. Kantor and Alex L. Kolodkin, 2003

Introduction:

Proper nervous system development involves progressive and regressive events (Low and Cheng 2006). Early progressive events such as neural proliferation, neurite outgrowth and synapse formation establish a rough approximation of the final mature pattern of connectivity. Later in development, precise circuitry is sculpted by regressive events that include cell death, axon pruning and synapse elimination (Figure 1.11). These degenerative incidents are essential for normal development (Figure 1.12); mutations in the apoptosis pathway cause early gross morphological and lethal defects in the brain (Figure 1.13)(Kuida, Zheng et al. 1996; Cecconi, Alvarez et al. 1998; Kuida, Haydar et al. 1998; Yoshida, Kong et al. 1998), and disrupting death and pruning pathways later in development results in a chaotic neuronal network and brain dysfunction (Lewis and Levitt 2002; Johnston 2004; Pardo and Eberhart 2007).

Until very recently, axon pruning and neuronal apoptosis were thought to be controlled by distinct mechanisms (Bredesen, Rao et al. 2006; Buss, Sun et al. 2006; Low and Cheng 2006). Neuron death eliminates the entire cell, including all neurites belonging to the dying parent neuron. In contrast, axon pruning removes exuberant or misprojecting axon branches while the soma and other appropriately connected axons of the same neuron remain functionally intact. As will be discussed at greater length in the Discussion, growing evidence demonstrates that apoptotic machinery acts at different levels within a

single cell, ranging from mediating the sub-lethal degeneration of a specific cellular compartment and tempering synaptic transmission in individual synapses to destroying the entire cell during nervous system development and disease (Dekkers, Nikoletopoulou et al. 2013).

Axon loss in injury and disease:

Axon elimination occurs during nervous system injury and degeneration (Raff, Whitmore et al. 2002; Coleman 2005; Luo and O'Leary 2005; Low and Cheng 2006). When axons are severed (an injury termed Wallerian degeneration), the distal separated portion of the axon quickly degenerates while the parent neuron survives and usually regrows the proximal injured axon (Figure 1.14). In contrast, the pace of axon removal is much slower in neurodegenerative situations. Here the axon slowly degenerates over time in a distal-to-proximal manner (a process called "dying back") and ultimately results in the death of the parent neuron.

The model of axotomy-induced axon degeneration has received more attention than the model of developmental pruning due to the early discovery that degeneration of transected axons is significantly delayed in Wallerian degeneration slow (Wlds) mice (Lunn, Perry et al. 1989). Wlds mice overexpress a fusion protein that contains the enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT) linked to the ubiquitination factor Ube4a (Mack, Reiner et al. 2001; Coleman and Freeman 2010; Yan, Feng et al. 2010). While the exact mechanism of axotomy-induced axon degeneration remains unclear, nicotinamide adenine dinucleotide (NAD+) metabolism (Araki, Sasaki et al. 2004), the ubiquitin-proteasome system (Zhai, Wang et al. 2003), disruption of Ca+ homeostasis

(George, Glass et al. 1995), and the recently identified Sarm1 protein (Osterloh, Yang et al. 2012) have all been implicated in this pathway.

The vast majority of literature refers to nearly every incidence of axon-specific removal as "axon pruning", and this loose use of terminology is far too general and fails to highlight the complex, nuanced nature of axon elimination in the nervous system. In this dissertation, axon pruning strictly refers to the physiological axon elimination that takes place during normal development. Wallerian degeneration occurs when the severed axon degenerates following axotomy, and pathological axon degeneration refers to axon loss that occurs during clinical degenerative states. The axonal destruction that happens secondarily to cell death (e.g. both soma and axon experiences an apoptotic insult such as global NGF deprivation) is also not axon pruning and will henceforth be referred to as apoptosis-associated axon degeneration.

Models of axon pruning:

A. Large-scale axon pruning in the CNS:

Large-scale axon pruning removes entire axon branches and projecting collaterals. One example of this process is the activity-mediated remodeling of cortical callosal axons that project to the contralateral side of the brain (Innocenti and Price 2005). Another well-studied example is the stereotyped pruning of layer V pyramidal neurons in the developing visual and motor cortices, which segregate their initially overlapping projections to the superior colliculus and spinal cord, respectively (O'Leary and Koester 1993); this pruning is believed to be transcriptionally regulated as the loss of the transcription factor *Otx1* results in profound pruning defects of layer 5 projections (Weimann, Zhang et al. 1999).

Large-scale pruning also occurs in infrapyramidal bundle (IPB) of the hippocampus (Bagri, Cheng et al. 2003). During development, granule cells in the dentate gyrus extend two mossy fiber axon bundles to the CA3 region (clearly seen in P5 mice), and the transient long IPB is pruned back in late developmental stages (removed in P40 mice)(Bagri, Cheng et al. 2003). Lastly, large-scale pruning is critical for establishing topographic maps such as those seen in the retinotectal system. Axons of retinal ganglion cells initially overshoot their targeted placements along the anterior-posterior axis of the tectum but extend elaborate interstitial branches at appropriate locations (Nakamura and O'Leary 1989). The correct pattern of connectivity is ultimately attained once the overshooting axon segments are eliminated by caspase-mediated pruning mechanisms (Simon, Weimer et al. 2012) and visual activity further refines the axon arbors (McLaughlin and O'Leary 2005).

While this thesis focuses on the events mediating NGF deprivation-induced pruning of peripheral neurons, the role of various guidance cues in CNS axon pruning deserves mention. Developmental guidance cues such as semaphorins (which mediate axon attraction) and ephrins (which mediate axon repulsion) have been implicated in axon pruning events of the hippocampus (Bagri, Cheng et al. 2003; Kantor and Kolodkin 2003), suggesting that such cues are first utilized to establish axon pathways and then again when those pathways are refined.

B. Small scale pruning events:

Axon pruning events also occur on a much smaller scale, and these tinier incidents of axon removal appear to be mediated by neural activity. For example, in the neuromuscular junction (NMJ), an initially broad pattern of polyinnervation is massively

scaled down to a refined pattern of monoinnervation in which one muscle fiber is innervated by the terminal arbor of a single motor axon (Lichtman and Colman 2000). The removal of exuberant terminal arbors in the NMJ is associated with synapse elimination and axosome shedding (Bishop, Misgeld et al. 2004), which is linked to lysosomal activity (Song, Misgeld et al. 2008). Similarly, in the developing cerebellum, pruning of climbing fiber inputs involves synapse elimination and the formation of axosome-like structures (Eckenhoff and Pysh 1979), indicating that small-scale pruning events in the CNS and PNS may utilize similar mechanisms (Hashimoto, Ichikawa et al. 2009). Although neural activity is recognized as an important factor across small-scale pruning events, the molecular factors that mediate this type of axon arbor elimination still remain largely unknown (Vanderhaeghen and Cheng 2010).

C. Insect metamorphosis is highly selective:

The precision of axon pruning is best illustrated by the extensive modifications that take place in the larval nervous system during insect metamorphosis (Figure 1.15)(Lee, Lee et al. 1999). Fly mushroom bodies (structures important for learning and memory) are populated by two neuron populations, γ neurons and α'/β' neurons. During metamorphosis, γ neurons lose their dendritic arbor as well as their dorsal and lateral axon branches while the neurites of α'/β' neurons remain unchanged. Studies reveal that activation of both ecdysone receptors (which are expressed in γ but not α'/β' neurons) and the ubiquitin-proteasome system are required for proper metamorphic pruning of γ neurons (Lee, Marticke et al. 2000; Watts, Hoopfer et al. 2003).

Axon pruning is achieved not only at the level of individual neurons but also at the level of individual axon branches; γ neurons eliminate their dorsal and medial axon branches while retaining the axon peduncle, and previous work has shown that intrinsic cell factors (such as GTPase activity) contribute to this process by regulating the stability of individual axon branches (Billuart, Winter et al. 2001; Kantor and Kolodkin 2003). How the precise discriminatory event of individual axon branch elimination is initiated and mediated remains unclear. Intrinsic programs may govern selective branch removal or, alternatively, extrinsic cues may trigger strictly localized destructive events.

D. Similarities between Drosophila pruning and Wallerian degeneration:

One commonly highlighted observation in the field of axon pruning is the striking similarity between pruning mechanisms in *Drosophila* and the destructive events underlying Wallerian degeneration in vertebrates. The following similarities between Wallerian degeneration and *Drosophila* pruning have been observed:

a. breaks in the microtubule network precede neurofilament breakdown and complete morphological axon fragmentation (Watts, Hoopfer et al. 2003; Zhai, Wang et al. 2003);
b. involvement of the ubiquitin-proteasome system, as inhibiting this system in either scenario robustly protects axons (Watts, Hoopfer et al. 2003; Zhai, Wang et al. 2003);

c. glial cells are actively recruited to the site of degeneration to clear away axon debris (Awasaki and Ito 2004; Watts, Schuldiner et al. 2004; Awasaki, Tatsumi et al. 2006; Hoopfer, McLaughlin et al. 2006; MacDonald, Beach et al. 2006).

Metamorphosis is a global, simultaneous overhaul of nervous system circuitry (Truman 1990), which highly contrasts with mammalian pruning that occurs locally at various developmental stages. Wallerian degeneration is directly caused by injury, and the fact that *Drosophila* pruning events appear to share similarities with this pathological mechanism of axon destruction suggests that *Drosophila* pruning pathways are likely different from those that mediate stereotyped pruning in vertebrates. Indeed, mice defective for Wallerian degeneration (those overexpressing the Wallerian degeneration slow (Wlds) gene (Lunn, Perry et al. 1989)) still undergo normal large-scale axon pruning during development (Hoopfer, McLaughlin et al. 2006). Interestingly, however, molecular manipulations that delay the degeneration of severed axons also robustly protect degenerating axons in both *in vitro* and *in vivo* models (Deckworth and Johnson, 1994; Sagot et al., 1995; Mack et al., 2001; Ferri et al., 2003; Samsam et al., 2003; Araki et al., 2004; Sasaki et al., 2006; Kaneko et al., 2006; Howell et al., 2007, Sasaki et al., 2009; Meyer zu Horste et al., 2011; Verghese et al., 2011; Wang et al., 2012). These observations suggest that the signals that initiate the elimination of axons during various situations (developmental and clinical) versus Wallerian degeneration after injury may converge onto similar downstream pathways to execute axon removal.

Pruning diversity and key questions:

In summary, it is well appreciated that different neuron populations utilize a diversity of axon remodeling processes to establish their final patterns of connectivity. The elimination of neuronal processes during regressive developmental phases ranges from small-scale pruning to large-scale removal of large axon branches that can extend up to millimeters in length (Luo and O'Leary, 2005). These examples demonstrate the diverse circumstances of pruning in the mammalian nervous system that likely arise from different biological processes in distinct regions, and such diversity raises several important questions: Are distinct mechanisms of axon pruning involved in different destructive contexts, or do shared events mediate axon pruning in every circumstance of axon elimination? Are these mechanisms intrinsic or extrinsic to axons? How does a neuron localize destructive events to axons without putting the rest of the cell at risk? And lastly, do the normal physiological processes of developmental axon pruning provide insight into the pathological axon degeneration that occurs during neurodegenerative disease?

Caspases mediate the selective pruning of dendrites and axons:

Evidence from multiple model systems indicates that the pruning of axons and dendrites is mediated by caspases. This finding was unexpected because caspase activation can put neurons at risk of undergoing apoptosis. During metamorphosis of *Drosophila melanogaster*, for example, dendritic pruning requires localized caspase activation (in this case, the upstream caspase Dronc) that is tightly and spatially regulated by the proteasome-mediated degradation of DIAP1 (Drosophila inhibitor of apoptosis protein, the fly homolog of XIAP)(Kuo, Zhu et al. 2006; Williams, Kondo et al. 2006; Rumpf, Lee et al.

2011). Dendritic caspase activation also appears to play a key role in learning and memory in zebra finch songbirds (Huesmann and Clayton 2006). Specifically, active Caspase-3 is located in dendritic spines of the auditory forebrain. Here, pre-activated Caspase-3 is held in check by being bound to XIAP, and appropriate stimuli trigger its release to induce synaptic remodeling.

Axon pruning removes individual axons and, when necessary, occurs on a larger scale to remove entire collateral branches. While developmental axon degeneration is central to the process that generates the adult patterns of projections in the nervous system, it is particularly important for facilitating overall system flexibility and the progressive processes of neuronal specification (Luo and O'Leary 2005; Low and Cheng 2006). As the induction of specific signaling and fate-determining pathways is highly regulated by which factors are encountered by a projecting axon during development, removing misguided axons is just as important as maintaining correctly projecting ones to establish proper neuronal identity.

Apoptotic proteins mediate axon pruning and synaptic plasticity:

Interestingly, the Bcl-2 family proteins have recently been shown to modulate the axon degeneration that occurs during pruning; deficiency of Bax or overexpression of anti-apoptotic protein Bcl-XL both protect axons following axon-specific nerve growth factor (NGF) deprivation (Nikolaev, McLaughlin et al. 2009; Schoenmann, Assa-Kunik et al. 2010; Vohra, Sasaki et al. 2010). Mice deficient for another anti-apoptotic Bcl-2 family member, Bcl-w, exhibit progressive degeneration of small fiber innervations in the skin without cell body loss, suggesting that Bcl-w is selectively induced in the axons of sensory neurons in

response to trophic factor stimulation (Pazyra-Murphy, Hans et al. 2009). Furthermore, consistent with the idea that apoptosis and axon pruning pathways overlap and are strictly spatially regulated, several critical caspases - specifically caspase-9, caspase-3, and caspase-6 - have now all been implicated in axon-specific degeneration (Nikolaev, McLaughlin et al. 2009; Schoenmann, Assa-Kunik et al. 2010; Vohra, Sasaki et al. 2010; Akpan, Serrano-Saiz et al. 2011; Simon, Weimer et al. 2012; Uribe, Wong et al. 2012). (Notably, at the beginning of this research project, only caspase-6 had been shown to mediate axon pruning (Nikolaev et al., 2009).)

The idea that caspase activation occurs in the absence of cell death is further supported by the recent finding that low levels of Caspase-3 activation are necessary for long-term depression (LTD)(Li, Jo et al. 2010), a synaptic change underlying memory that causes the removal of synapses and the spines in which they reside. Indeed, Bax, Bad, Caspase-9, and Caspase-3 have all been shown to be required for LTD in mice (Figure 1.16)(Li, Jo et al. 2010; Jiao and Li 2011). Therefore, there appears to be an evolutionarily conserved role for site-specific and tightly regulated caspase activation in post-synaptic remodeling.

These observations raise several interesting points. If the effectors of apoptosis and axon degeneration are identical, how do neurons activate axon-selective degeneration or synapse-specific elimination without triggering apoptosis? Alternatively, are there key differences that remain unidentified that allow neurons to precisely activate one but not the other pathway? Such critical questions are partially addressed in this thesis and remain under intense investigation as the number of non-death functions of classically apoptotic proteins continues to grow.

1.7 Figures and Legends

Figure 1.1: Apoptosis in the developing mouse forelimb.

Normal developmental apoptosis results in the formation of interdigital spaces. (**a-c**) Scanning electron microscope and (**d-f**) acridine orange staining of wildtype forelimb at embryonic stages (**a,d**) E12.5, (**b,**e) E13.5), and (**c,f**) E14.5.

Figure 1.1

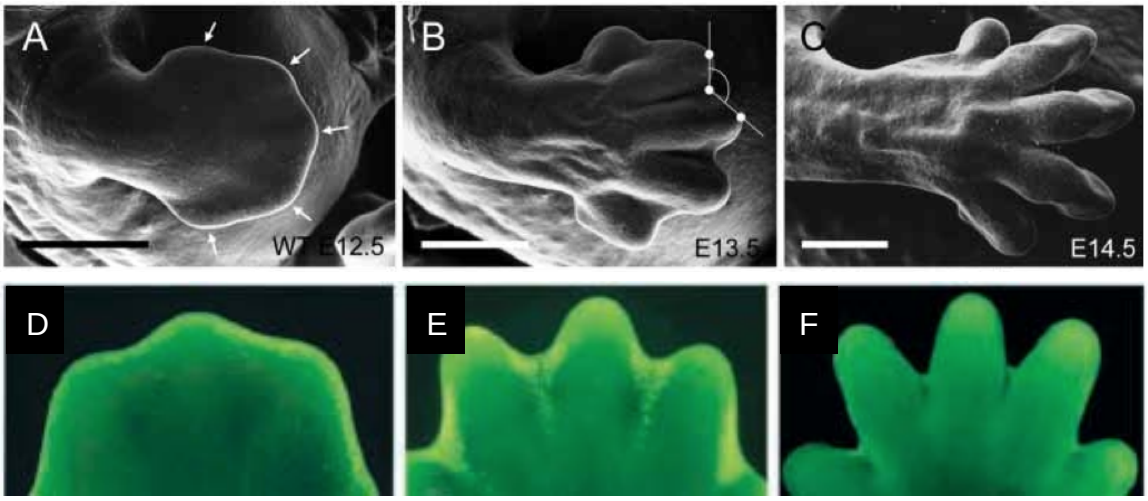
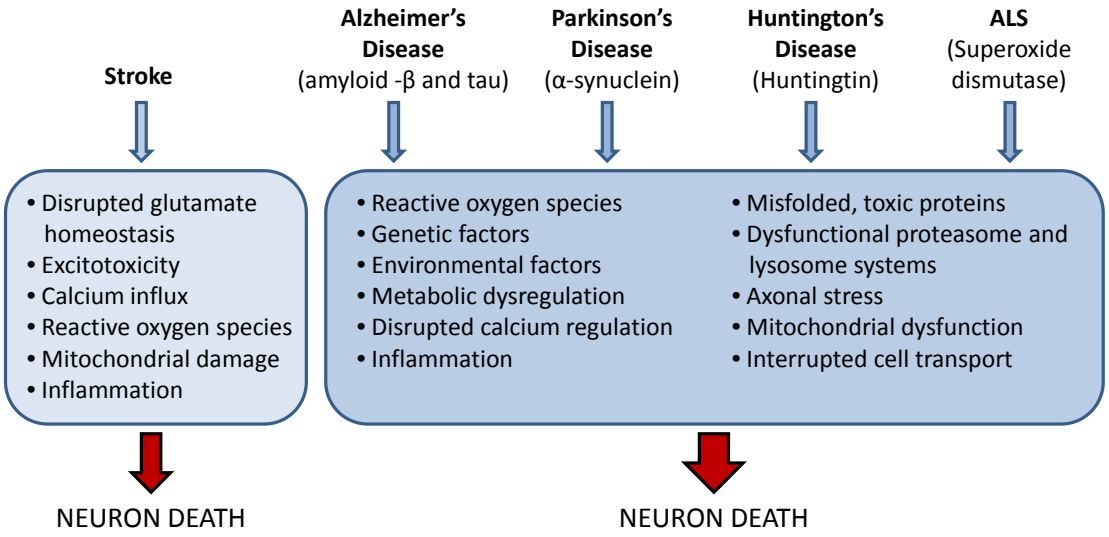


Figure adapted from Wood et al., *Development* (2000)

Figure 1.2: Insults associated with neurodegenerative disease and stroke that lead to neuron death.

Diverse events contribute to the pathologies and neuron death observed in neurodegenerative situations and stroke.

Figure 1.2

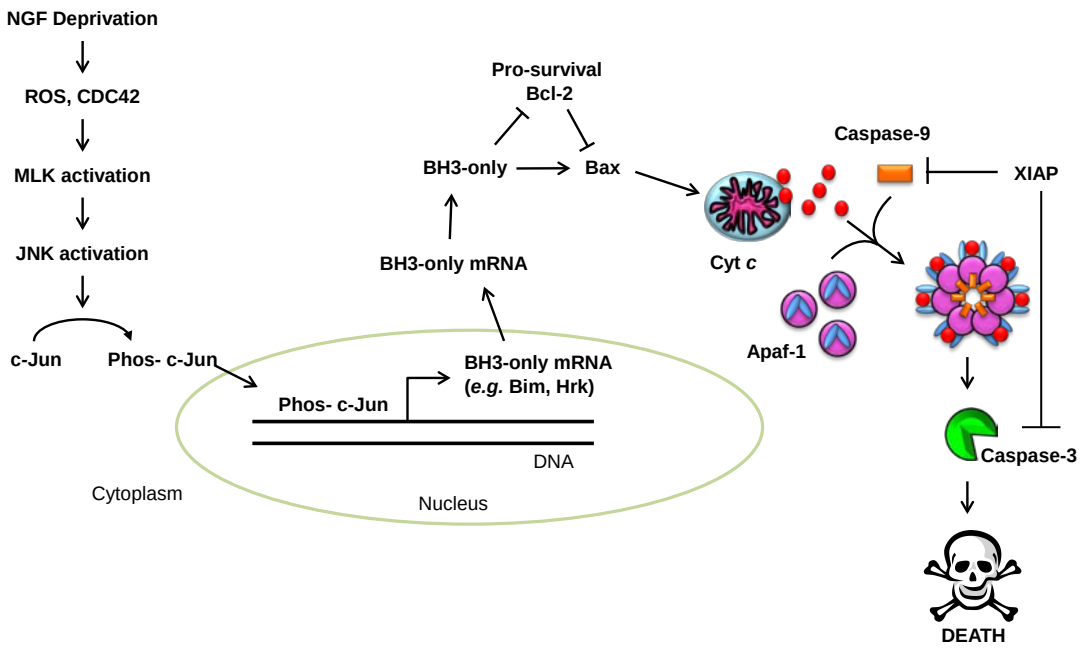


From Cusack et al., *Cell Death: Mechanisms and Disease* (2013)

Figure 1.3: Apoptosis pathway induced in sympathetic neurons following NGF deprivation.

Following NGF deprivation, there is an early increase in reactive oxygen species (ROS) and Cdc42 activity, leading to the activation of mixed lineage kinases (MLKs). In a kinase cascade, MLKs phosphorylate the c-Jun N-terminal kinase (JNK), which in turn phosphorylates the transcription factor c-Jun. Upon phosphorylation, c-Jun translocates to the nucleus where it drives transcription of pro-apoptotic BH3-only genes such as Bim and Hrk. Bim and Hrk either directly or indirectly activate Bax, a protein which oligomerizes and forms pores through which cytochrome *c* passes into the cytoplasm. The presence of cytosolic cytochrome *c* initiates the formation of the apoptosome complex, which activates caspase-9 and caspase-3, and ultimately causes cell death. In healthy neurons, the endogenous caspase inhibitor XIAP binds to cleaved caspase-9 and caspase-3 to prevent a lethal caspase cascade.

Figure 1.3

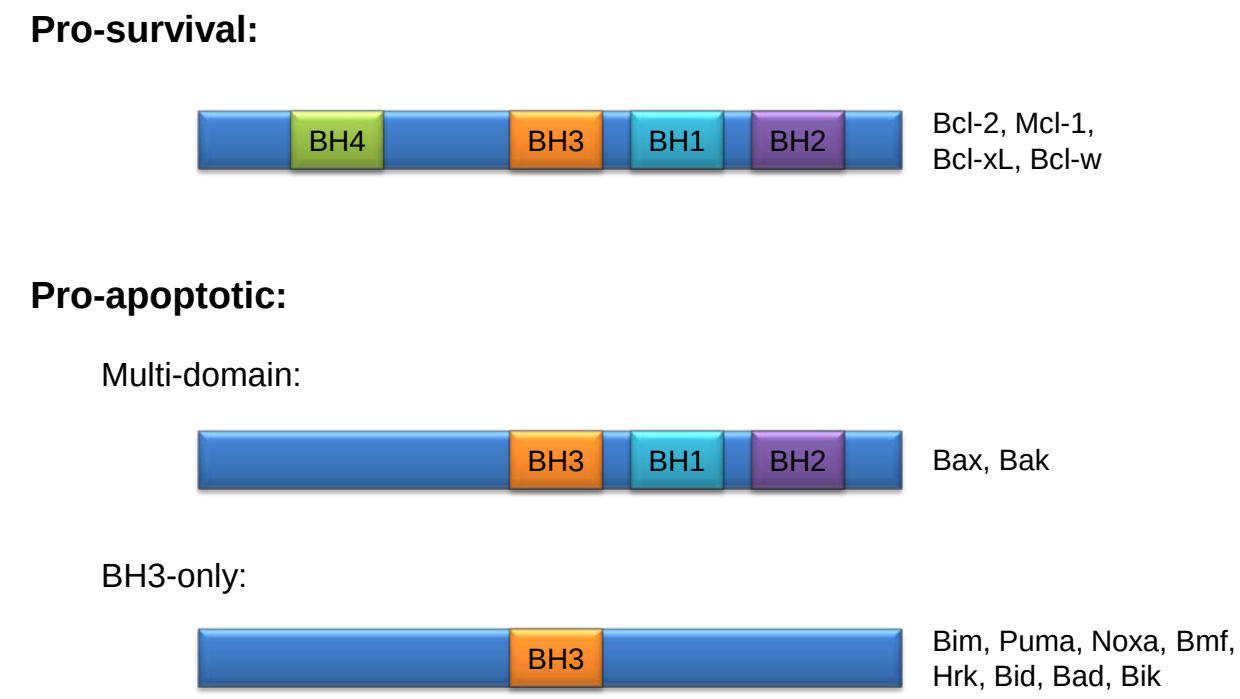


Adapted from Dr. Adam J. Kole

Figure 1.4: Member of the Bcl-2 family of proteins.

The Bcl-2 family of proteins contains both pro-survival and pro-apoptotic members, which are subdivided based on their activity and the number of Bcl-2 homology (BH) domains present. The pro-survival proteins Bcl-2, Bcl-XL, Bcl-w, and Mcl-1 contain four BH domains (BH1-4). The pro-apoptotic members are divided into two subgroups: the multidomain pro-apoptotic proteins Bax and Bak contain BH1-3 domains while the BH3-only pro-apoptotic proteins contain only the BH3 domain.

Figure 1.4

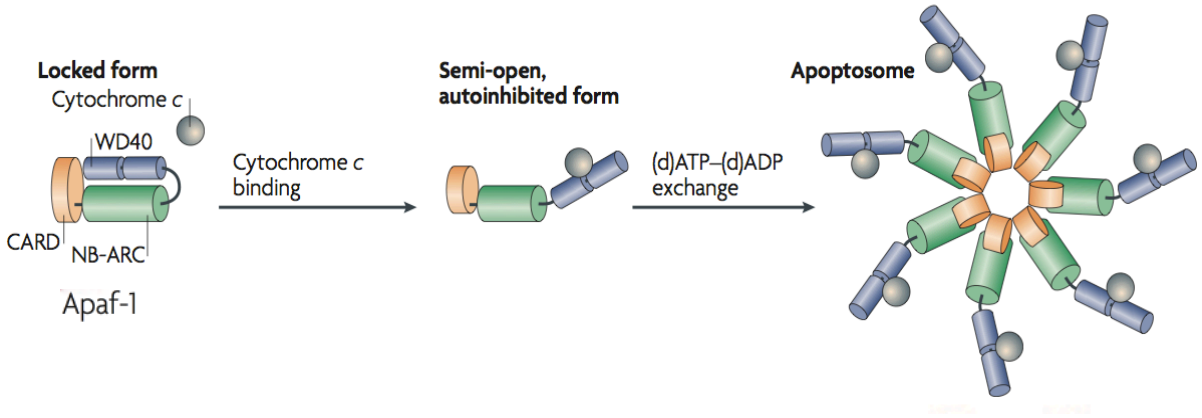


Adapted from Parsons and Green, *Essays in Biochemistry* (2010)

Figure 1.5: Formation and activation of the apoptosome complex.

Inactive Apaf-1 exists in a locked state in which the WD40 repeats fold to block access of dATP and caspase-9 to the NB-ARC and CARD domains, respectively. Upon activation of apoptosis, cytochrome *c* binds to the WD40 repeats of Apaf-1, which opens its structure to allow binding of dATP and caspase-9. Upon Apaf-1 oligomerization, caspase-9 and Apaf-1 bind via their CARDs, resulting in caspase-9 activation.

Figure 1.5

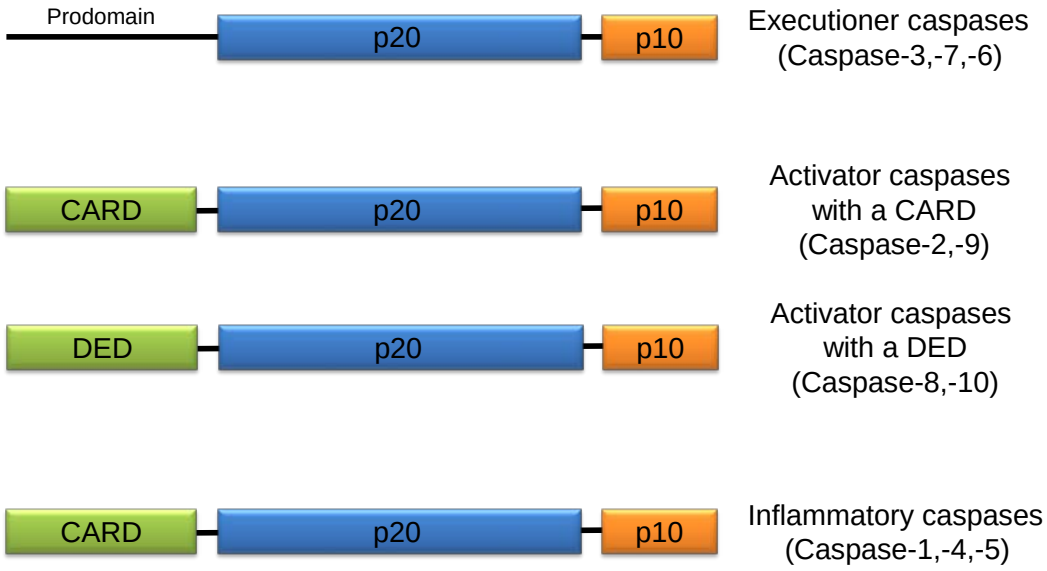


Adapted from Riedl and Salvesen, *Nat Rev Mol Cell Biol* (2007)

Figure 1.6: Basic overview of caspase domains.

Caspases (cysteine aspartic acid-specific proteases) can be subdivided into initiators, which can auto-activate and initiate the proteolytic processing of other caspases, and effectors, which are activated by other caspase molecules. Effector caspases cleave the vast majority of substrates during apoptosis. All caspases have a similar domain structure consisting of a pro-domain followed by a large (p20) and small (p10) subunit. The pro-peptide can be of variable length and, in the case of initiator caspases such as caspase-9, can recruit the enzyme to activation scaffolds such as the apoptosome. Two distinct pro-peptides that facilitate interactions with other proteins have been identified on initiator caspases: the caspase recruitment domain (CARD) and the death effector domain (DED). Caspase activation is usually initiated by cleavage between the large and small subunits to form a heterodimer, and the dimerization of two caspase heterodimers forms the mature active heterotetramer.

Figure 1.6

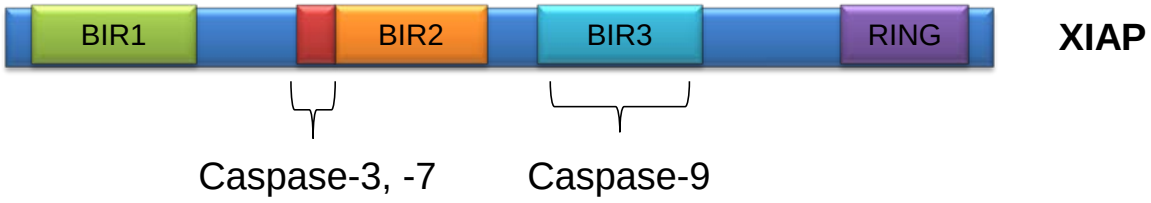


Adapted from Riedl and Shi, *Nat Revs Mol Cell Bio* (2004)

Figure 1.7: Domains of XIAP.

XIAP (X-linked inhibitor of apoptosis protein) binds directly to cleaved caspases to block their proteolytic activity. XIAP contains three BIR domains, the third of which binds to cleaved and activated caspase-9. The small linker region on the N-terminal side of XIAP's BIR2 domain forms a reversible steric occlusion of substrates from caspase-3 and caspase-7.

Figure 1.7

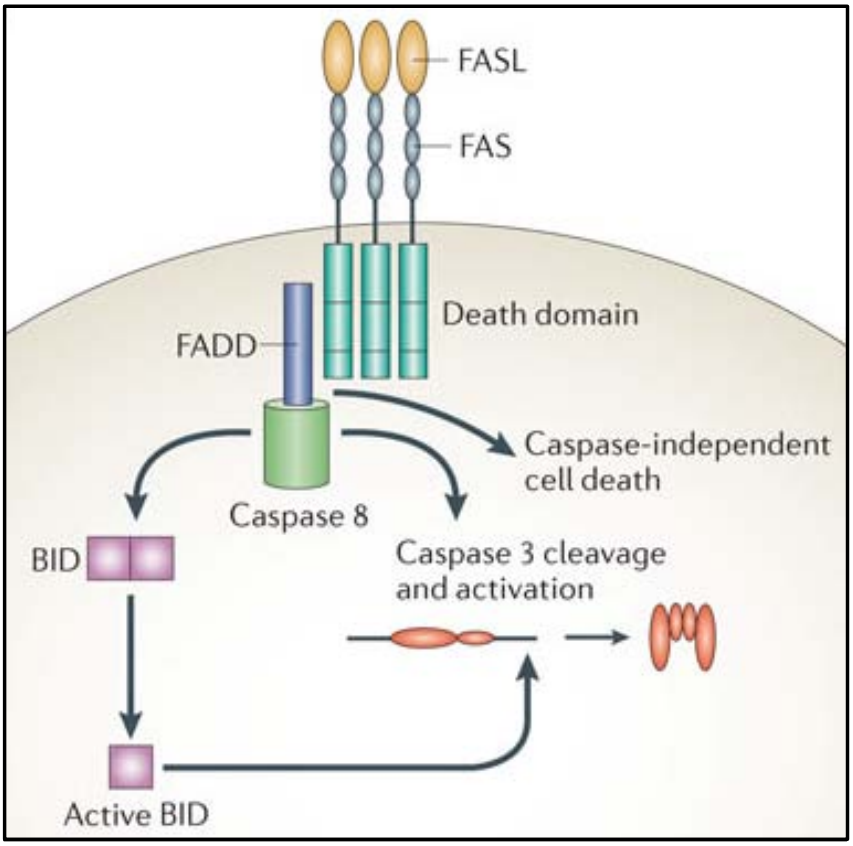


Adapted from Holcik and Korneluk, *Nat Revs Mol Cell Bio* (2001)

Figure 1.8: Overview of the extrinsic pathway of apoptosis.

The extrinsic cell death pathway begins outside of the cell with the activation of specific death receptors such as FAS (shown here). Upon ligand binding (FASL) and activation of the death receptor, a signaling complex called the death-inducing signaling complex (DISC) forms that includes a FAS-associated death domain protein (FADD) and leads to caspase-8 activation. Active caspase-8 can cleave and activate caspase-3 or cleave the BH3-only protein, Bid, into its Bax-activating form, tBid, resulting in mitochondrial cytochrome *c* release and a deadly caspase cascade.

Figure 1.8

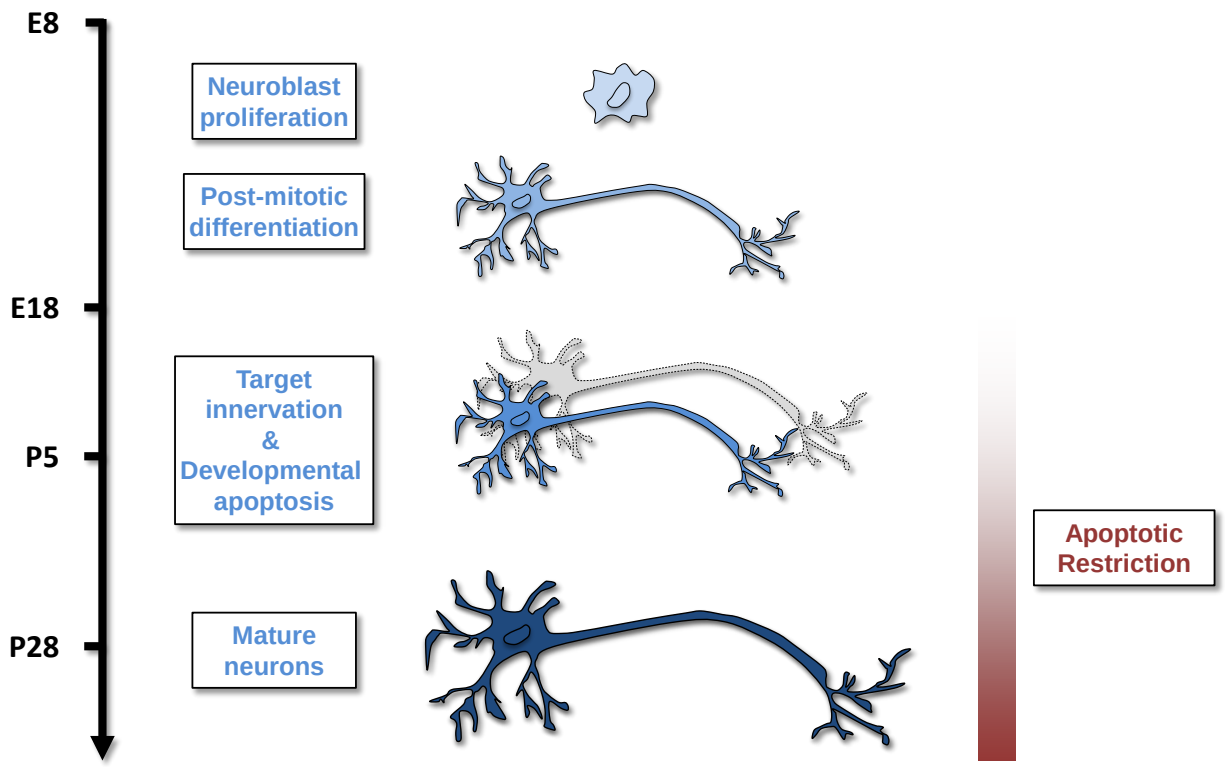


From Hyman and Yuan, *Nat Rev Neurosci* (2012)

Figure 1.9: Neurons become increasingly resistant to apoptosis with maturation.

During development, neurons move from an early state of high apoptotic sensitivity to a later state of apoptotic resistance. We have established that cultured sympathetic neurons become resistant to apoptosis after 28 DIV due to the development of multiple apoptotic brakes, such as restricted Apaf-1 expression and the upregulation of the anti-apoptotic microRNA, miR-29.

Figure 1.9

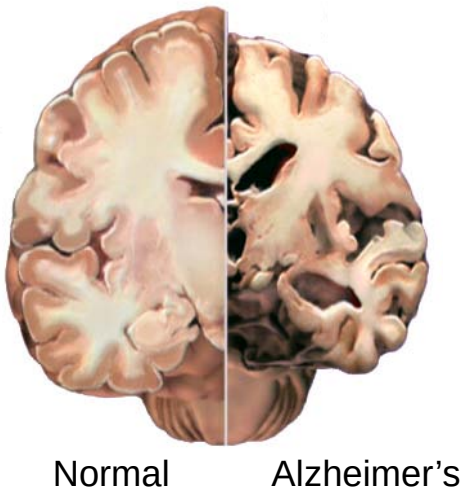


Adapted from Dr. Adam J. Kole

Figure 1.10: Comparison of a healthy adult brain to a highly atrophied brain of an Alzheimer's disease patient.

Alzheimer's disease is characterized by the loss of neurons and synapses in the cerebral cortex and subcortical regions, resulting in massive brain atrophy.

Figure 1.10

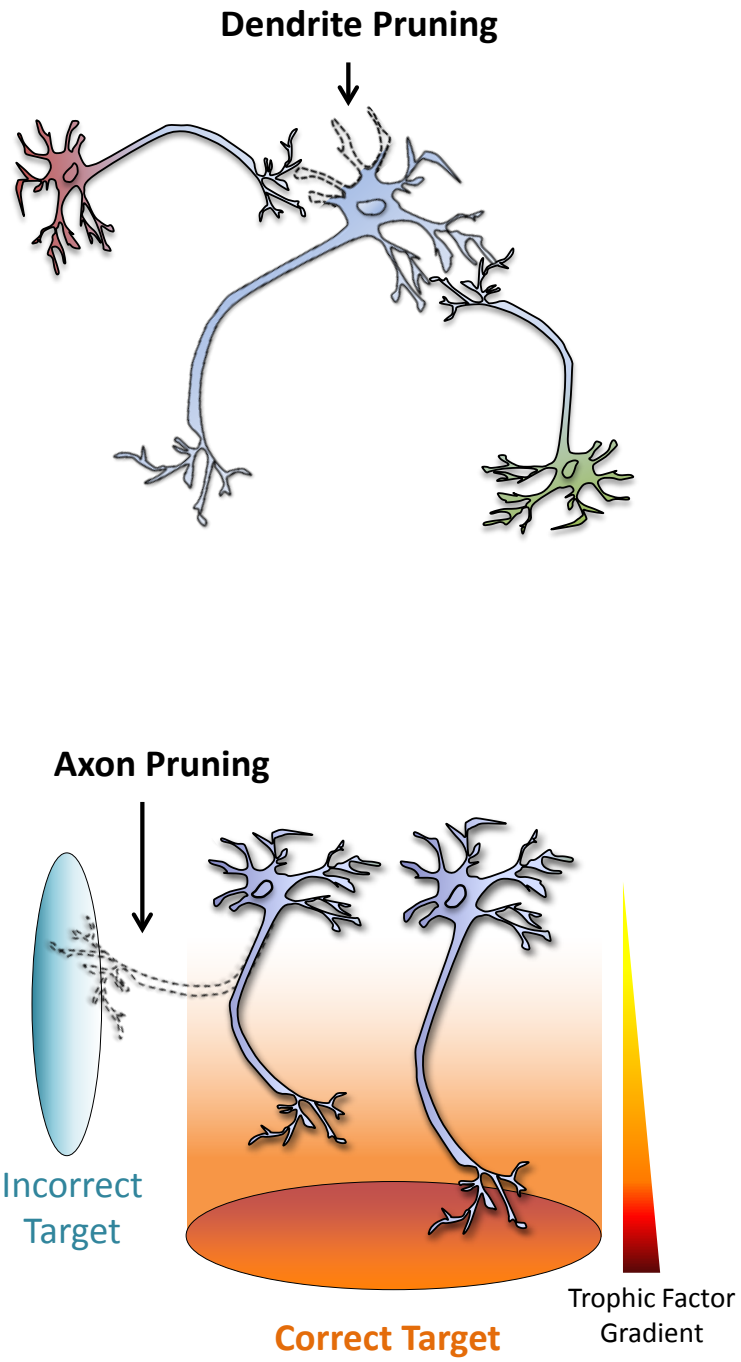


Adapted from the Alzheimer's Association

Figure 1.11: Localized regressive events occur during normal development.

Precise neuronal circuitry is sculpted by localized regressive events such as synapse removal and dendrite pruning (top) and axon-specific pruning (bottom). Growing axons compete for limited amounts of neurotrophic factor secreted by innervation targets. Outcompeted or misprojecting axons are removed by axon pruning mechanisms whereas successful axons are maintained.

Figure 1.11

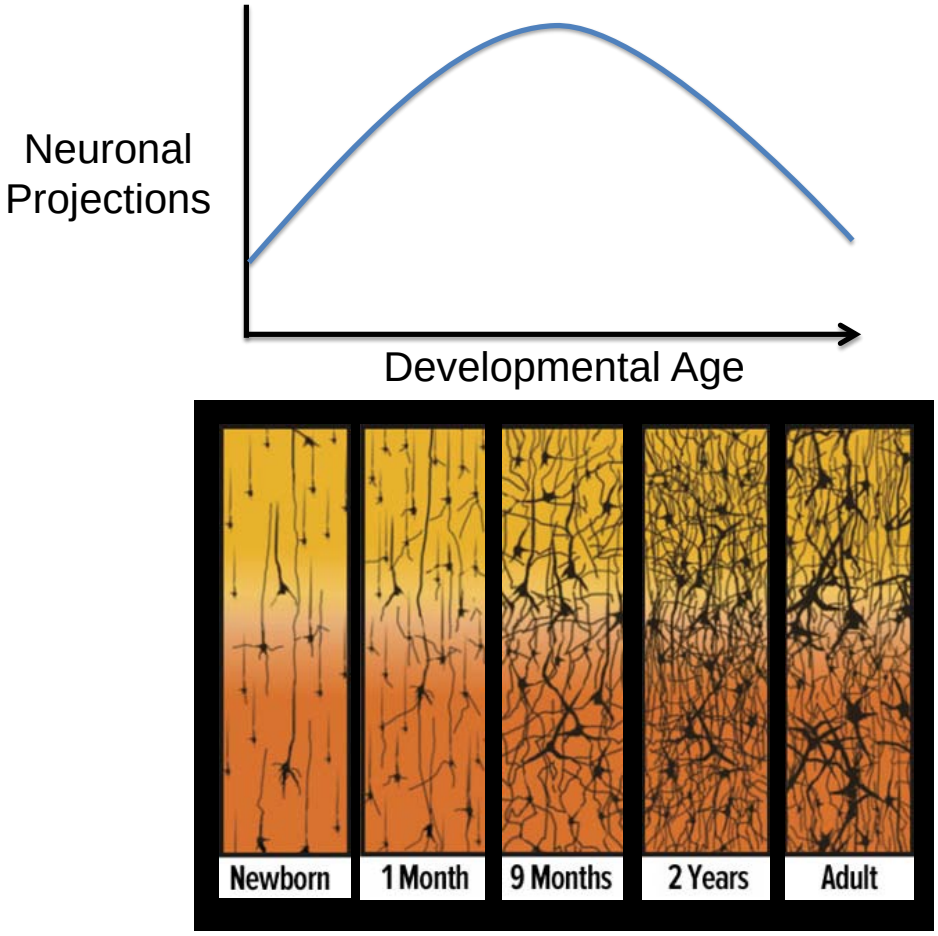


From Cusack et al., *Cell Death: Mechanisms and Disease* (2013)

Figure 1.12: Massive pruning events sculpt the mature nervous system.

The number of neuronal projections is highest during childhood, with children ages 2-7 possessing approximately 50% more connections than adults. As humans age, regularly stimulated connections are strengthened while lesser used connections weaken and may be removed completely. This process explains, for example, why adults so often struggle to remember the foreign language they studied as children once the foreign language use stops. Continuous use and practice of the language, however, ensure that the circuitry responsible for bilingualism remain intact.

Figure 1.12

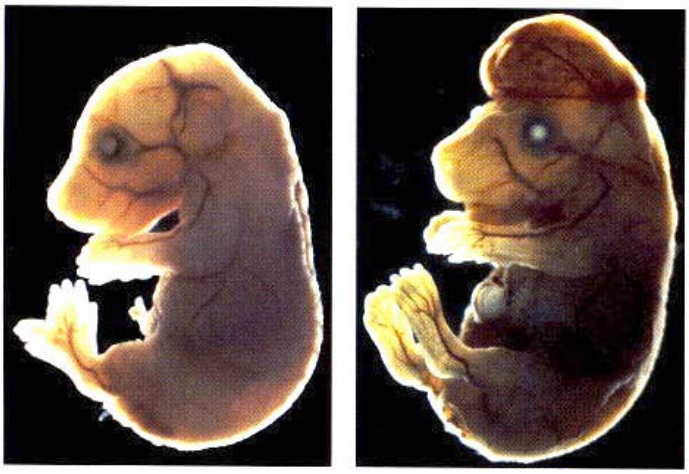


Adapted from Urban Child Institute

Figure 1.13: Loss of key apoptotic proteins during development causes gross morphological and lethal defects in the brain.

Wildtype or caspase-9 knockout mouse embryos at embryonic day 16 (E16). Mice lacking the pro-apoptotic caspase-9 gene develop with large brain protrusions, known as exencephaly.

Figure 1.13



Wildtype Caspase-9 -/-

Adapted from Kuida et al., *Cell* (1998)

Figure 1.14: Distal portions of severed axons degenerate following axotomy during Wallerian degeneration.

When axons are severed (an injury termed Wallerian degeneration), the distal axon degenerates while the parent neuron and connecting axon segment survive.

Figure 1.14

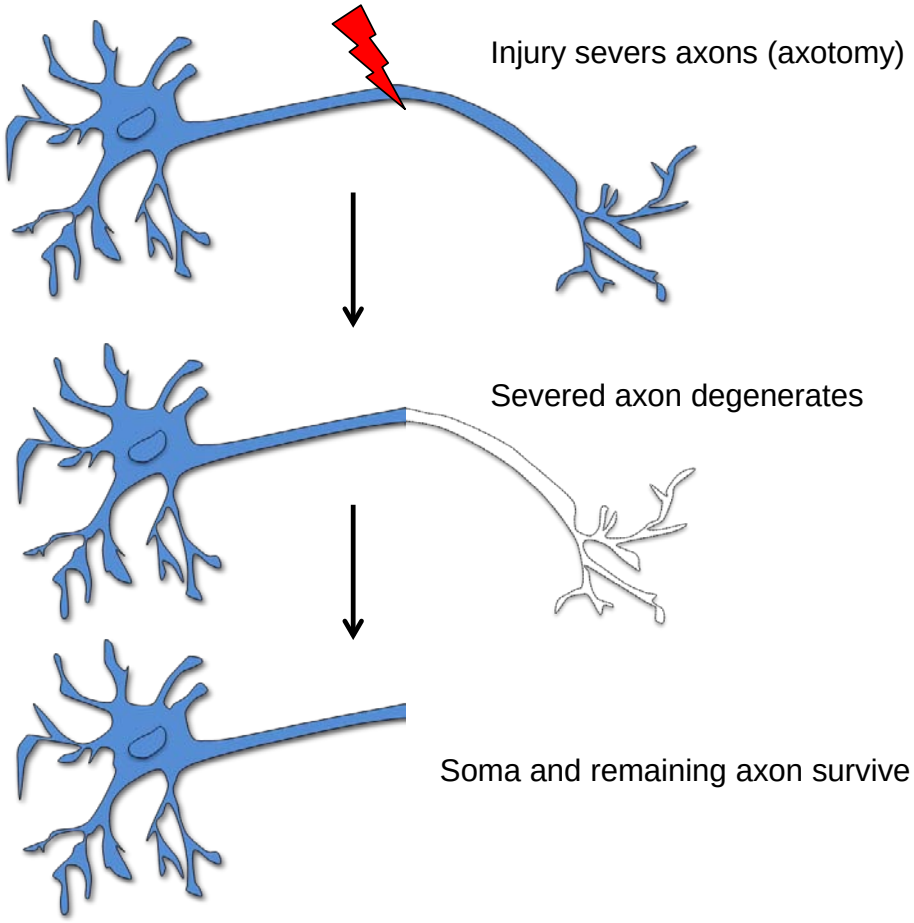
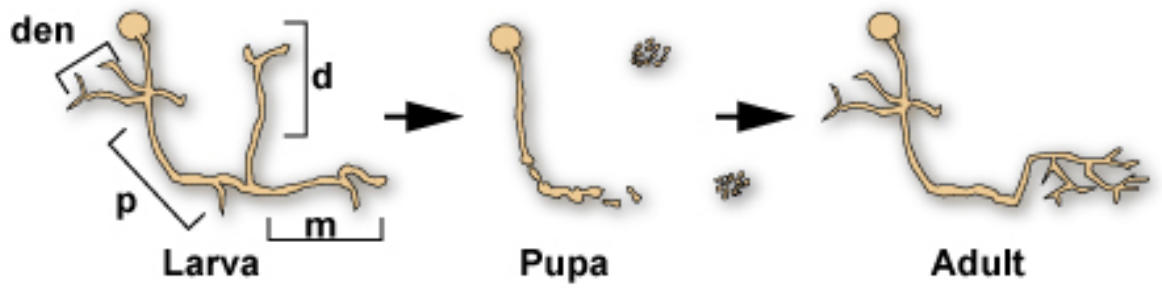

Injury severs axons (axotomy)
Severed axon degenerates
Soma and remaining axon survive

Figure 1.15: Schematic of developmental mushroom body neurite pruning in *Drosophila*.

During metamorphosis, γ neurons of the fly mushroom body structure undergo dendritic (den) and axonal pruning (p, d, m). New axon projections grow from the same neuron later in development.

Figure 1.15

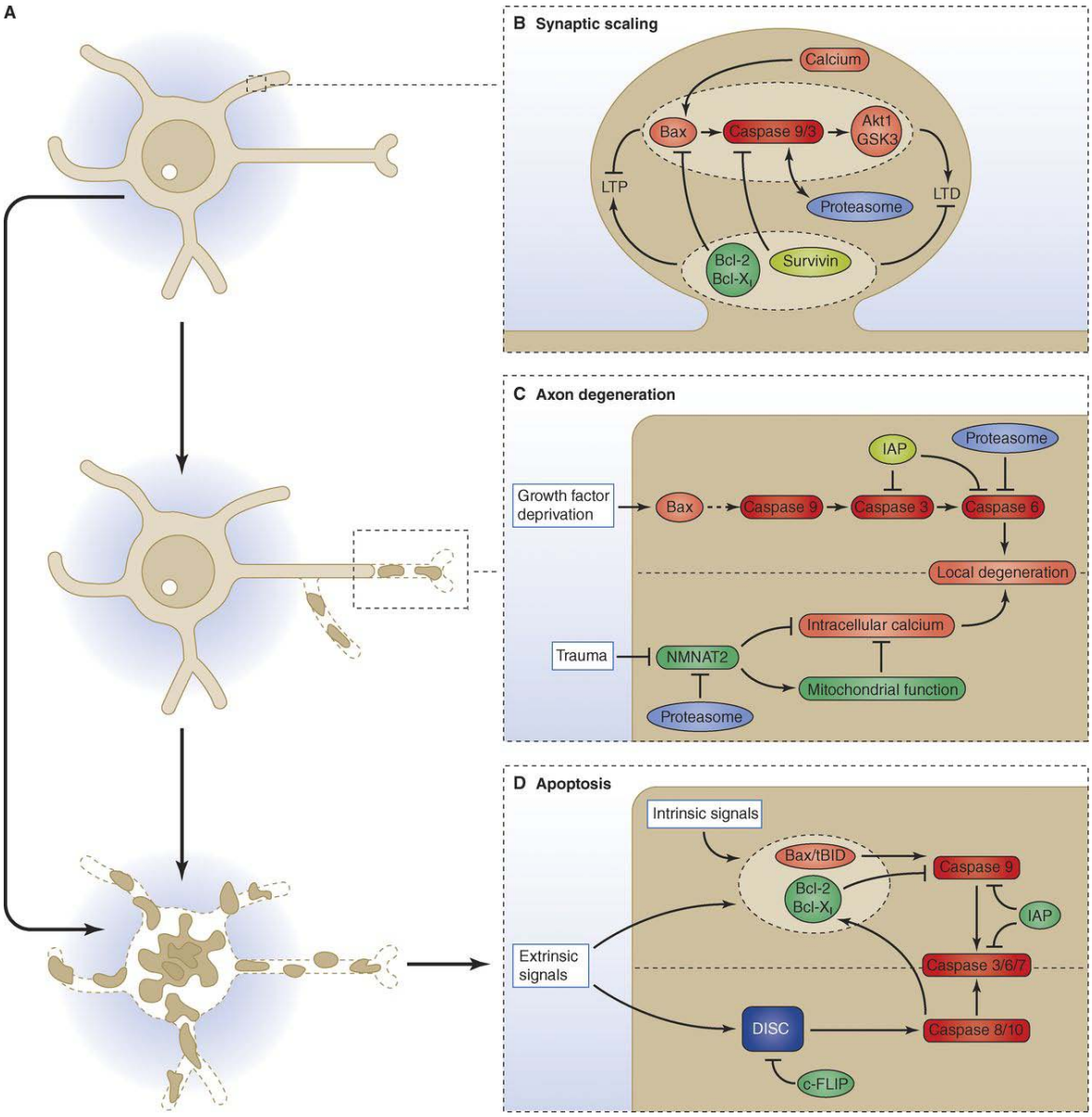


Adapted from Watts et al., *Neuron* 2003

Figure 1.16: Current models of non-canonical and canonical functions of apoptotic molecules in neurons.

Growing evidence indicates that apoptotic machinery is involved in critical, non-apoptotic neuronal functions such as synaptic plasticity and axon pruning. (**a**) In the adult nervous system, Bax, caspase-9, and caspase-3 are required for LTD while anti-apoptotic Bcl-XL and the IAP survivin promote LTP. (**b**) Axon-specific growth factor deprivation leads to the activation of Bax, caspase-9, caspase-3, and caspase-6 during axon pruning. In contrast, none of these apoptotic components appear to be required for injury-induced axon degeneration (Wallerian degeneration). (**c**) A simplified schematic of intrinsic and extrinsic neuronal death pathways are shown for comparison.

Figure 1.16



From Dekkers et al., *J Cell Bio* (2013)

CHAPTER TWO: ESTABLISHING MICROFLUIDIC CHAMBER TECHNOLOGY TO ISOLATE AXON-SPECIFIC DEGENERATION FROM WHOLE-CELL DEGENERATION

I provide a brief history of my experience with microfluidic chambers below because establishing and troubleshooting microfluidic technology in the lab occupied a significant amount of time and effort during my graduate school career. I have also identified personal recommendations, potential problem sources and their solutions (in italics) for future researchers who encounter difficulties with their own microfluidic systems.

2.1 Microfluidics and neurons: Enabling fluidic isolation of soma and axon

Microfluidics and neurons: A perfect match:

The field of microfluidics uses devices with minute reservoirs and channels to manipulate very small amounts of fluid (Whitesides 2006). Compared to conventional large-scale methods, microfluidic devices offer a miniaturized platform for faster and more sensitive chemical and biochemical analyses. The recently increased popularity of microfluidic technology in neuroscience research is largely due to the in-house ability to fabricate custom-designed devices using photolithography and polymers (i.e., poly(dimethylsiloxane) (PDMS), a translucent biocompatible polymer). Microfluidic devices have widespread applications in neuroscience, from stem-cell-to-neuron

differentiation and cell migration to drug screens and, as described here, the examination of differences between a neuron's soma and axon. The ability to precisely control neuronal microenvironments using microfluidic technology creates opportunities for new biological insights that were previously impossible using conventional macro-scale methods.

One of the most unique characteristics about neurons are their extensive processes that traverse – and survive – extremely long distances across diverse extracellular environments. For example, the peripheral axon that innervates the big toe starts at the neuron cell body located in the base of the spinal cord and extends to the hip, down the thigh, through the knee, and down the lower leg into the foot where it innervates the toe. Likewise, in the central nervous system, 200-250 million axons project from one side of the brain to the other by navigating across the corpus callosum, the "bridge" between the two hemispheres of the brain. Rarely is an entire neuron – the soma, dendrites, and axonal branches – exposed to the same environment at any given time. Importantly, this multi-environment issue means that neurons have developed stimuli- and location-specific mechanisms to maintain proper cell functions; thus, the ability to environmentally isolate different parts of neurons during experiments is essential for achieving a more accurate understanding of basic neuronal mechanisms. Mechanisms that affect distinct parts of neurons between different environments cannot be studied using conventional culturing methods (i.e., dissociated neurons grown in a culture dish) because the entire cell is exposed to the same stimuli, which includes media, pharmaceutical treatments, and factors released by the cells in culture, among others. Even the use of mesh inserts that physically separate cell bodies from their axons in culture do not enable axon-specific treatments (Figure 2.1).

Microfluidic technology provides a fluid, adaptable system to more adequately model neuronal responses to differences between somatic and axonal environments *in vitro*. The microfluidic chamber design used for the experiments described in this work (originally invented and verified by Dr. Anne Marion Taylor during her post-doctoral career in Dr. Noo Li Jion lab at the University of California Irvine) contains two vertical and parallel compartments connected by tiny grooves (Figure 2.2)(Taylor, Blurton-Jones et al. 2005; Taylor and Jeon 2011). Neuronal cell bodies are cultured in one compartment and grow their axons through the grooves into the second compartment (Figure 2.3). Small differences in media volume between the two main compartments create hydrostatic pressure inside the device that fluidically isolates one compartment from the other for up to 20 hours (Figure 2.2). Re-establishing the volume differential between the two compartments maintains fluidic isolation for as long as needed.

The predecessor: Campenot chambers:

The concept of physically separating and fluidically isolating neuronal cell bodies from their axons is not new; Robert Campenot first demonstrated the differential treatment of cell bodies and axons in 1982 using his (now household-name) Campenot chambers (Figure 2.4)(Campenot 1982; Campenot, Lund et al. 2009). Campenot chambers consist of a reusable Teflon piece applied with a grease layer that is placed on a Petri dish. The grease layer forms a hydrophobic barrier that prevents the exchange of soluble factors between compartments, and axons of long projection neurons such as dorsal root and superior cervical ganglion neurons can grow through the grease into adjacent compartments. Though effective when successfully constructed (particularly for acquiring enough axonal

material for Western blot analysis and other biochemical assays), Campenot chambers were initially discouraged by colleagues due to a reported 50% leakage rate following assembly. During my third year of graduate school, I spent one week in Dr. Barry Kaplan's lab at the National Institutes of Health learning Campenot chamber technique from Anthony Gioio, a Campenot chamber expert with over a decade of experience and a 90% chamber success rate. I tried to assemble Campenot chambers in the Deshmukh lab and, similar to my peers, experienced a consistent failure rate due to leakage (40-50%). I decided to continue using microfluidic chambers in my experiments to best maintain a strong pace of research progress and minimize animal waste as much as possible. However, I encourage others to use Campenot chambers when given adequate time to practice and perfect the technique; the ability to collect and analyze sufficient amounts of protein from axon-only lysates is unquestionably valuable for dissecting axon-specific mechanisms from whole-cell or soma-specific mechanisms.

2.2 First steps: Collaboration, production and instruction

After speaking with several axon researchers during the formation of this thesis project, I decided to establish microfluidic chamber technology in the lab as the primary method for separating soma and axon. Speed and ease of assembly are the top advantages provided by microfluidic chambers; once the master mold is made, microfluidic chambers can be quickly mass-produced and assembled in the lab. The most difficult part of the process was imprinting the master mold design onto a silicon wafer in the CHANL (Chapel Hill Analytical Nanofabrication Laboratory). Notably, I spent six months working with Dr. Henley in the UNC Chemistry Department (Dr. J. Michael Ramsey's lab) drafting the chamber design in ICAD (a knowledge-based engineering system), getting the design masks printed by Infinite Graphics, Inc. (Minneapolis, MN), and troubleshooting the novel deep reactive ion etching (DRIE) wafer fabrication process. Please note that our DRIE approach deviates from the standard photolithography approach for fabricating the master wafer mold. Standard photolithography uses light to transfer a pattern from a photomask to a light-sensitive chemical called photoresist on the substrate (in this case, a silicon wafer). Exposing the photoresist to ultraviolet light through the patterned photomask results in the formation of extremely strong chemical bonds (a useful artistic analogy involves putting a desired pattern on paper using spraypaint and a stencil), and any non-exposed photoresist is then removed by a series of chemical washes. While we used photolithography to imprint the larger side compartments onto the wafer, we decided to pioneer DRIE for the microscopic grooves to achieve a higher quality and significantly (3-6 times) longer-lasting pattern.

The original chamber design was based on the one previously published by Taylor and colleagues (Taylor, Blurton-Jones et al. 2005), but we customized the design for axon degeneration experiments by increasing the length of the side compartments (to permit more axon growth and imaging opportunities) and adding numbered grooves. After using a mask aligner (Karl Suss MA6/BA6) to imprint our groove pattern on the silicon wafer, we used deep reactive ion etching (DRIE; Alcatel AMS 100 Deep Reactive Ion Etcher) to achieve the following critical details:

1.) smooth groove edges, which are far more amenable to neurite outgrowth than jagged or rough groove edges (personal observation);
2.) exact groove height and width measurements, which are required to achieve and maintain fluidic isolation between the two side compartments; measurements were confirmed using a stylus profiler (KLA Tencor P-6) and an electron beam evaporator (Thermionics VE-100);
3.) an imprinted number system on the central microgrooves to precisely identify and measure the same axons as they degenerate over time.

Standard photolithography and the mask aligner were then used to add the two large side compartments along the etched grooves to complete the silicon wafer mold. Lastly, the wafers were silane-coated overnight in a sealed chamber, a step that enables poured and cured polymer to easily peel away from the wafer during the chamber production process.

After creating the master mold, I spent one week in Dr. David Ginty's lab at Johns Hopkins University under the tutelage of two postdocs, Drs. Anthony Harrington and Christopher Deppman, to learn how to assemble and use microfluidic chambers with mouse sympathetic neurons. Over the years I have come to appreciate how microfluidic chambers are both incredibly useful and extremely sensitive ("finicky" is a term widely used in the field by those who regularly use the chambers). For example, the chambers suddenly stopped working for six months during my fourth year of graduate school. After replacing every single component of the chambers, I learned that the problem was the air in the lab, which was affecting how the polymer cured at room temperature; moving the chambers to another building (and later a dry oven) immediately fixed the problem. My personally modified protocol for fabricating and troubleshooting microfluidic chambers is described in greater detail below.

2.3 Fabrication of microfluidic devices using DRIE

Standard photolithography was used to fabricate channel array devices for axon growth. Negative photoresist (KMPR 1010, MicroChem Corp., Newton, MA) was used to pattern arrays of 10-μm-wide features spaced 40 μm apart onto a standard 152-mm-diameter silicon wafer (Silicon Quest International, San Jose, CA). The bare silicon around the pattern was etched approximately 3-μm deep using deep reactive ion etching (Alcatel AMS 100 Deep Reactive Ion Etcher) with a low-roughness Bosch process. The photoresist was then stripped using 2800 W oxygen plasma. The features for the large side compartments (750-μm long, 100-μm-deep, 1.0-mm-wide on each side) at either end of the channel array were fabricated onto the wafer using negative photoresist (SU8-100, MicroChem Corp., Newton, MA) and standard photolithography using the manufacturer's recommended procedure. Wafers were then transferred to a vacuum desiccator containing a vial with 0.5 mL of trichlorooctylsilane to form a low-energy surface coating. After evacuation of the air, the wafers were left in the desiccator overnight to react. A mold form was built around the wafer for PDMS casting. Sylgard 184 (Dow Corning) was prepared according to the manufacturer's recommended procedure and poured into the mold form. After curing at 65°C overnight, the PDMS was removed from the mold. A tool for puncturing leather material (purchased at a local craft supply store) was used to punch out the individual reservoirs, and chambers were then cut into individual chips using a standard razor blade. Industrial packaging tape (3M-471; ULine) was gently applied to both sides of the chambers to remove all dust particles. Chambers were then sterilized in 70% EtOH for 1-3 minutes and left to air dry at least 1 hour in the hood.

Recommendations:

1.) *3M-471 tape does not leave behind any sticky residue, which can cause both sealing issues and toxicity to cells.*
2.) *Do not leave chambers in ethanol longer than a few minutes; longer time periods will result in culture toxicity (personal observation).*
3.) *Beware: Silicon wafers can explode in the DRIE machine, which will require you to spend several hours vacuuming the inside chamber with a pipette tip to remove all miniscule shards.*
4.) *Mix the polymer for at least 5 minutes (10 minutes is best, and some recommend 30 minutes) to reduce toxicity caused by any free-floating polymer agents.*
5.) *Recoat the wafer with silane approximately every 30 uses to keep the wafer and pattern in good optimal condition.*
6.) *Store wafers in an airtight freezer bag in between uses. Do not store wafers in the dry oven as this will quicken the photoresist's degradation.*
7.) *Consecutively rinse the wafers with 95% and then 70% ethanol every few uses to remove dust particles and any potential sources of contamination.*
8.) *Humidity matters! Chambers do not cure (they will feel stickier than usual) or seal to glass as well in highly humid weather conditions. Minimize the time the chambers spend outside of the dry oven as much as possible.*
9.) *Air quality matters! Pour and cure chambers in a clean, toxin- and fume-free location.*

2.4 Preparation steps and assembly

Coverslip preparation:

Coverslips were cleaned in a sonicator (two 30-minute cycles in sterile water and one 30-minute cycle in 70% ethanol; Sentry Digital Ultrasonic Cleaner) and air-dried completely in a sterile hood. Using sterile forceps, coverslips were placed in a non-overlapping pattern in a 10-cm culture dish filled with 30 mL of 40 ug/mL poly-D-lysine (PDL; BD Biosciences). Parafilm was used to seal the culture dish to prevent evaporation and coverslips were incubated at 37°C for at least one night. Next, each coverslip was placed into a 60-mm culture dish and gently rinsed three times with sterile water. Coverslips were then coated with 0.1 ug/mL mouse laminin (Invitrogen) for a minimum of 2 hours, rinsed gently three times with sterile water, and left in the hood to dry.

Recommendations:

1.) *Dilute both the PDL and laminin in molecular grade sterile water.*
2.) *Always use the highest molecular weight of PDL (500-550 kD; I used BD Biosciences catalog# 354210). Aliquot and store diluted PDL at -20°C for up to 3 months. PDL older than this does not work well. Thawed PDL can be reused for up to 2 weeks at 37°C.*
3.) *Aliquot the mouse laminin stock (10 uL per tube; Invitrogen catalog# 23017-015) and use one aliquot per 10 mL water.*
4.) *Be very careful when handling the coverslips to avoid scratching or accidentally removing the PDL and/or laminin coating. Only apply forceps to the coverslip edge and do not blast water onto the coverslips during rinsing.*
5.) *Places to pause: Any rinsing or laminin-coating steps can extend overnight without negative consequences.*

Chamber assembly:

Once both the PDL/laminin-coated coverslips and sterilized microfluidic chambers were completely dry in the hood, chambers were sealed to the coverslips (patterned-side down) by placing each chamber in the center of a coverslip, applying steady gentle pressure above the grooves and then pressing outwards to the edges of the chamber. Extra pressure was applied around each reservoir to ensure a strong seal, and the back of a pair of sterile forceps was sometimes used to apply pressure at this step. Culture dishes were covered, placed in 10-cm dishes (three 60-mm dishes plus one 35-mm dish filled with sterile water per large 10-cm dish), and stored in a 37°C incubator until neuron plating.

Recommendations:

1.) *Identify and discard any "bad" (i.e., non-sticking, uneven, wet, poorly sealed) chambers now. Bad chambers will lift off the coverslip almost immediately.*
2.) *Double-check all chambers right before you plate cells to ensure a well-maintained seal. Sometimes chambers seal initially but lift off in places after a few minutes.*
3.) *Pressing too hard on the groove pattern can crush it. Be cautiously firm.*
4.) *An uneven polymer surface will prevent a good seal. Make sure that the wafer mold surface is free of any dust particles, fuzz, or polymer pieces before you pour and cure the polymer as these items can create unwanted air pockets between the polymer and glass.*
5.) *Do not reuse or autoclave chambers. Previously used and autoclaved chambers do not seal well and tend to leak.*

2.5 Figures and Legends

Figure 2.1: Diagram of the mesh insert approach to physically (but not fluidically) separating cell bodies from axons.

Culturing neurons on a tiny mesh screen inserted into a culture well allows neuronal cell bodies to settle on top while their axons extend downwards through the mesh insert. This technique permits the physical separation of soma and axon; however, it does not permit differential treatment of axon and soma compartments because media moves freely through the mesh divider.

Figure 2.1

Side view of well with mesh insert:

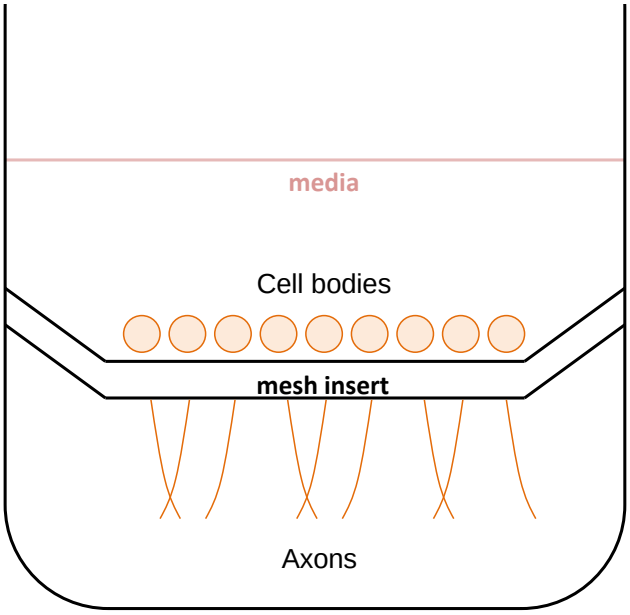
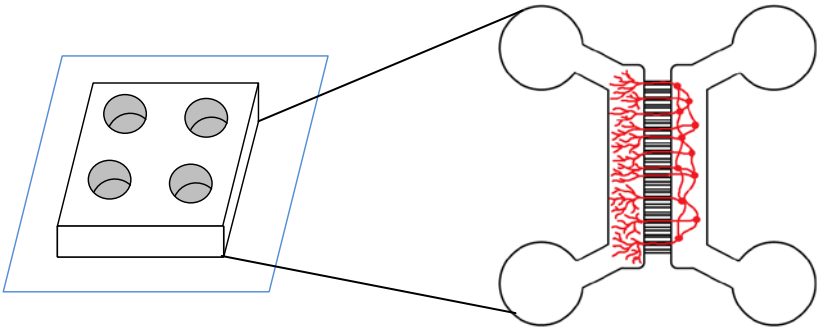


Figure 2.2: Diagram of a microfluidic chamber and the fluidic isolation between soma and axon compartments.

The microfluidic chambers used here consist of a patterned polymer chamber (cast and cured on a wafer mold) sealed to a glass coverslip coated with PDL and laminin. (**a**) Cell bodies are plated in one compartment and their axons grow through tiny microgrooves into the other compartment. (**b**) A side view of a microfluidic chamber and how different volumes of media are applied during experiments to fluidically isolate the two compartments.

Figure 2.2

a.



b.

View from side:

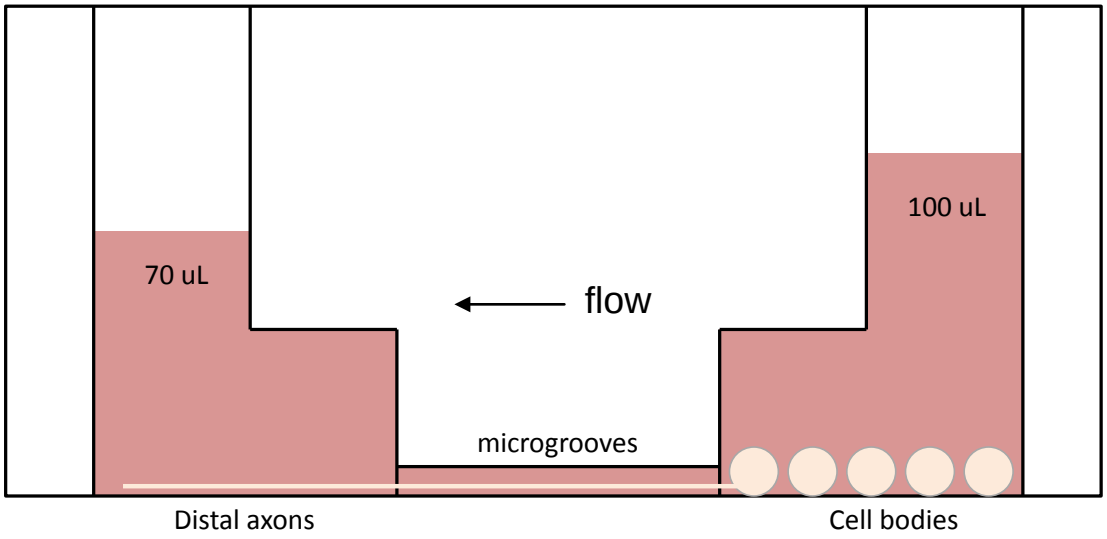


Figure 2.3: Sample of an ideal microfluidic chamber containing sympathetic neurons after 5 days in culture.

Wildtype neurons (5 DIV) cultured in a microfluidic chamber were fixed and immunostained for tubulin (red) and the nuclear stain Hoechst (blue). The image (a composite of 40 individual 10X images stitched together) captures a beautiful, all-encompassing glimpse of healthy neurons cultured in a chamber.

Figure 2.3

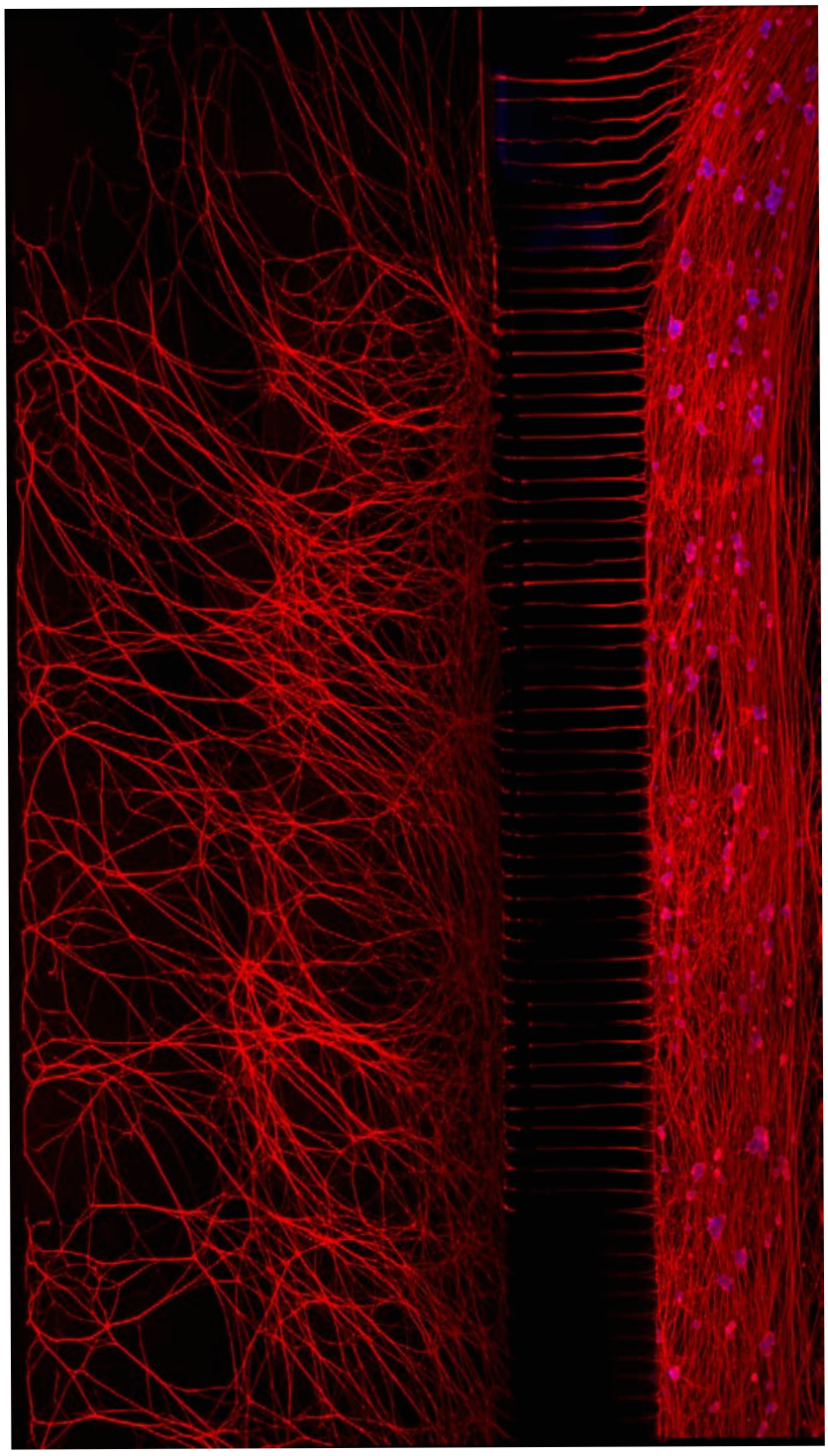
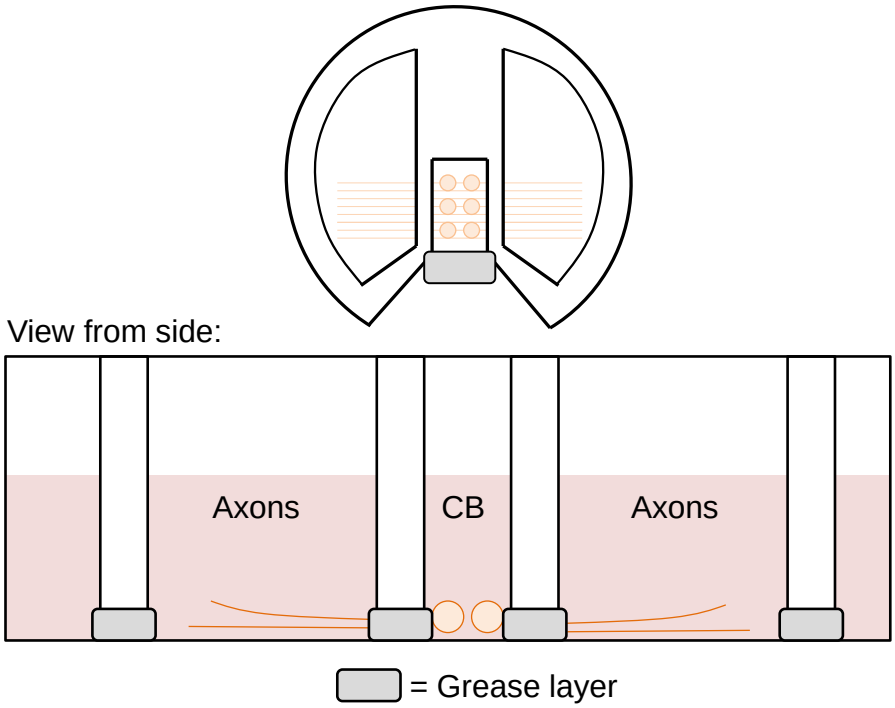


Figure 2.4: Campenot chambers permit the fluidic isolation of soma and axon.

Campenot chambers utilize a Teflon divider sealed to the bottom of a culture dish by a thin grease layer to fluidically isolate distal axons from cell bodies. Dissociated neurons are plated in the center compartment and their axons grow outwards, extending through the dividing grease layer into the side compartments.

Figure 2.4



CHAPTER THREE: DISTINCT PATHWAYS MEDIATE AXON DEGENERATION DURING APOPTOSIS AND AXON-SPECIFIC PRUNING

3.1 Overview

Neurons can activate pathways that destroy the whole cell *via* apoptosis or selectively degenerate only the axon (pruning). Both apoptosis and axon degeneration require Bax and caspases. Despite this overlap, we demonstrate that the pathways mediating axon degeneration during apoptosis *versus* axon pruning are distinct. While caspase-6 is activated in axons following nerve growth factor (NGF) deprivation, microfluidic chamber experiments reveal that caspase-6 deficiency only protects axons during axon-specific but not whole-cell (apoptotic) NGF deprivation. Strikingly, axon-selective degeneration requires the apoptotic proteins Caspase-9 and Caspase-3 but, in contrast to apoptosis, not Apaf-1. Additionally, cell bodies of degenerating axons are protected from caspase activation by proteasome activity and XIAP. Also, mature neurons restrict apoptosis but remain permissive for axon degeneration, further demonstrating the independent regulation of these two pathways. These results reveal how neurons allow for precise control over apoptosis and axon-selective degeneration pathways, thereby permitting long-term plasticity without risking neurodegeneration.

3.2 Introduction

Neurons execute one of two critical self-destruct programs to properly shape the developing nervous system: apoptosis, which destroys the entire neuron, and axon degeneration, which removes unnecessary axon branches to refine neuronal connections (Raff, Whitmore et al. 2002; Wang, Medress et al. 2012). While extensive neuronal apoptosis occurs during development (Yuan and Yankner 2000), the apoptotic pathway becomes highly restricted once neurons are integrated in the nervous system, enabling neurons to survive for the lifetime of the organism (Lazarus, Bradshaw et al. 1976; Easton, Deckwerth et al. 1997; Yakovlev, Ota et al. 2001; Kole, Swahari et al. 2011). In contrast, the selective pruning of axons is critical not only for establishing specific neuronal circuitry during development but also for permitting plasticity in the adult nervous system (Bagri, Cheng et al. 2003; Luo and O'Leary 2005; Low and Cheng 2006). Although axons are destroyed during both apoptosis and axon-specific degeneration, whether exactly the same pathway mediates axonal destruction in these two contexts has not been specifically examined.

The key mediators of the apoptosis pathway are caspases, a family of cysteine proteases that cleave specific cellular substrates to cause rapid cell death (Denault and Salvesen 2002). Apoptotic signaling cascades converge on and activate Bax, a proapoptotic member of the Bcl-2 family, resulting in the release of cytochrome c (cyt *c*) from the mitochondria. Released cyt *c* binds to Apaf-1 (Apoptotic protease activating factor 1), which recruits procaspase-9 to form the apoptosome complex. Autoactivation of caspase-9 (Casp9) leads to the cleavage and activation of caspase-3 (Casp3), resulting in cell death. Caspase activation in neurons is strictly regulated by XIAP (X-linked inhibitor of apoptosis

protein), which binds to and inhibits cleaved Casp9 and Casp3. Thus, inactivation of XIAP is necessary for caspase-mediated apoptosis in neurons (Potts, Singh et al. 2003).

Any overlap between neuronal apoptosis and axon-specific degeneration was initially dismissed due to the lack of active Casp3 detection in degenerating axons and the inability of Casp3-specific inhibition to protect axons (Finn, Weil et al. 2000; Saxena and Caroni 2007; Nikolaev, McLaughlin et al. 2009). Interestingly, however, recent studies point to significant overlap between these two pathways. For example, the Bcl-2 family proteins have been shown to modulate axon degeneration as deficiency of Bax or overexpression of anti-apoptotic protein Bcl-XL both protect axons following axon-specific nerve growth factor (NGF) deprivation (Nikolaev, McLaughlin et al. 2009; Schoenmann, Assa-Kunik et al. 2010; Vohra, Sasaki et al. 2010). Furthermore, several caspases - specifically Casp9, Casp3, and caspase-6 (Casp6) - have now all been implicated in axon degeneration (Guo, Albrecht et al. 2004; Nikolaev, McLaughlin et al. 2009; Park, Grosso et al. 2010; Vohra, Sasaki et al. 2010; Akpan, Serrano-Saiz et al. 2011; Graham, Ehrnhoefer et al. 2011; Simon, Weimer et al. 2012; Uribe, Wong et al. 2012). These results raise several interesting points. If the effectors of apoptosis and axon degeneration are identical, how do neurons activate axon-selective degeneration without triggering apoptosis? Alternatively, are there key differences that remain unidentified that allow neurons to precisely activate one but not the other pathway?

In this study, we examine the molecular intersection between axon degeneration and apoptosis. We report that the pathways mediating axon degeneration in the context of apoptosis *versus* localized, axon-specific degeneration are distinct. We also find that activities of the proteasome and XIAP are important for restricting caspase activation to the

axons during axon-selective degeneration. Importantly, we show that mature neurons, which restrict apoptosis, are still capable of undergoing axon-specific degeneration, further supporting our conclusion that neuronal apoptosis and axon degeneration are mediated by different mechanisms. The distinct regulation of these two pathways is essential for maintaining life-long neuronal plasticity without risking neurodegeneration.

3.3 Results

Casp6 is required for axon-selective pruning but not apoptosis:

As Casp6 has emerged as an important mediator of axon degeneration, we examined whether Casp6 is activated in multiple models of axon degeneration. We subjected whole cultures of primary mouse sympathetic neurons to various insults *in vitro*, including NGF deprivation, DNA damage with etoposide, ER stress with tunicamycin, and microtubule destabilization with vinblastine. Consistent with recent reports (Vohra, Sasaki et al. 2010), despite the ability of all treatments to induce extensive axon degeneration (Fig. 3.1a), we detected robust axonal Casp6 staining selectively following NGF deprivation but not other insults (Fig. 3.1b). NGF deprivation is known to induce apoptosis in neurons resulting in both soma and axon degeneration. To test whether Casp6 was required for axon degeneration in this model, we isolated neurons from wildtype and Casp6-deficient mice and deprived them of NGF. Surprisingly, Casp6 deficiency did not prevent axon degeneration in NGF-deprived neurons; Casp6-deficient neurons underwent apoptosis and exhibited soma and axon degeneration just as seen with wildtype neurons (Fig. 3.2a).

These results were unexpected because recent studies have shown that Casp6 is required for axon degeneration (Nikolaev, McLaughlin et al. 2009; Simon, Weimer et al. 2012). However, in these studies, neurons were cultured in compartmentalized chambers and NGF was withdrawn from only the axons. Thus, we examined whether neurons activate a Casp6-dependent or -independent pathway to degenerate axons based on the spatial localization of NGF deprivation signal. We generated microfluidic chambers that permit the physical separation and fluidic isolation of soma and distal axons (Taylor, Blurton-Jones et al. 2005). The microfluidic chambers allow local NGF withdrawal from distal axons in the

axon compartment while maintaining NGF on the cell bodies and proximal axons in the soma compartment (Fig. 3.2b). This axon-specific NGF withdrawal, termed *local deprivation*, results in axon-specific degeneration in wildtype neurons within 24-48 hours. Alternatively, we can also withdraw NGF from both compartments, termed *global deprivation*, to induce whole-cell degeneration *via* apoptosis.

Using microfluidic chambers to clearly distinguish axon degeneration caused by apoptosis *versus* that during axon-specific NGF deprivation, we subjected wildtype and Casp6-deficient neurons to global and local deprivation. Our results show that while Casp6 deficiency is indeed able to block axon degeneration with local deprivation, it is incapable of doing so with global deprivation (Figs. 3.2c,d; Supplemental Fig. 3.1). These results indicate that the Casp6-mediated pathway of axon degeneration induced during local deprivation is distinct from the pathway utilized by dying neurons to degenerate their axons. Thus, neurons appear to have Casp6-dependent and Casp6-independent pathways to degenerate their axons, and the specific location of NGF withdrawal (axon *versus* whole cell) determines which pathway becomes activated.

Axon pruning is Apaf-1-independent but requires Casp9 and Casp3:

The requirement of Casp6 in the axon-selective degeneration pathway but not apoptosis indicates that these two pathways are distinct. However, deficiency of Bax protects against *both* neuronal apoptosis and local deprivation-induced axon degeneration (Nikolaev, McLaughlin et al. 2009; Schoenmann, Assa-Kunik et al. 2010). Thus, the apoptotic pathway intersects with the axon degeneration pathway. Since death receptor 6 (DR6) has been implicated in Casp6 activation in axon degeneration (Nikolaev, McLaughlin

et al. 2009), we first examined whether the death receptor apoptotic pathway, also known as the extrinsic pathway, was important for axon degeneration. In the extrinsic pathway, activation of caspase-8 can either directly activate Casp3 or cleave Bid, a proapoptotic member of the Bcl-2 family of proteins, which then activates Bax (Thorburn 2004). However, neither Bid-deficiency nor caspase-8-specific inhibition blocked axon degeneration during local deprivation (Supplemental Figs. 3.2a,b).

We next tested whether components of the mitochondrial apoptotic pathway are critical for axon-selective degeneration using mice deficient in the key components of this pathway. As anticipated, Bax-deficient axons were protected during local deprivation and lacked Casp6 activation (Figs. 3.3a-c). Both Casp9 and Casp3 were also required for axon degeneration following local deprivation as axons deficient for these respective caspases were protected (Figs. 3.3a-c). Indeed, we detected active Casp3 in locally deprived wildtype axons, indicating that Casp3 is activated during axon-selective degeneration (Fig. 3.3b). Interestingly, we also examined the status of cleaved Casp6 in these knockout neurons that were locally deprived of NGF. While no active Casp6 staining was detected in locally-deprived Bax- or Casp9-deficient neurons, we observed positive staining in the Casp3-deficient neurons, albeit to a reduced extent than seen in wildtype neurons (Fig. 3.3a). These results suggest that Casp6 cleavage occurs downstream of Bax and Casp9. Importantly, Casp3 appears to be required for full Casp6 activation in locally deprived axons.

Next, we examined the importance of the key apoptotic protein Apaf-1 in axon degeneration. Similar to neurons deficient for Bax, Casp9, and Casp3, neurons deficient for Apaf-1 are protected from apoptosis induced by NGF deprivation (Wright, Smith et al.

2007). Strikingly, Apaf-1 deficiency did not block axon-selective degeneration following local deprivation (Fig. 3.3, Supplemental Fig. 3.2c). Similar to wildtype neurons, robust Casp6 activation was also seen in Apaf-1-deficient neurons (Fig. 3.3a). These results indicate that while axon-selective degeneration is Casp9- and Casp3-dependent, it is Apaf-1-independent.

As active Bax is known to trigger mitochondrial cyt *c* release during apoptosis, we also examined the status of cyt *c* in deprived axons. Control axons in the presence of NGF maintained cyt *c* in the mitochondria and global deprivation triggered its release (as indicated by the loss of cyt *c* staining). Our results show that local deprivation also induced the release of cyt *c* from mitochondria in the axon compartment (Fig. 3.4).

Together, these results show that axon degeneration during apoptosis *versus* axon-selective pruning have some overlap but also key differences. While both pathways require Bax, Casp9, and Casp3, they diverge at the requirements of Casp6 (required only for axon-selective pruning) and Apaf-1 (required only for axon degeneration during apoptosis).

XIAP and the proteasome protect the soma during axon pruning:

The unexpected involvement of proapoptotic caspases in axon degeneration raises a key question of how neurons can limit caspase activity to axons without increasing the risk of apoptosis at the soma. While some have reported proteasome inhibition to inhibit axon degeneration (Zhai, Wang et al. 2003; MacInnis and Campenot 2005) others have reported the opposite effect (Laser, Mack et al. 2003; Lang-Rollin, Vekrellis et al. 2004). Recently, Casp6 activity was reported to be enhanced by proteasome inhibition (Tounekti, Zhang et al. 2004; Gray, Mahrus et al. 2010). Indeed, we detected Casp6 activation and axon

degeneration at an earlier timepoint when proteasome activity was inhibited with Bortezomib (Fig. 3.5a; Supplemental Fig. 3.3a) or MG132 (data not shown). Equally striking, proteasome inhibition made the cell bodies of locally deprived axons more permissive for caspase activation; significantly more Casp3- and Casp9-positive cell bodies were observed in locally deprived, proteasome-inhibited chambers than those that had been locally deprived without proteasome inhibitor (Figs. 3.5b,c). Proteasome inhibition alone did not induce Casp3 activation in NGF-maintained neurons (Fig. 3.5a). Also, the increased axon degeneration seen with proteasome inhibition after local deprivation was Casp3-dependent, indicating that this degeneration was not due to non-specific toxicity of proteasome inhibition (Fig. 3.5d).

Another factor that can restrict caspase activation during axon-specific degeneration is XIAP, which is a potent endogenous inhibitor of caspases in neurons (Potts, Singh et al. 2003). Due to the requirement of two critical targets of XIAP (Casp9 and Casp3) for axon degeneration, we predicted that XIAP-deficient neurons may be more vulnerable to caspase-mediated degeneration during local deprivation. Indeed, XIAP-deficient neurons degenerated their axons earlier than wildtype neurons (Fig. 3.6a; Supplemental Fig. 3.3b). Also, significantly more Casp3 activation was observed in the cell bodies of locally deprived XIAP-deficient neurons compared to wildtype neurons (Fig. 3.6b). These results show that caspase activation during axon-selective degeneration is spatially restricted by the activities of the proteasome and XIAP.

Mature neurons inhibit apoptosis but permit axon pruning:

Our results thus far show that the axon degeneration and apoptosis pathways are distinct but share several key components. One question this raises is whether neurons can shut off apoptosis while maintaining the ability to undergo axon-selective degeneration. Mature neurons provide an excellent model to examine this point because neurons are known to restrict the apoptotic pathway with maturation, in part by turning off the expression of Apaf-1 (Wright, Smith et al. 2007). Specifically, we and others have shown that global NGF deprivation induces apoptosis in young (5 DIV) but not mature (28 DIV) neurons (Fig. 3.7a)(Lazarus, Bradshaw et al. 1976; Easton, Deckwerth et al. 1997; Yakovlev, Ota et al. 2001; Kole, Swahari et al. 2011).

To examine whether mature neurons remain permissive for axon-selective degeneration despite shutting down their apoptosis pathway, we subjected young and mature neurons to local NGF deprivation. Our results show that mature neurons are fully capable of activating Casp6 and undergoing axon-selective degeneration as removal of NGF from just the axons triggered axon degeneration (Figs. 3.7b-d). These results are particularly striking since removal of NGF from the entire mature neuron (soma and axons) induces neither apoptosis nor axon degeneration (Fig. 3.7a). Thus, the ability of mature neurons to be permissive or resistant to axon degeneration depends not only on whether NGF is deprived from the axons, but also whether it is present or absent in the soma.

Taken together, our results have identified key differences in the axon degeneration pathway depending on whether the context is apoptosis or axon-selective pruning (Fig. 3.8).

3.4 Discussion

Neurons have the unique capability to activate pathways that cause degeneration of either the entire cell or only the axons (Hyman and Yuan 2012). The picture that is emerging is that neurons engage multiple pathways for triggering axon degeneration that is stimuli-specific. Our results now show that even in the context of one stimulus (*e.g.* NGF deprivation), the spatial origin of the stimuli is a key determining factor for activating a specific axon degeneration pathway. We find Casp6 to be activated during axon degeneration but selectively in the context of NGF deprivation. Indeed, axon degeneration after axotomy, termed Wallerian degeneration (Coleman and Freeman 2010), and axon degeneration induced by other insults such as microtubule destabilization are likely mediated by an alternate, Casp6-independent mechanism (Vohra, Sasaki et al. 2010; Simon, Weimer et al. 2012). The observation that neurons activate a specific pathway for axon degeneration that responds selectively to NGF deprivation is consistent with the relevance of NGF as a physiological factor that regulates sympathetic neuron innervations (Glebova and Ginty 2005). Importantly, we find that even in the context of NGF deprivation, Casp6 is essential for axon degeneration only during axon-specific but not whole cell breakdown. Thus, axon degeneration appears to be critically dependent on Casp6 when the insult is physiologically relevant and spatially localized to axons.

Our results also make the critical distinction between axon degeneration that occurs during apoptosis *versus* axon-selective pruning. While we and others show that Bax, Casp9, and Casp3 are required for axon degeneration (Nikolaev, McLaughlin et al. 2009; Schoenmann, Assa-Kunik et al. 2010; Vohra, Sasaki et al. 2010; Akpan, Serrano-Saiz et al. 2011; Simon, Weimer et al. 2012), we report the novel finding that Apaf-1 is not required

for this process. These results were unexpected because the major known mechanism for activating Casp9 is *via* Apaf-1. Incidences of Apaf-1-independent Casp9 activation have been reported in non-neuronal models, yet the mechanism remains unknown (Ho and Zacksenhaus 2004; Nagasaka, Kawane et al. 2010; Manns, Daubrawa et al. 2011). Our results showing that mature neurons, which lack Apaf-1 (Wright, Smith et al. 2007), were fully capable of axon degeneration further supports the model of axon-selective pruning as an Apaf-1-independent process. Triggering the complete apoptotic pathway in axons can be especially dangerous for neurons, which are post-mitotic cells that must survive long-term. Thus, neurons may utilize a non-apoptosome mechanism of activating Casp9 during axon pruning to tightly restrict the direct activation of Casp3, therefore avoiding the risk of a lethal and widespread apoptotic caspase cascade.

Increasing evidence indicates that neurons utilize Bax, Casp9, and Casp3 for synaptic plasticity (Li and Sheng 2012). We predict that caspase activation under these non-apoptotic conditions may also occur by a pathway that is Apaf-1-independent. Also, while we find cyt *c* to be released from the mitochondria in degenerating axons, determining whether it is required for Apaf-1-independent Casp9 activation needs further investigation. Experiments that knock-down cyt *c* are problematic due to the essential function of cyt *c* in mitochondrial respiration. However, one potential alternative approach would be to introduce neutralizing antibodies to cyt *c* into axons and assess whether axonal degeneration is prevented.

The exact non-apoptosome mechanism by which Casp9 activates the downstream caspases, Casp6 and Casp3, remains unclear. A recent study found Casp3 deficiency to block Casp6 activation, placing Casp6 downstream of Casp3 in this pathway (Simon,

Weimer et al. 2012). While we found Casp6 activation to be reduced in Casp3-deficient axons during local deprivation, it was nevertheless detectable (Fig. 3.3a). Importantly, this reduced level of Casp6 activation was incapable of inducing axon degeneration as axonal integrity was maintained in the locally-deprived Casp3-deficient neurons. Thus, while Casp3 appears to be needed for the full activation of Casp6 during axon pruning, the exact mechanism of this caspase activation appears more complex than a linear pathway.

Our results also identify the proteasome and XIAP as two mechanisms by which neurons limit caspase activity during axon-selective degeneration. Casp6 is a known target of the proteasome (Tounekti, Zhang et al. 2004; Gray, Mahrus et al. 2010) and endogenous XIAP is a potent inhibitor of caspases in neurons (Potts, Singh et al. 2003). The protective function of XIAP in the cell body during axon-specific degeneration is similar to the mechanism in Drosophila where DIAP (the insect homolog of XIAP) regulates caspase activity during developmental dendritic pruning (Kuo, Zhu et al. 2006; Williams, Kondo et al. 2006). Our results indicate that neurons have evolved multiple mechanisms for restricting axonal caspase activity in order to remodel axons without risking long-term survival.

Extensive neuronal apoptosis and axon pruning are both required to establish specific neuronal circuitry during development (Yuan and Yankner 2000; Low and Cheng 2006). However, whereas the apoptotic pathway becomes highly restricted once neurons are integrated into the nervous system to enable neurons to survive for the lifetime of the organism (Lazarus, Bradshaw et al. 1976; Easton, Deckwerth et al. 1997; Yakovlev, Ota et al. 2001; Kole, Swahari et al. 2011), the selective pruning of axons is continually required to permit plasticity in the adult nervous system (Bagri, Cheng et al. 2003; Luo and O'Leary

2005; Low and Cheng 2006). Indeed, we find mature neurons to fully restrict apoptosis yet remain capable of undergoing axon-specific degeneration. Aberrant activation of axon pruning mechanisms in the mature nervous system could contribute to pathological axon degeneration in neurodegenerative disease. While inhibiting caspases could be a potential therapy in these situations, one concern is that chronic administration of caspase inhibitors may also inhibit physiological pruning and synaptic plasticity.

Together, our results reveal that neurons are exquisitely capable of discerning the spatial location of NGF signal loss and activating an appropriate response. Neurons appear to utilize essentially the same components for whole cell and axon-selective degeneration yet have evolved distinct mechanisms to allow precise spatial and temporal control over the activation of these pathways.

3.5 Materials and Methods

Mice:

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). Casp6-deficient mice were obtained from Jackson Labs. Mice deficient for Casp9, Apaf-1, or Casp3 were dissected at E16.5 due to the embryonic lethality often associated with these gene deficiencies at later ages. All knockout mice were on a C57BL/6 background, and wildtype littermates served as controls for all experiments.

Primary sympathetic neuronal cultures:

Primary sympathetic neurons were cultured as previously described (Potts, Singh et al. 2003). Briefly, sympathetic neurons were dissected from the superior cervical ganglia (SCG) of postnatal day (P) 0-1 CD1 mice. Cells were plated on collagen-coated dishes at a density of 10,000 cells per well or in microfluidic chambers (see section below) and maintained for 5 days *in vitro* (DIV) in NGF-containing medium (AM50). For NGF deprivation, cultures were rinsed three times with medium lacking NGF (AM0) and then maintained in NGF-deficient media containing a neutralizing antibody to NGF. Some neuronal cultures were treated with various compounds in the presence of NGF: 20 μM etoposide; 2.5 μM tunicamycin; 1 μM vinblastine. All reagents were obtained from Sigma unless otherwise indicated. All experiments were performed in triplicate.

Fabrication and use of microfluidic devices:

Standard photolithography was used to fabricate channel array devices for axon growth. Negative photoresist (KMPR 1010, MicroChem Corp., Newton, MA) was used to pattern arrays of 10-µm-wide, 3-um-deep grooves spaced 40 µm apart onto a standard silicon wafer (Silicon Quest International). The bare silicon around the pattern was etched approximately 3-µm deep using deep reactive ion etching (Alcatel AMS 100 Deep Reactive Ion Etcher) with a low-roughness Bosch process. The photoresist was then stripped using 2800 W oxygen plasma. The features for the large side compartments (750-µm long, 100-µm-deep, 1.0-mm-wide on each side) at either end of the central grooves were fabricated onto the wafer using negative photoresist (SU8-100, MicroChem Corp.) and standard photolithography using the manufacturer's recommended procedure. Sylgard 184 (Dow Corning) was prepared according to the manufacturer's recommended procedure and poured into the mold form. After curing at 65^0C overnight, individual PDMS chambers were cut out, sterilized in 70% EtOH, and placed upon glass coverslips coated with 40 µg/ml poly-D-lysine (BD Biosciences) and 0.1 µg/mL mouse laminin (Invitrogen).

Culture and treatment of primary neurons in microfluidic chambers:

Neurons were plated in microfluidic chambers as described previously (Taylor, Blurton-Jones et al. 2005) and maintained in AM50 until 5 DIV (or 28 DIV for mature experiments). To achieve *global deprivation* of NGF, both compartments were carefully rinsed three times with AM0 and then maintained in AM0 containing NGF-neutralizing antibodies. For *local deprivation* only the axon compartment was rinsed and deprived of NGF whereas the soma compartment was maintained in AM50. Chambers containing AM50

in both compartments served as controls. A 30 µL volume differential was established between the two compartments at the start of treatment and reset every 12-20 hours to maintain fluidic isolation (Taylor, Blurton-Jones et al. 2005). Where indicated, 25 µM zIETD-FMK (Casp8-specific inhibitor, SM Biochemicals) or 25 µM QVD (pan-caspase inhibitor, SM Biochemicals) was added at time of treatment. Also, 10 µM Bortezomib was added 8-10 hours into treatment to preclude any toxic effects resulting from longer proteasome inhibition.

Quantification:

The length of intact axons was measured both before and after treatment. The left edge of the chamber's microgrooves (where axons first enter the axon compartment) served as the starting point of measurement. Distinct markers were imprinted on the microgrooves during the chamber design and fabrication process to allow identification of the same axons and chamber regions over time. It is important to note that fragmented axons do not remain adhered to the glass coverslip; very slow media flow within the chamber compartment, coupled with the absence of a 'sticky' matrix such as collagen or methylcellulose, removes away the degenerated axon pieces. The microfluidic chamber system permits very clear detection of maintained, adherent, and intact axons versus degenerating and non-adherent axons (see phase time-course images in Fig. 3d and Fig. 7b). Therefore, we compared the length of intact axons both before and after treatment to determine the percentage of each measured axon that was maintained and, inversely, the percentage of each measured axon that was lost as a result of treatment-induced degeneration.

Immunofluorescence:

Immunofluorescence staining was carried out as previously described (Potts, Singh et al. 2003). Briefly, cultures were rinsed in phosphate-buffered saline, fixed in fresh 4% paraformaldehyde at room temperature (RT) for 20 minutes, and rinsed three times with Tris-buffered saline (TBS). After a 1 hour exposure to blocking solution (TBS containing 5% donkey serum and 0.3% Triton X-100), cells were treated with primary and secondary antibodies using standard methods and nuclei were labeled with Hoechst 33258 (1 μg/ml, Invitrogen). For knockout experiments, cells were fixed when neurons from wildtype littermates showed extensive axon degeneration following treatment. The following primary antibodies were used for immunostaining: cleaved Caspase-6 (SantaCruz, 1:200), cleaved Caspase-3 (Cell Signaling, 1:400), cleaved Caspase-9 (Cell Signaling, 1:400), cytochrome *c* (Molecular Probes, 1:400), Tom20 (Santa Cruz, 1:400), and tubulin (1:400). Tyramide Signal Amplification (TSA; Molecular Probes) was used according to manufacturer's instructions to visualize cleaved Casp3 in axons and cleaved Casp9.

Image acquisition and processing:

Images were acquired by an ORCA-ER digital B/W CCD camera (Hamamatsu) mounted on a DMIRE2 inverted fluorescence microscope (Leica) using Metamorph version 7.6 software (Molecular Devices).

Statistical Analysis:

All p values were calculated using an unpaired t-test.

3.6 Figures and Legends

Figure 3.1: Casp6 activation in axons is selective to NGF deprivation.

(**a**) Wildtype (WT) sympathetic neurons (5 DIV) were treated with the following conditions to induce axon degeneration, respectively: NGF deprivation (-NGF), 20 μM etoposide (DNA Damage), 2.5 μM tunicamycin (ER Stress), and 1 μM vinblastine (to induce microtubule destabilization). NGF-maintained neurons served as controls. Neurons were fixed and immunostained for tubulin upon axon beading and fragmentation. (**b**) Neurons were treated as described in (**a**) and immunostained for cleaved Casp3 and cleaved Casp6. Nuclei were labeled with Hoechst. Scale bar, 20 μm.

Figure 3.1

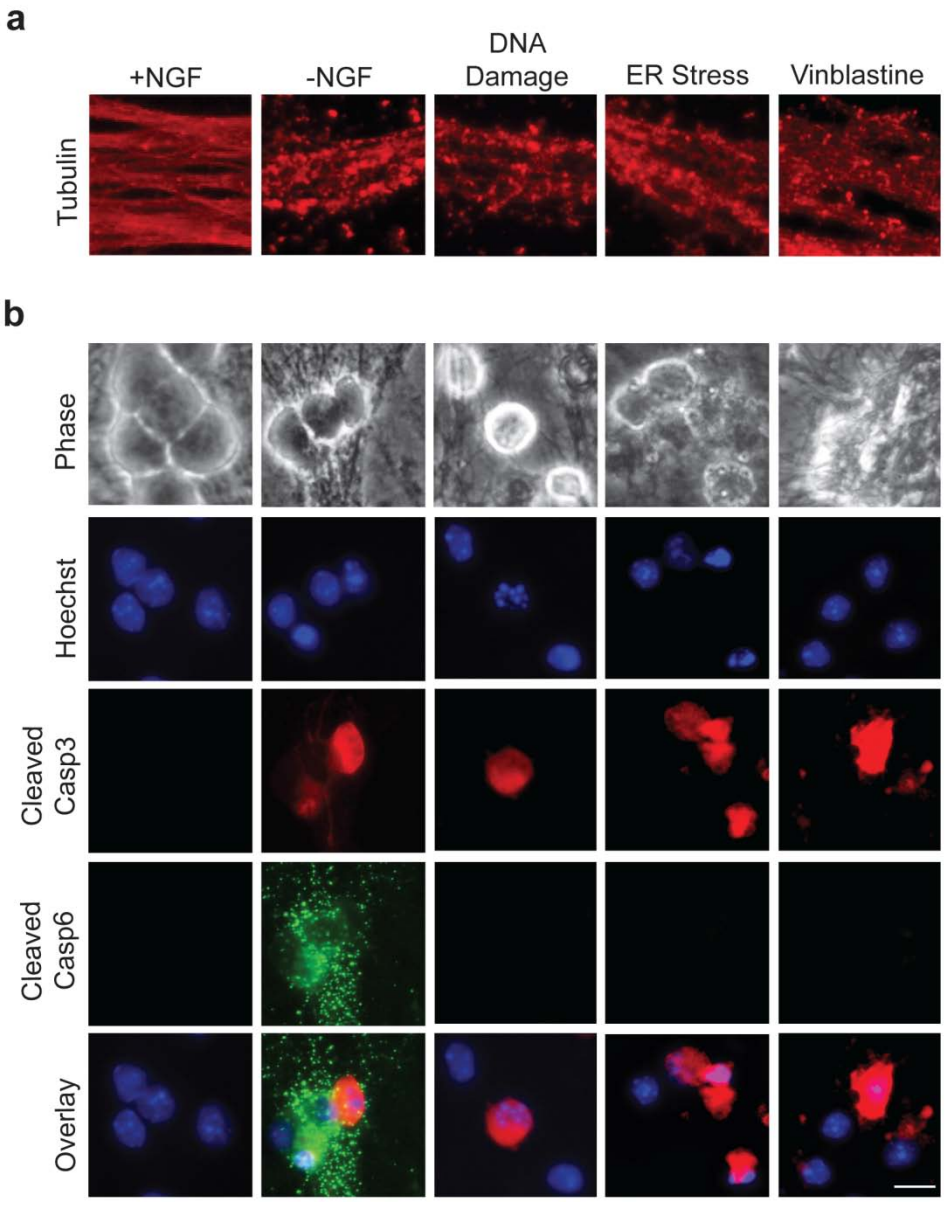

a
+NGF
-NGF
DNA Damage
ER Stress
Vinblastine
Tubulin
b
Phase
Hoechst
Cleaved Casp3
Cleaved Casp6
Overlay

Figure 3.2: Casp6 is required for axon degeneration during axon pruning but not apoptosis.

(**a**) WT and Casp6-deficient (Casp6 KO) neurons (5 DIV) were NGF-deprived for 24 hours. WT neurons maintained in NGF served as a control. Scale bar, 10 μm. (**b**) Microfluidic chamber model of global deprivation, which induces soma and axon degeneration, and local deprivation, which results in axon-selective degeneration. (**c**) WT and Casp6-deficient neurons were NGF-maintained, globally deprived, or locally deprived. Neurons were immunostained for tubulin and nuclei were labeled with Hoechst. Scale bar, 50 μm. (**d**) Quantification of axon degeneration for conditions shown in (**c**). Data represent the mean ± s.e.m. (n=3).

Figure 3.2

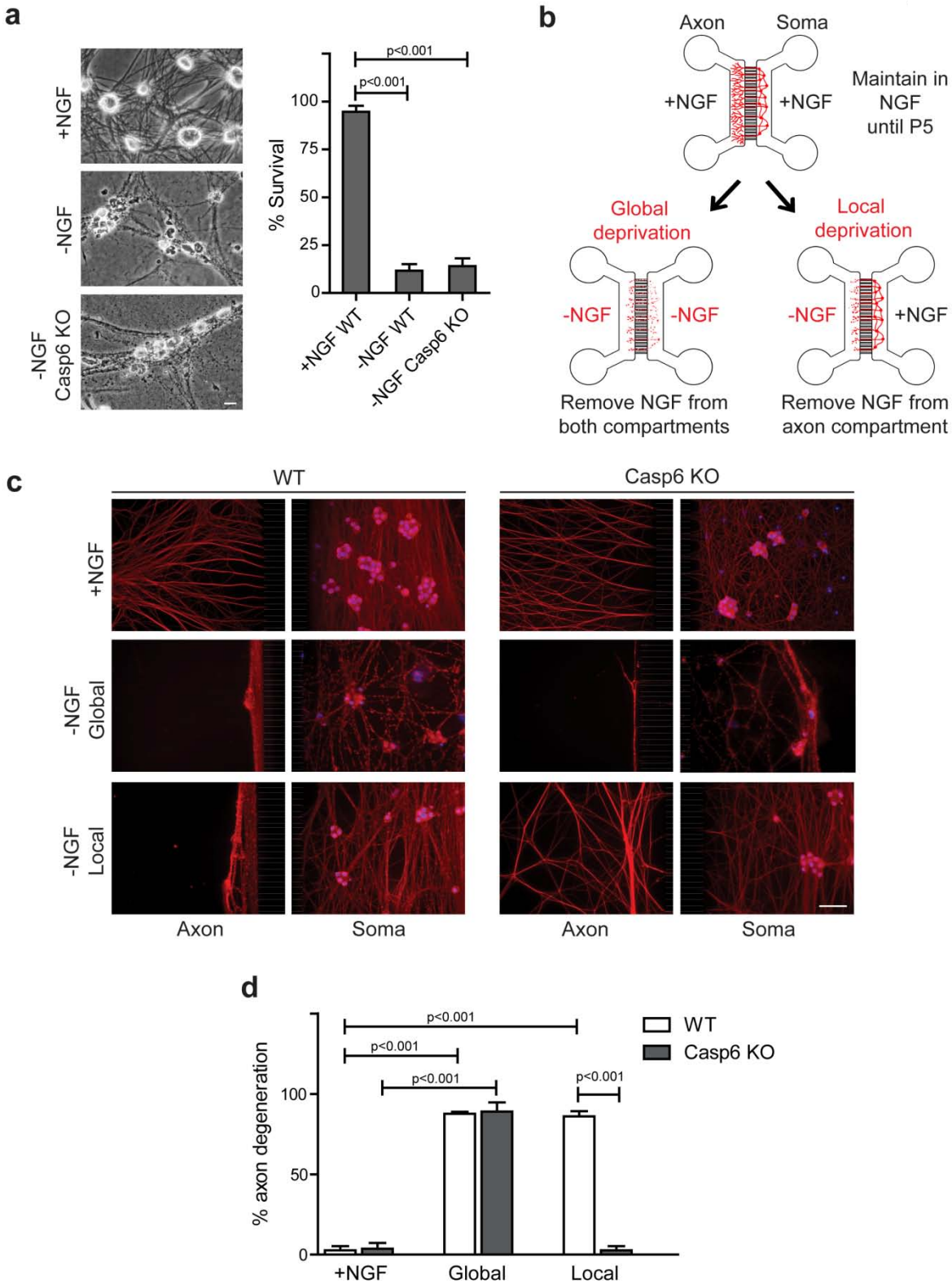

a
+NGF
-NGF
-NGF Casp6 KO
p<0.001
p<0.001
% Survival
100
75
50
25
0
+NGF WT
-NGF WT
-NGF Casp6 KO
b
Axon
Soma
+NGF
+NGF
Maintain in NGF until P5
Global deprivation
Local deprivation
-NGF
-NGF
-NGF
+NGF
Remove NGF from both compartments
Remove NGF from axon compartment
c
WT
Casp6 KO
+NGF
-NGF Global
-NGF Local
Axon
Soma
Axon
Soma
d
p<0.001
p<0.001
p<0.001
p<0.001
WT
Casp6 KO
% axon degeneration
100
50
0
+NGF
Global
Local

Figure 3.3: Axon-selective degeneration is Apaf-1-independent but requires Casp9 and Casp3.

(**a**) Sympathetic neurons (5 DIV) from mice deficient for Bax, Apaf-1, Casp9, Casp6, and Casp3 were locally deprived in microfluidic chambers. NGF-maintained and locally deprived WT littermate neurons served as controls. Neurons were immunostained for tubulin and cleaved Casp6. Scale bar, 50 μm. (**b**) WT neurons were locally deprived for 24 hours and probed for cleaved Casp3. NGF-maintained neurons served as controls. (**c**) Quantification of axon degeneration for conditions shown in (**a)**. (**d**) Quantification of maintained axon length over time during local deprivation for all conditions shown in (**a**). All data represent the mean ± s.e.m. (n=3). (**e**) Phase images of the same axons over a 36-hour timecourse of local deprivation demonstrate similar degeneration kinetics between WT and Apaf-1-deficient axons.

Figure 3.3

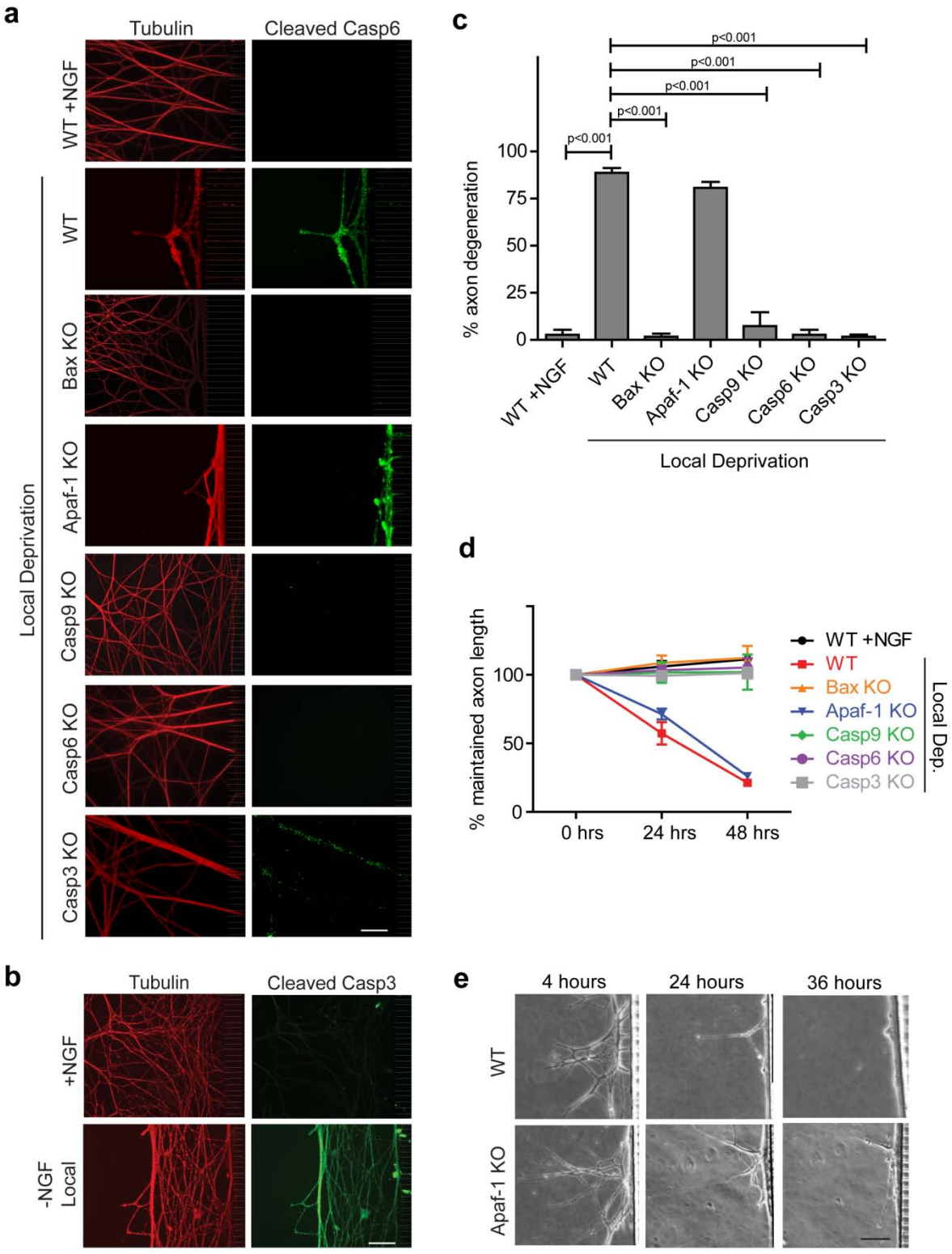

a
Tubulin
Cleaved Casp6
WT +NGF
WT
Bax KO
Apaf-1 KO
Casp9 KO
Casp6 KO
Casp3 KO
Local Deprivation
b
Tubulin
Cleaved Casp3
+NGF
-NGF
Local
c
p<0.001
p<0.001
p<0.001
p<0.001
p<0.001
% axon degeneration
100
75
50
25
0
WT +NGF
WT
Bax KO
Apaf-1 KO
Casp9 KO
Casp6 KO
Casp3 KO
Local Deprivation
d
% maintained axon length
100
50
0
0 hrs
24 hrs
48 hrs
WT +NGF
WT
Bax KO
Apaf-1 KO
Casp9 KO
Casp6 KO
Casp3 KO
Local Dep.
e
4 hours
24 hours
36 hours
WT
Apaf-1 KO

Figure 3.4: Local deprivation triggers cyt *c* release in axons.

Neurons (5 DIV) were NGF-maintained, globally deprived, or locally deprived in the presence of pan-caspase inhibitor (25 μM QVD) for 36 hours and probed for cyt *c* and the mitochondrial marker Tom20. Quantification of the colocalization index (ImageJ) for cyt *c* and Tom20 is shown below. Data represent the mean ± s.e.m. (n=3).

Figure 3.4

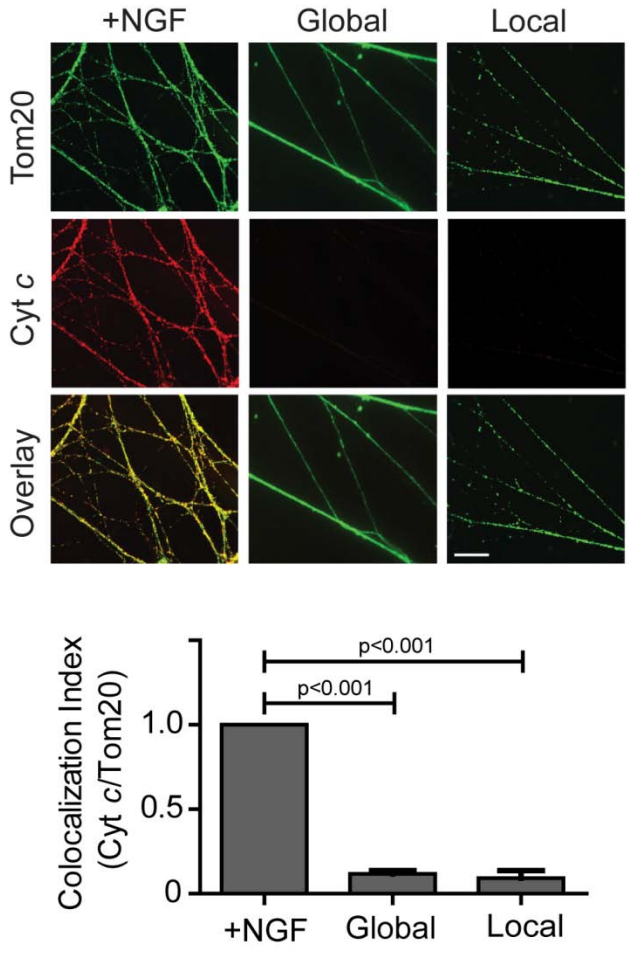

+NGF
Global
Local
Tom20
Cyt c
Overlay
Colocalization Index
(Cyt c/Tom20)
p<0.001
p<0.001
1.0
0.5
0
+NGF
Global
Local

Figure 3.5: The proteasome restricts caspase activation to axons during axon-specific degeneration.

(**a**) WT neurons were NGF-maintained or locally deprived in the absence (-) or presence (+) of the proteasome inhibitor Bortezomib. Neurons were immunostained for cleaved Casp6 and tubulin (inset). Scale bar, 50 μm. Quantification of axon degeneration is shown on the right. (**b**) Cell bodies of neurons treated as described in (**a**) were immunostained for cleaved Casp3. Nuclei were labeled with Hoechst. Scale bar, 20 μm. Quantification of cleaved Casp3-positive cell bodies is shown on the right. (**c**) Cell bodies of neurons treated as described in (**a**) were immunostained for cleaved Casp9 and nuclei were labeled with Hoechst. Scale bar, 20 μm. Quantification of cleaved Casp9-positive cell bodies is shown on the right. (**d**) WT and Casp3-deficient neurons were locally deprived with (+) and without (-) Bortezomib and probed for tubulin. NGF-maintained neurons served as controls. Scale bar, 50 μm. Quantification of axon degeneration is shown on the right. All data represent the mean ± s.e.m. (n=3).

Figure 3.5

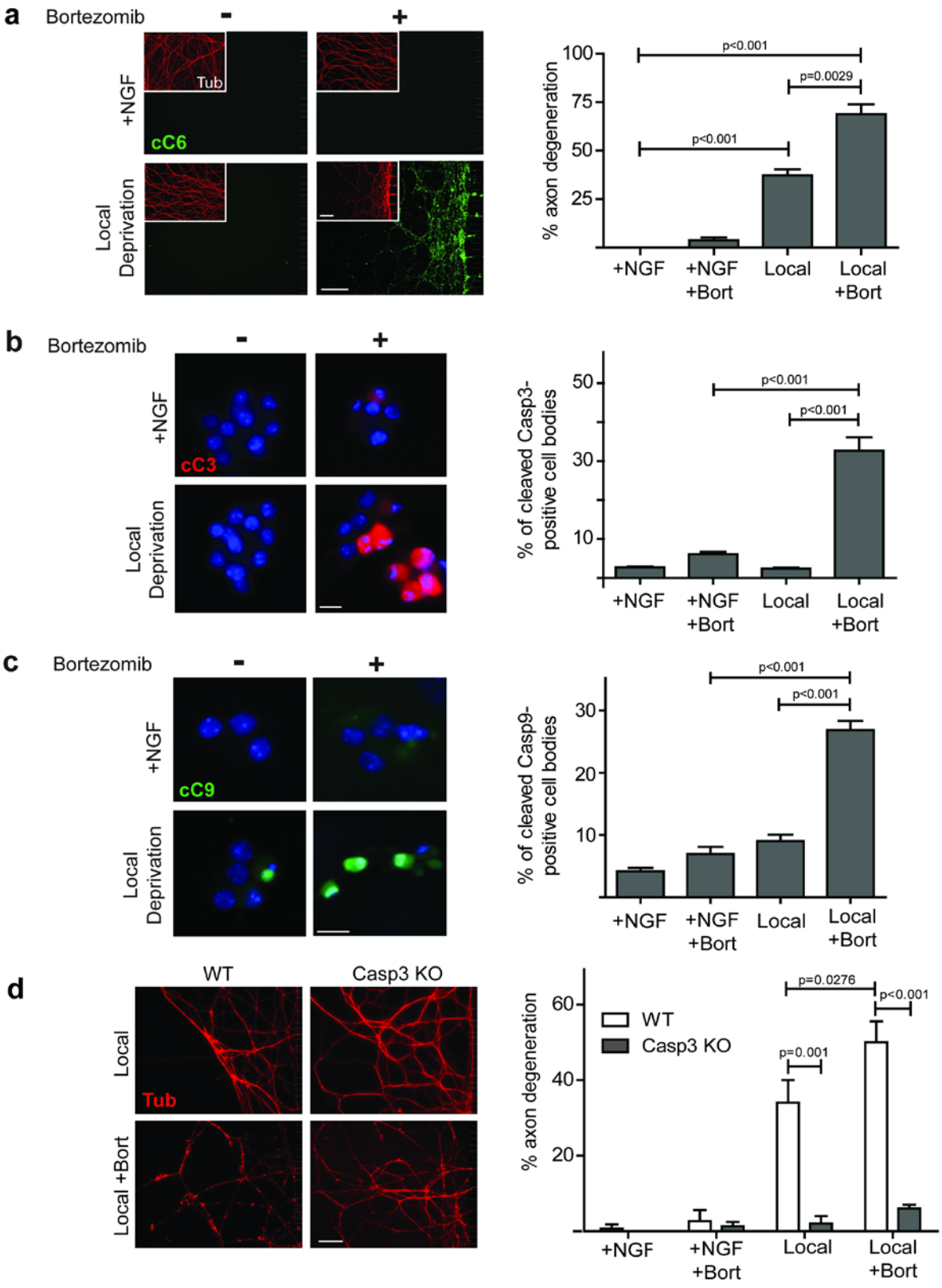

a
Bortezomib
-
+
+NGF
Local Deprivation
Tub
cC6
% axon degeneration
p<0.001
p=0.0029
p<0.001
+NGF
+NGF +Bort
Local
Local +Bort
b
cC3
% of cleaved Casp3-positive cell bodies
c
cC9
% of cleaved Casp9-positive cell bodies
d
WT
Casp3 KO
Local
Local +Bort
Tub
p=0.0276
p<0.001
p=0.001

Figure 3.6: XIAP protects the soma from caspase activation during axon-specific degeneration.

(**a**) WT or XIAP-deficient neurons were locally deprived and probed for cleaved Casp6. Arrows indicate early signs of axonal collapse. Scale bar, 50 μm. Quantification of axon degeneration is shown on the right. (**b**) WT and XIAP-deficient neurons were NGF-maintained or locally deprived and cell bodies were probed for cleaved Casp3. Nuclei were labeled with Hoechst. Scale bar, 20 μm. Quantification of the percentage of cell bodies positively labeled for cleaved Casp3 is shown on the right. All data represent the mean ± s.e.m. (n=3).

Figure 3.6

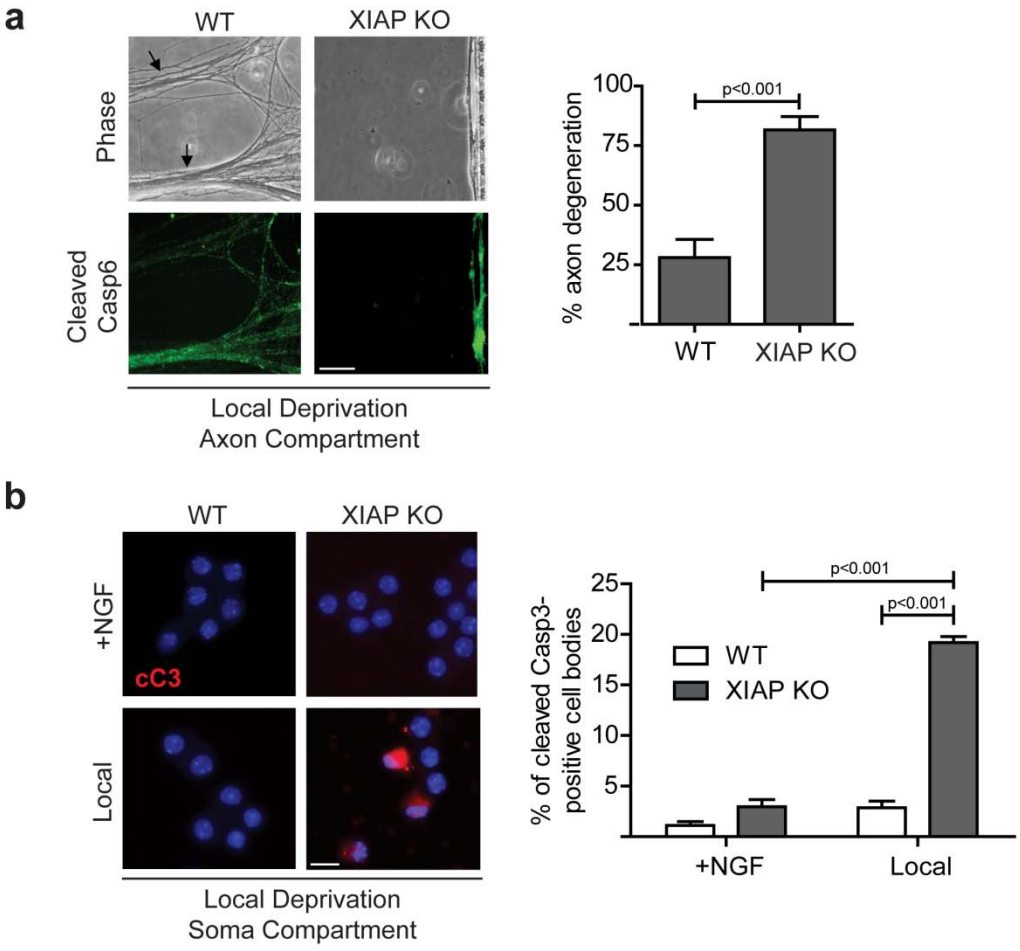

a
WT
XIAP KO
Phase
Cleaved Casp6
Local Deprivation
Axon Compartment
% axon degeneration
p<0.001
100
75
50
25
WT
XIAP KO
b
WT
XIAP KO
+NGF
cC3
Local
Local Deprivation
Soma Compartment
% of cleaved Casp3-positive cell bodies
p<0.001
p<0.001
25
20
15
10
5
WT
XIAP KO
+NGF
Local

Figure 3.7: Mature neurons inhibit apoptosis during global deprivation but degenerate axons during local deprivation.

(**a**) Young (5 DIV) and mature (28 DIV) sympathetic neurons were either NGF-maintained or NGF-deprived for 48 hours. Scale bar, 20 μm. (**b**) The same axons of young and mature neurons were imaged every 24 hours during local deprivation. NGF-maintained neurons at both ages served as controls. Scale bar, 50 μm. (**c**) Quantification of axon degeneration for conditions shown in (**b**). Data represent the mean ± s.e.m. (n=3). (**d**) Cleaved Casp6 immunostaining and corresponding phase images (inset) in the axon compartments of conditions shown in (**b**).

Figure 3.7

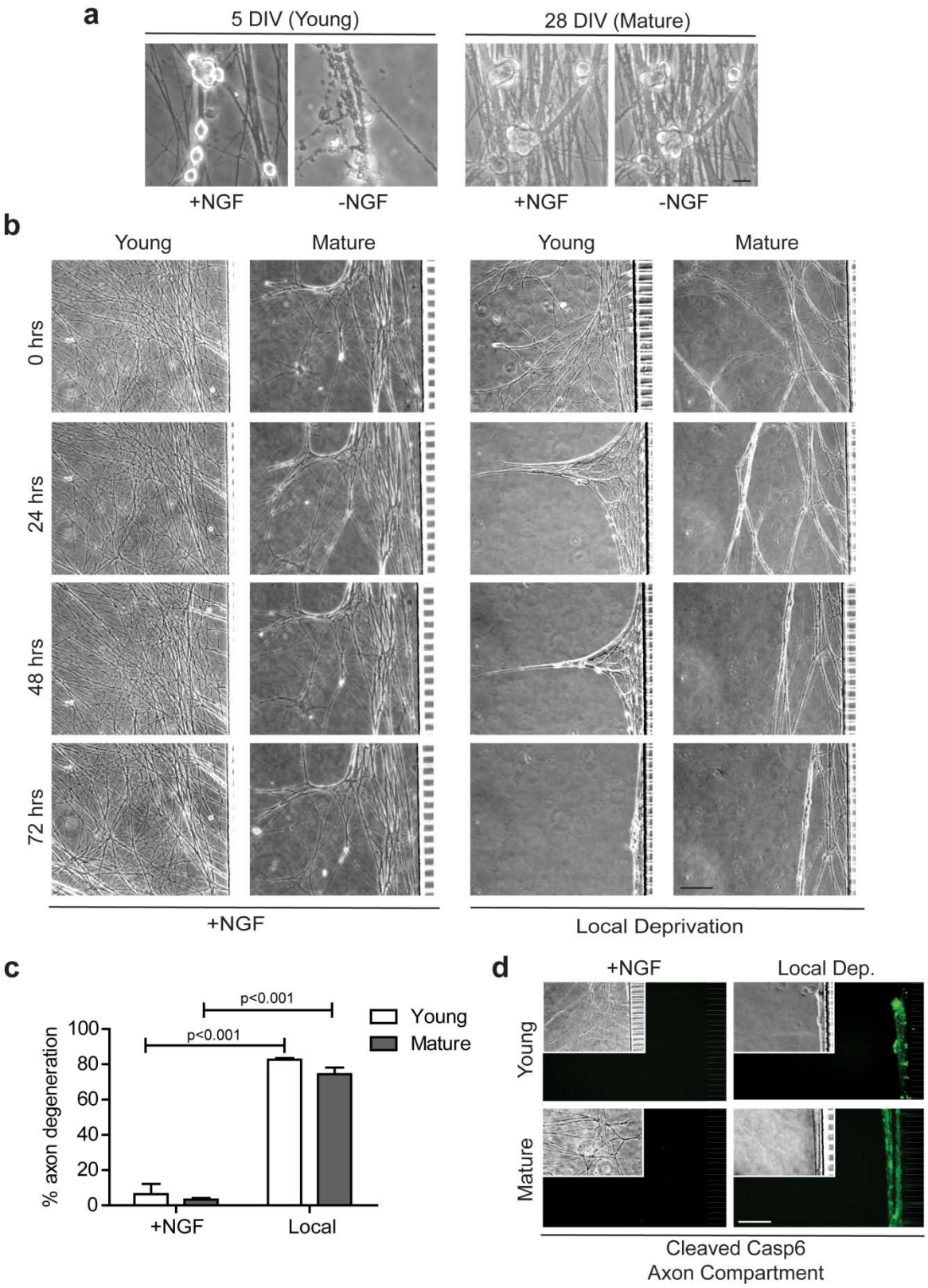
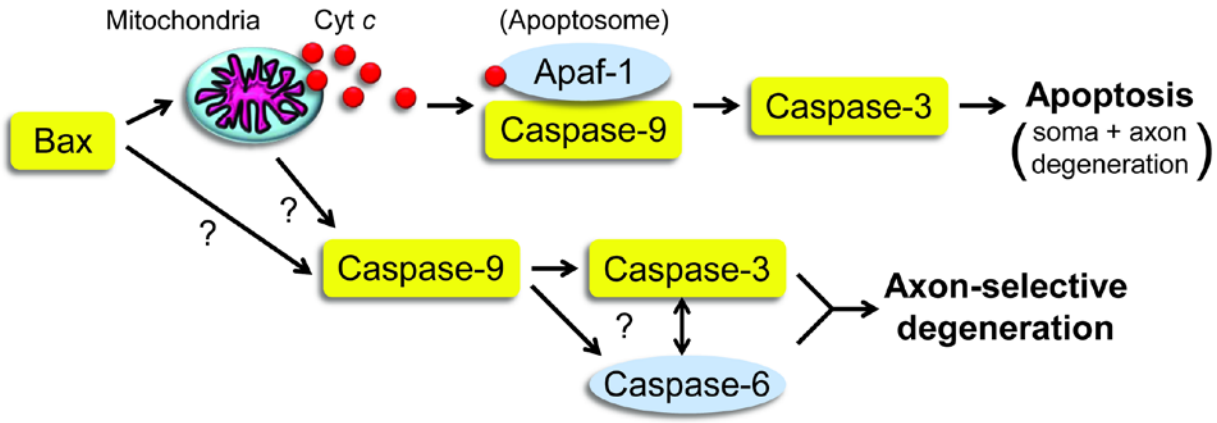

a
5 DIV (Young)
28 DIV (Mature)
+NGF
-NGF
+NGF
-NGF
b
Young
Mature
Young
Mature
0 hrs
24 hrs
48 hrs
72 hrs
+NGF
Local Deprivation
c
p<0.001
p<0.001
Young
Mature
% axon degeneration
100
80
60
40
20
0
+NGF
Local
d
+NGF
Local Dep.
Young
Mature
Cleaved Casp6
Axon Compartment

Figure 3.8: Working model of the pathways mediating apoptosis *versus* axon pruning.

Proteins highlighted in yellow have been shown to be required for both apoptosis and axon pruning pathways. Our results indicate that Apaf-1 and Caspase-6 (highlighted in blue) are two points of divergence. Future experiments will be required to determine what role, if any, is played by the mitochondria and cyt *c* release in axon-selective degeneration. In addition, how Caspase-9 is able to activate Caspase-6 and Caspase-3 without Apaf-1 remains to be determined. While Casp6 and Casp3 are both required for axon pruning, their exact order of activation during axon pruning in neurons is still unclear.

Figure 3.8

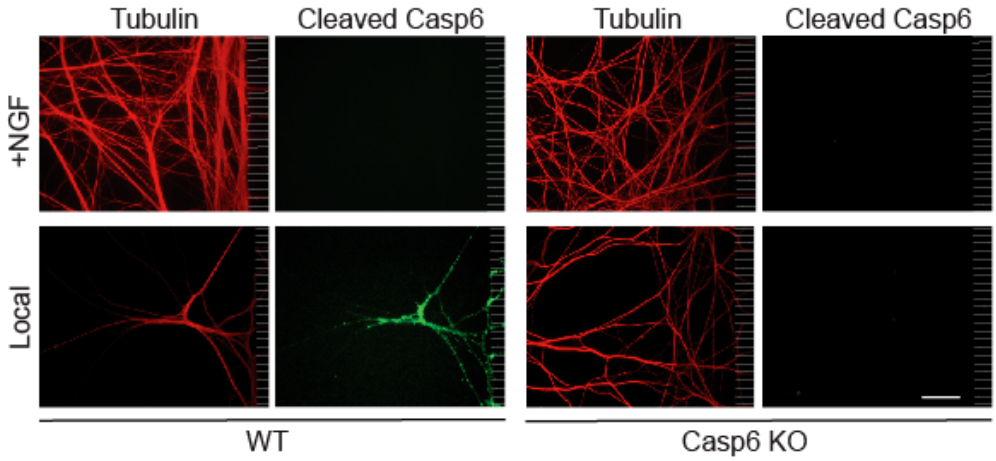

Mitochondria
Cyt c
(Apoptosome)
Apaf-1
Caspase-9
Caspase-3
Apoptosis
soma + axon
degeneration
Bax
?
?
Caspase-9
Caspase-3
?
Caspase-6
Axon-selective
degeneration

3.7 Supplemental Figures

Supplemental Figure 3.1: Casp6 activation in axons is selective to NGF deprivation.

WT and Casp6-deficient neurons (5 DIV) were locally deprived and probed for cleaved Casp6 and tubulin. Scale bar, 50 μm.

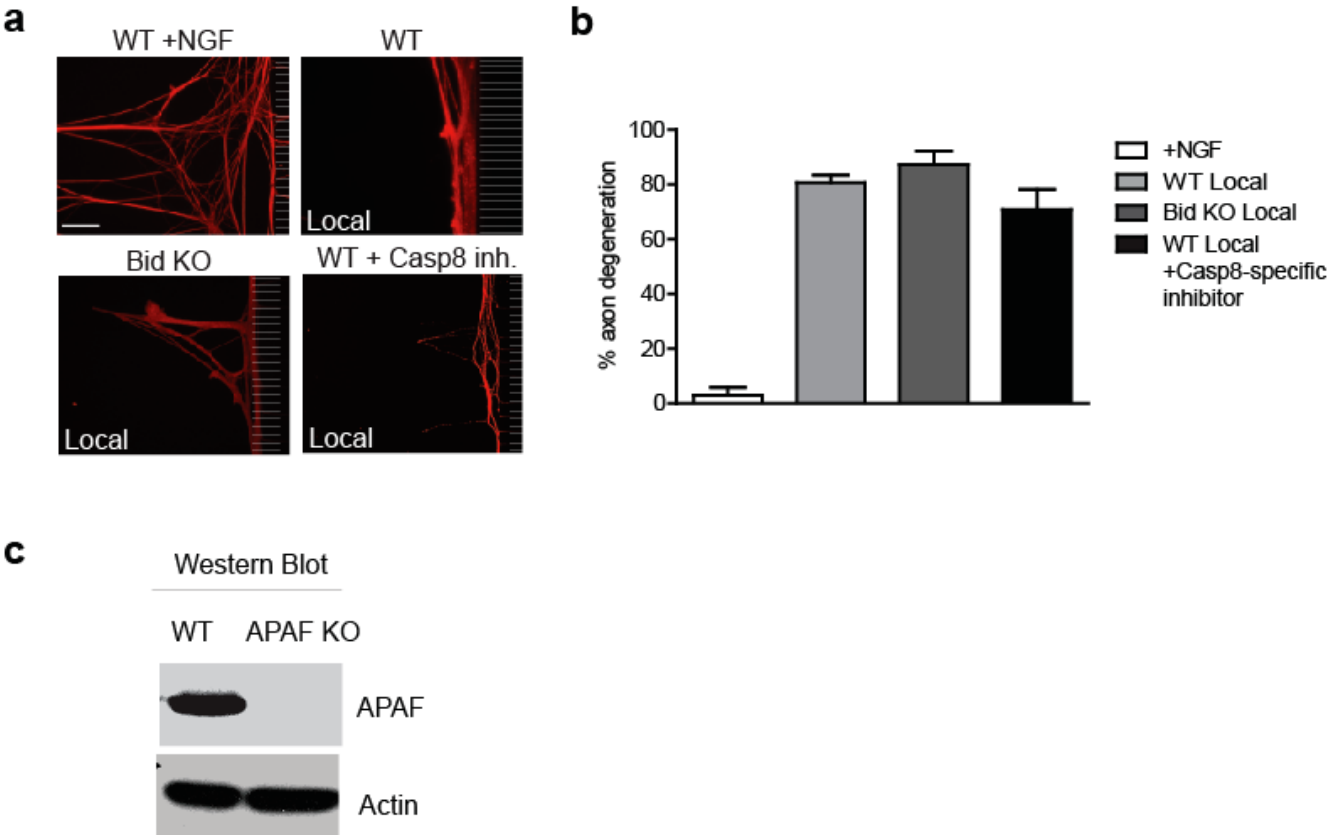
Supplemental Figure 3.1



Supplemental Figure 3.2: The extrinsic pathway is not involved in axon-selective degeneration induced by local deprivation.

(**a**) WT, Bid-deficient, and Casp8-inhibited neurons (5 DIV) were locally deprived and probed for tubulin. NGF-maintained WT neurons served as controls. Scale bar, 50 μm. (**b**) Quantification of the conditions shown in (**a**). All data represent the mean ± s.e.m. (n=3). The difference in the percentage of axon degeneration is significant ($p<0.005$) for all treatments compared to NGF-maintained control neurons. (**c**) Western blot for Apaf-1 using lysates made from mouse embryonic fibroblasts obtained from WT and Apaf-1-deficient (E13) mice. Apaf-1-deficient mice are complete nulls.

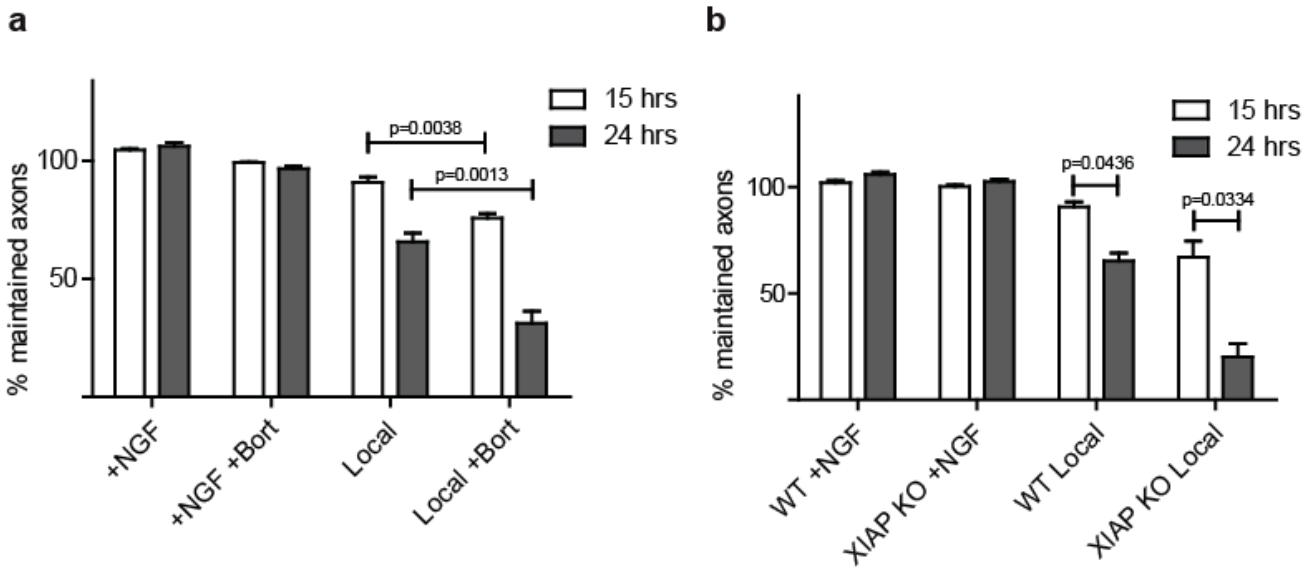
Supplemental Figure 3.2



Supplemental Figure 3.3: Time course of axon degeneration in proteasome-inhibited and XIAP-deficient neurons during local deprivation.

(**a**) Quantification of maintained axon length over time during local deprivation with and without the proteasome inhibitor Bortezomib. NGF-maintained neurons (also with and without Bortezomib) served as controls. (**b**) Quantification of maintained axon length over time during local deprivation for WT and XIAP-deficient neurons. NGF-maintained neurons served as controls. Data represent the mean ± s.e.m. (n=3).

Supplemental Figure 3.3



CHAPTER FOUR: EXPLORING BAX ACTIVATION IN AXON PRUNING

4.1 Introduction

At the beginning of this research project, a paper from Marc Tessier-Lavigne's group at Genentech (Nikolaev, McLaughlin et al. 2009) surprised the field of axon pruning. Nikolaev and colleagues demonstrated that two proteins typically associated with apoptosis – Bax and caspase-6 – are required for developmental axon pruning. The observation that Bax is required for both apoptosis and axon degeneration points to Bax activation as a key point of intersection between these two pathways. While Bax activation during apoptosis is mediated by the transcriptional and translation upregulation of BH3-only proteins (Ham, Towers et al. 2005; Shamas-Din, Brahmbhatt et al. 2011; Happo, Strasser et al. 2012), virtually nothing is known about how Bax is activated during axon-specific degeneration.

The critical observation that Bax-deficient neurons are robustly protected during axon-specific NGF deprivation has been widely reproduced over the past five years (Schoenmann, Assa-Kunik et al. 2010; Vohra, Sasaki et al. 2010; Simon, Weimer et al. 2012; Cusack, Swahari et al. 2013), and the idea that components of the intrinsic apoptotic pathway regulate developmental axon pruning is now accepted in certain experimental contexts. However, there remains a striking lack of information about how axon-specific NGF withdrawal leads to the precise, spatially restricted activation of Bax in degenerating

axons. Diverse events that lie upstream of Bax activation – kinase activation, transcription, translation, BH3 protein family members, and microRNAs – have been linked to axon pruning and are individually explored in the following sections.

4.2 Overview of pre-mitochondrial players in axon pruning

Kinases

A. JNK and DLK

The JNK pathway is required for both neuronal apoptosis and axon pruning but also regulates growth and homeostasis (Chang, Jones et al. 2003; Bjorkblom, Ostman et al. 2005). Healthy neurons maintain high levels of activated JNK, which are kept in check by the ability to discriminate between pro-survival basal activity and pro-apoptotic signaling (Coffey, Hongisto et al. 2000). JNK selectivity is partly achieved by assigning specific functions to each of the three mammalian JNK genes. For example, mice deficient for JNK2 and/or JNK3 are protected from neuronal apoptosis and display significantly reduced phosphorylation of downstream stress-induced targets such as c-Jun; in contrast, JNK1 null animals show no protection (Yang, Kuan et al. 1997; Chang, Jones et al. 2003; Chang, Doherty et al. 2003; Hunot, Vila et al. 2004). JNK-interacting proteins (JIPs) also contribute to the selectivity of JNK function, theoretically by forming highly specialized complexes of JNK, JIPs, and upstream kinases that mediate JNK signaling with tightly defined outputs (Waetzig and Herdegen 2005; Whitmarsh 2006). However, very few of these types of complexes have been identified.

The family of mixed lineage kinases (MLKs) is well known to play a role in neuronal apoptosis and acts as a major regulator of JNK activation in neurons (Maroney, Glicksman et al. 1998), yet the specific MLKs that mediate axon pruning remain largely undefined, with one exception: the MLK dual leucine zipper kinase (DLK). DLK has demonstrated roles in injury-induced axon degeneration (Miller, Press et al. 2009), axonal regeneration after injury (Hammarlund, Nix et al. 2009), and mediates JNK activation and/or P38MAPKs in a

pathway that regulates axon growth and synapse formation (Nakata, Abrams et al. 2005; Collins, Wairkar et al. 2006; Hirai, Cui de et al. 2006; Lewcock, Genoud et al. 2007). How DLK is regulated to perform such diverse and often opposing functions remains unclear.

Joseph Lewcock's group at Genentech has made significant progress in defining the role of DLK in axon pruning. Specifically, DLK selectively regulates the JNK-mediated stress pathway in locally deprived neurons by interacting with the scaffolding protein JIP3 (Ghosh, Wang et al. 2011). This DLK/JIP3 JNK signaling complex redistributes JNK to the soma to induce c-Jun phosphorylation. Although c-Jun phosphorylation occurs within several hours of axon-specific NGF deprivation (Mok, Lund et al. 2009; Ghosh, Wang et al. 2011), this signaling event is not required for axon pruning as c-Jun -/- axons still degenerate during local deprivation (Ghosh, Wang et al. 2011).

The protection bestowed upon DLK -/- neurons during NGF-induced apoptosis and axon pruning is surprising given the amount of crosstalk observed between MAPK pathways. For example, multiple MAPKKKs are capable of activating JNK in multiple contexts (Xu, Maroney et al. 2001), leading one to predict that knocking out a single gene within the JNK activation pathway would not have much of a saving effect. The protective effect of DLK may be due to different factors such as specific DLK-interacting proteins or the precise localization of DLK protein to distal axonal sites where stress is initially encountered. Interestingly, long term NGF deprivation (>72 hours) of DLK-deficient neurons *does* eventually induce degeneration, creating a striking contrast with NGF-deprived Bax-deficient neurons that survive for extended periods (Deckwerth, Elliott et al. 1996; Ghosh, Wang et al. 2011). Thus, neurons that endure prolonged NGF withdrawal are

able to circumvent DLK to initiate degenerative mechanisms, possibly by utilizing a different MAPKKK or a completely different pathway.

B. GSK3β

Another critical kinase involved in axon pruning is glycogen synthase kinase 3β (GSK3β). Several signaling cascades protect neurons from death (e.g. PI3K/Akt signaling) by negatively regulating GSK3β, and this kinase has been shown to phosphorylate and regulate Bax activity in cerebellar granule neurons to induce apoptosis (Linseman, Butts et al. 2004). GSK3β has also been shown to regulate a large number of transcription factors, including β-catenin, c-Jun, and CREB (cAMP response element-binding protein), among others (Hur and Zhou 2010). With specific regard to axon pruning, somatic GSK3β becomes dephosphorylated (active) during local NGF deprivation (Chen, Maloney et al. 2012). Inhibiting GSK3β in the soma (but not axons) during local deprivation robustly protects axons from degeneration, and expressing constitutively active GSK3β is sufficient to induce degeneration of healthy axons (Chen, Maloney et al. 2012). Chen and colleagues demonstrate that p38MAPK, CamKK, and ErbB all lie upstream of GSK3β during local deprivation, and p38MAPK was shown to transduce the local NGF deprivation signal from axon to soma (Chen, Maloney et al. 2012). Interestingly, GSK3β in the soma appears to act in parallel to JNK in axons during local deprivation; JNK inhibition in axons failed to block GSK3β activation in the soma, and somatic GSK3β inhibition had no effect on JNK activation (Chen, Maloney et al. 2012). These finding provide evidence that a.) various (and potentially distinctly localized, simultaneously-acting) kinase pathways transduce degeneration cues from axon to soma to regulate axon-specific degeneration, and b.) GSK3β

acts centrally to modulate gene expression that regulates distally restricted axon degeneration.

Transcription

A. Transcription-independent axon removal

The role of transcription in axon pruning is a topic of ongoing discussion. In Wallerian degeneration, post-axotomy axon degeneration occurs via an intrinsic axonal pathway, thus dismissing any transcriptional contributions originating from the soma. DLK deficiency, JNK inhibition, proteasome inhibition, and calpain inhibition protect against Wallerian degeneration after axotomy, respectively (Wang, Medress et al. 2012), suggesting that each of these degenerative components can be activated in axons *without* transcriptional influences from the soma. As Wallerian degeneration has been widely used to model axon pruning over the past several decades, there is an overwhelming amount of literature declaring that "pruning""(placed in quotations because axotomy-induced axon degeneration is distinct from the naturally occurring axon degeneration that happens during developmental pruning) does not require somatic input, transcriptional or otherwise. This conclusion may be true for Wallerian degeneration, but transcriptional contributions during NGF-deprivation induced pruning have barely been explored.

The shared requirement of DLK and JNK in both developmental axon pruning and Wallerian degeneration indicates that their soma-free mechanisms of activation may also be shared between these two axon degeneration pathways. For example, several cytosolic pro-degenerative JNK targets have been identified, including doublecortin, SCG10, and Tau (Goedert, Hasegawa et al. 1997; Gdalyahu, Ghosh et al. 2004; Tararuk, Ostman et al. 2006).

Additionally, JNK can phosphorylate the BH3-only proteins Bad and Bim to contribute to degeneration in other systems (Donovan and Cotter 2002; Putcha, Le et al. 2003); while activation of these two proteins are mediated by dephosphorylation in neurons undergoing apoptosis (Zha, Harada et al. 1996; Puthalakath and Strasser 2002), the evidence supports a potential mechanism of caspase modulation that is DLK/JNK-mediated and transcription-independent.

B. Transcription-dependent axon removal

A very simple experiment indicates that transcription does indeed play a role in NGF deprivation-induced axon pruning: when the potent transcription inhibitor Actinomycin D (ActD) is added to the cell bodies of locally deprived neurons in a compartmentalized chamber, deprived axons are protected (Figure 4.1)(Chen, Maloney et al. 2012). In a more focused approach, a screen comparing the somata and axons of locally deprived cortical neurons discovered the upregulation of two transcription factors, *dleu2* and *tbx6*. *dleu2* is a long non-coding RNA and host gene for two microRNAs, miR-15a and miR-16-1, and t*bx6* is a T-box transcription factor (Chen, Maloney et al. 2012). Simultaneously upregulating these two transcription factors is not sufficient to induce axon degeneration in healthy neurons, suggesting that additional signaling events (such as those that 'prime' the axon to degenerate) are required to complete axon pruning. Intriguingly, miR-15 and miR-16 target the anti-apoptotic gene Bcl-2, an oncogene that drives B-cell lymphoma (Pegoraro, Palumbo et al. 1984; Calin, Dumitru et al. 2002; Cimmino, Calin et al. 2005). While no one has overexpressed Bcl-2 in the axon-specific NGF deprivation model of axon pruning, overexpressing the closely related proteins Bcl-XL and Bcl-w, respectively, strongly

protects NGF-deprived axons *in vitro* (Vohra, Sasaki et al. 2010; Cosker, Pazyra-Murphy et al. 2013). Collectively, these observations suggest that a.) anti-apoptotic BH3 proteins are involved in some situations of axon loss but not others, and b.) axon-specific NGF deprivation may induce the upregulated transcription of specific miRNAs that target anti-apoptotic BH3 genes, resulting in the loss of a critical protective brake on Bax activation. The latter hypothesis predicts that decreasing the expression of anti-apoptotic BH3 proteins will make insulted axons more vulnerable and enhance degeneration; indeed, this appears to be the case (at least for Bcl-w) as genetic deletion of Bcl-w accelerates axon degeneration of locally deprived sympathetic neurons and induces an adult-onset axonopathy *in vivo* (Courchesne, Karch et al. 2011).

C. Translation-dependent axon removal

Axon-specific translation is well known to be essential for axon growth, guidance, maintenance and regeneration, but its role in axon removal remains poorly established. Several studies show that blocking the translation of certain mRNAs in healthy axons induces axon degeneration. For example, NGF-maintained sympathetic axons degenerate upon axon-specific loss of either Bcl-w (Cosker, Pazyra-Murphy et al. 2013) or Impa1 (myo-inositol monophosphatase-1)(Andreassi, Zimmermann et al. 2010), demonstrating that NGF stimulation of distal axons induces axon-specific targeting of these mRNAs as well as their local translation. Cosker and colleagues also demonstrate that axonal Bcl-w binds to Bax in axons to prevent both caspase-6 activation and axon degeneration (Cosker, Pazyra-Murphy et al. 2013), which suggests that the Bcl-w brake on Bax activation needs to be removed during local deprivation-induced pruning.

Does axonal translation also play a role in NGF deprivation-induced pruning? One study implicates translation in Drosophila axon pruning; specifically, loss of the mRNA-binding translation regulator FMRP causes severe axon pruning deficits during late brain development (Tessier and Broadie 2008). In mouse sympathetic neurons, however, do the transcription products created in response to local deprivation also need to be translated, and, if so, where (soma or axon)? I explored this question by locally depriving neurons in the presence of the translation inhibitor cycloheximide (CHX) on either the soma or axon compartment. Surprisingly, addition of CHX to the soma compartment did not prevent axon-specific degeneration, indicating that protein translation is not needed in the soma compartment for NGF deprivation-induced pruning. In contrast, addition of CHX to the axon compartment completely blocked axon-specific degeneration (Figure 4.1). Intriguingly, axon-specific addition of CHX also blocked c-Jun phosphorylation in cell bodies following local deprivation, indicating that axonal translation may be required for initially signaling the loss of NGF at distal axons (Figure 4.2).

These results reveal the surprising finding that local deprivation-induced axon degeneration requires mRNA expression in the soma but protein translation only in the axons. It appears that translation may lie upstream of transcription induced by local deprivation signals and, as will be addressed in greater detail in the next section, I hypothesize that axonal translation is also post-transcriptionally required during axon pruning. Specifically, I hypothesize that local deprivation induces the expression of BH3-only mRNAs that are transported to the site of NGF deprivation in the axons. Thus, axonal translation of BH3-only proteins may enable Bax activation only in the axons to trigger

axon-specific degeneration. This mechanism would efficiently compartmentalize Bax activation to permit axon-specific degeneration while preventing total cell death.

BH3 family of proteins

A. Anti-apoptotic BH3s

Several observations support the involvement of anti-apoptotic BH3 proteins in NGF deprivation-induced axon pruning. First, overexpression of Bcl-XL (Vohra, Sasaki et al. 2010) and Bcl-w (Cosker, Pazyra-Murphy et al. 2013) protects locally deprived axons from degeneration *in vitro*. Addition of ABT-737 (a compound that collectively inhibits Bcl-XL, Bcl-2 and Bcl-w) to NGF-maintained axons in a compartmentalized culture induces axon degeneration in wildtype neurons, suggesting that simply removing the endogenous brakes on Bax activation in axons is sufficient to trigger an axon degeneration pathway (Figure 4.3)(Simon, Weimer et al. 2012). Whether axon-specific application of ABT-737 triggers a selective pruning pathway as opposed to simply inducing the classical apoptotic pathway in axons remains unclear. A more complete understanding of how the other BH3 protein family members promote axon pruning (if at all) will be required to determine whether mechanisms of Bax activation during axon pruning are similar or distinct from those utilized during apoptosis.

B. Pro-apoptotic BH3s

Since NGF deprivation-induced pruning was shown to require a death receptor (DR6)(Nikolaev, McLaughlin et al. 2009), I initially explored whether axon pruning shares its mechanism of Bax activation with the death receptor-mediated (extrinsic) apoptotic

pathway. In this death pathway, Bax activation is dependent on Bid. I examined whether Bid was required for axon degeneration and found that Bid-deficient neurons still degenerate their axons during local deprivation (Supplemental Figure 3.2).

I then focused on the other BH3-only proteins that are known to be transcriptionally and translationally upregulated during neuronal apoptosis induced by global NGF deprivation (Freeman, Burch et al. 2004): Bim (Putcha, Moulder et al. 2001; Gilley, Coffer et al. 2003), DP5 (Imaizumi, Tsuda et al. 1997), Puma (Besirli, Wagner et al. 2005; Wyttenbach and Tolkovsky 2006), and Bmf (Kole, Swahari et al. 2011; Kristiansen, Menghi et al. 2011). A preliminary experiment examined levels of Bim and Bmf mRNA in soma and axon lysates collected from NGF-maintained, globally deprived, and locally deprived microfluidic chambers, respectively (5 DIV). I found that while both Bim and Bmf levels increased in the soma during global deprivation as expected, only Bim increased dramatically in axons during local deprivation (Figure 4.4). While nanobacteria and chamber difficulties have prevented me from determining whether the levels of other BH3-only mRNAs also increase in axons during local deprivation, I anticipate that Bim is not the only member of this family associated with axon pruning. This hypothesis is based on the preliminary observation that Bim -/- neurons still degenerate their axons during local deprivation (Figure 4.5). Thus, as the BH3-only proteins regularly act redundantly, it is very possible that one or more other BH3-only proteins besides Bim are locally upregulated to mediate Bax activation in deprived axons.

While lentiviral-mediated knockdown of BH3-only proteins, individually or in combination, during local deprivation is predicted to functionally illuminate which of these proteins are involved in axon pruning, the mechanism of axon-specific Bax activation likely

involves both *direct* activators and sensitizers. BH3-only activators (Bid, Bim, Puma) directly interact with Bax to induce a conformational change and promote its translocation to the mitochondria. Bax activation can also require BH3-only sensitizers (Bad, Bmf, DP5, Bik, Noxa) that bind and inhibit anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-w, Mcl-1). These anti-apoptotic proteins act by either directly binding to active Bax or by binding and sequestering the direct activators. Thus, depending on the context, Bax activation requires only activators, only sensitizers, or both types of BH3-only family proteins. The previous section describes how the activation of BH3-only sensitizers alone is sufficient to permit Bax activation during axon pruning, yet I propose that *optimal* axon pruning requires *both* sensitizers and activators because a.) Bim, a direct Bax activator, is induced in axons during local deprivation and b.) ABT-737-induced axon degeneration is enhanced by NGF deprivation (Figure 4.6). Attempts to directly activate Bax in axons using the cell-permeable small molecule activator, BAM7, have failed in both global and local deprivation scenarios using sympathetic neurons (results personally communicated by Ryan Annis and Matt Geden), suggesting that an NGF-deprivation signal is required to 'prime' axons for degeneration. Priming events might include transcription and translation of other necessary players in the pathway that enable Bax activation in axons. This idea of priming relates to how a neuron is able to differentiate between activating Bax to kill the whole cell versus activating Bax to degenerate only the axon. Is the location of Bax activation the key determining factor for which pathway is activated (i.e., Bax activation throughout the neuron triggers apoptosis whereas localized Bax activation in axons triggers the axon pruning pathway)? Or are neurons able to recognize the precise context of NGF deprivation? For example, global deprivation may generate cellular conditions that are

more permissive for apoptosis whereas local deprivation generates conditions that are more permissive for axon degeneration. To attack these questions, I propose the following future experiments:

1.) Add ABT-737 and BAM7 (alone and together) to the axon compartment of NGF-maintained neurons to determine whether axon pruning is achieved with both sensitizers and activators. Bax -/- neurons will serve as controls for drug specificity.
2.) Add ABT-737 and BAM7 together either globally or locally, using caspase-6 -/- neurons as controls. Treatment in these knockout neurons will determine whether the apoptotic (caspase-6 -/- neurons will degenerate) or axon pruning pathways (caspase-6 -/- axons will be protected) are activated.
3.) Neurons will be globally or locally deprived for 24 hours in the presence of ActD. ActD will block both apoptotic and axon pruning events that occur upstream of Bax activation, thereby permitting NGF deprivation without Bax activation. BAM7 and ABT-737 will then be added to the axon compartment to determine whether the specific pathway activated (apoptosis or axon pruning) is determined by the context of local versus global NGF deprivation. Again, caspase-6 -/- neurons will serve as controls to differentiate between the two pathways.

MicroRNAs

A. General background

MicroRNAs (miRNAs) are small, approximately 20-22 nucleotide RNAs that are generated by the processing of longer RNA precursors (Figure 4.7)(He and Hannon 2004). The initial RNA transcript encoding a miRNA (called primary-miRNA, or pri-miRNA) contains at least one short stem-loop structure that houses the future mature miRNA sequence. Pri-miRNAs are processed in the nucleus by Drosha, a nuclease that cleaves and removes the stem loop of the pri-miRNA; this excised stem-loop, called precursor miRNA (or pre-miRNA), is then exported to the cytoplasm where another nuclease, Dicer, cleaves the loop from the pre-miRNA to yield the short 20-22 nucleotide mature double stranded miRNA duplex (Bernstein, Caudy et al. 2001). One strand of this duplex is degraded while the other is incorporated into a complex called the RNA-induced silencing complex (RISC). Depending on the sequence of the miRNA within the RISC, this ribonuclease complex binds to mRNAs (usually at the 3' UTR) to negatively regulate their expression. In turn, the "seed region" at the 5' end of the miRNA must bind with perfect complementarity to achieve specific target recognition (Doench and Sharp 2004). The precise mechanism utilized by miRNAs to block gene expression remains unknown, yet studies suggest that miRNAs and RISC function together to promote mRNA degradation or to directly inhibit protein translation (Hammond, Bernstein et al. 2000; Khvorova, Reynolds et al. 2003; Du and Zamore 2005).

Today, we understand that miRNAs comprise an entire class of critical regulatory molecules (there are over 1,000 annotated miRNAs in the human genome) that control diverse cell processes, including apoptosis (Griffiths-Jones 2004; Griffiths-Jones, Grocock et

al. 2006). Intriguingly, a screen that profiled SCG miRNA expression between axons and cell bodies revealed that 200 miRNAs are significantly upregulated in axons compared to the soma (Natera-Naranjo, Aschrafi et al. 2010; Kaplan, Kar et al. 2013). These results suggest that neurons may utilize miRNAs to locally regulate mRNA synthesis in axons, and my preliminary findings support a potential link between miRNAs and axon pruning.

B. Differential miR-29 levels between mature soma and axon

My results show that that mature neurons are fully capable of degenerating axons following local deprivation just as seen in young neurons (Figure 3.7). These results are particularly striking because while local deprivation induces axon degeneration, global deprivation causes neither axon degeneration nor apoptosis. This finding indicates that neurons can acutely discern the spatial location of the loss of NGF signal and activate an appropriate response. As mentioned earlier, one mechanism that mature neurons utilize to restrict apoptosis is the microRNA (miRNA) miR-29, which is strikingly upregulated during sympathetic neuron maturation and targets multiple members of the BH3-only family that have been associated with neuronal apoptosis: Bim, Puma, DP5/Hrk, Bmf, and N-Bak (Kole, Swahari et al. 2011). These results can explain how high miR-29 levels in mature neurons inhibit apoptosis, but they do not explain how mature neurons selectively inhibit apoptosis yet remain permissive for axon degeneration. My preliminary results reveal the striking result that miR-29 levels are selectively elevated in the soma (and not the axons) in mature neurons (Figure 4.8). This differential miR-29 expression would allow the translation of Bim (and any other BH3-only proteins) in mature axons during local NGF deprivation while

inhibiting it in the soma, therefore permitting axon-specific Bax activation without risking somatic destruction.

Axon transport

There are known neurobiological processes in which distally located events require transcription and transport for complete execution. For instance, the immediate-early gene *Arc/Arg3.1* is rapidly transcribed after long-term potentiation-inducing stimuli and then transported to the dendrite, where it is critical for the maintenance of synaptic potentiation (Bramham, Worley et al. 2008).

Axon transport also appears to play a key role in axon pruning mechanisms induced by local NGF deprivation. Specifically, blocking retrograde transport in locally deprived neurons prevents GSK3β activation in the soma (Mok, Lund et al. 2009; Chen, Maloney et al. 2012), and deleting KIF2a, a kinesin motor protein that destabilizes microtubules, robustly protects axons from degeneration (Maor-Nof, Homma et al. 2013). Axonal transport may be required to first transduce the local NGF deprivation signal to the soma and then again to deliver newly transcribed, targeted mRNA to the precise site of NGF deprivation to trigger degeneration. Based on my preliminary findings regarding transcription, translation, and axonal BH3-only mRNA levels during local deprivation, I hypothesize that axonal transport of BH3-only mRNAs is required for axon pruning. I also hypothesize that globally deprived mature neurons do not degenerate their axons (whereas locally deprived mature neurons do) because transport becomes blocked under global NGF deprivation conditions. This could occur as a consequence of the marked decrease in cellular metabolism seen after global NGF deprivation (Deckwerth and Johnson 1993).

4.3 Materials and Methods

Cell culture

For BH3-only mRNA analysis experiments, primary sympathetic neurons (P0) were dissected and cultured for five days in microfluidic chambers (as described in Chapter Three) or grown as explants on collagen (for miR-29 analysis). Cell bodies and axons were collected and lysed separately in Trizol prior to analysis. Cycloheximide (CHX; Sigma) and actinomycin D (ActD; Sigma) were diluted in media at 1 μg/mL.

The following experiments and data analysis were performed by Dr. Vijay Swahari:

cDNA synthesis and qRT- PCR analysis for Bim and Bmf

For analysis of BH3-only mRNAs, cDNA was synthesized using 150-300 ng RNA. RNA samples were first treated with RQ1 DNase (Promega) for 30 min at 37^{o}C followed by a 10 minute incubation at 65^{o}C with DNase Stop Solution (Promega). DNase-treated RNA was mixed with 0.25 □g random hexamer primers (Invitrogen) and reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Each 25 μL PCR reaction contained 1 μL cDNA, each primer at a final concentration of 400 nM, and Power SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed using Primer Express software (Applied Biosystems; sequences in Table 4.1). Reactions were amplified in an ABI7500 system and relative quantification was carried out using the delta-delta Ct method. Sample variability was corrected by normalizing to GADPH levels.

MicroRNA qRT- PCR analysis

Mature miR-29 expression was assayed using TaqMan MicroRNA Assays (Applied Biosystems). Briefly, 10 ng of RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and specific RT primers for miR-29b or U6 RNA (Applied Biosystems). cDNA was amplified in an ABI7500 system using TaqMan Universal PCR Master Mix (Applied Biosystems). Relative quantification was carried out using the delta-delta Ct method. Sample variability was corrected by normalizing to U6 RNA levels.

4.4 Figures and Legends

Figure 4.1: Transcription and axon-specific translation are required for axon degeneration induced by local NGF deprivation.

Wildtype neurons (5 DIV) were treated with either the transcription inhibitor Actinomycin D (ActD; 1 μg/mL) or the translation inhibitor cycloheximide (CHX; 1 μg/mL) in microfluidic chambers for 24 hours during global or local deprivation. Neurons were fixed and immunostained for tubulin (red) and Hoechst (blue). As expected, addition of ActD or CHX to globally deprived neurons blocked apoptosis and axon degeneration. Intriguingly, addition of ActD to the soma compartment and CHX to the axon compartment, respectively, robustly protected axons during local deprivation. Addition of CHX to the soma compartment during local deprivation failed to inhibit axon degeneration, indicating that somatic translation is not required for NGF deprivation-induced pruning.

Figure 4.1

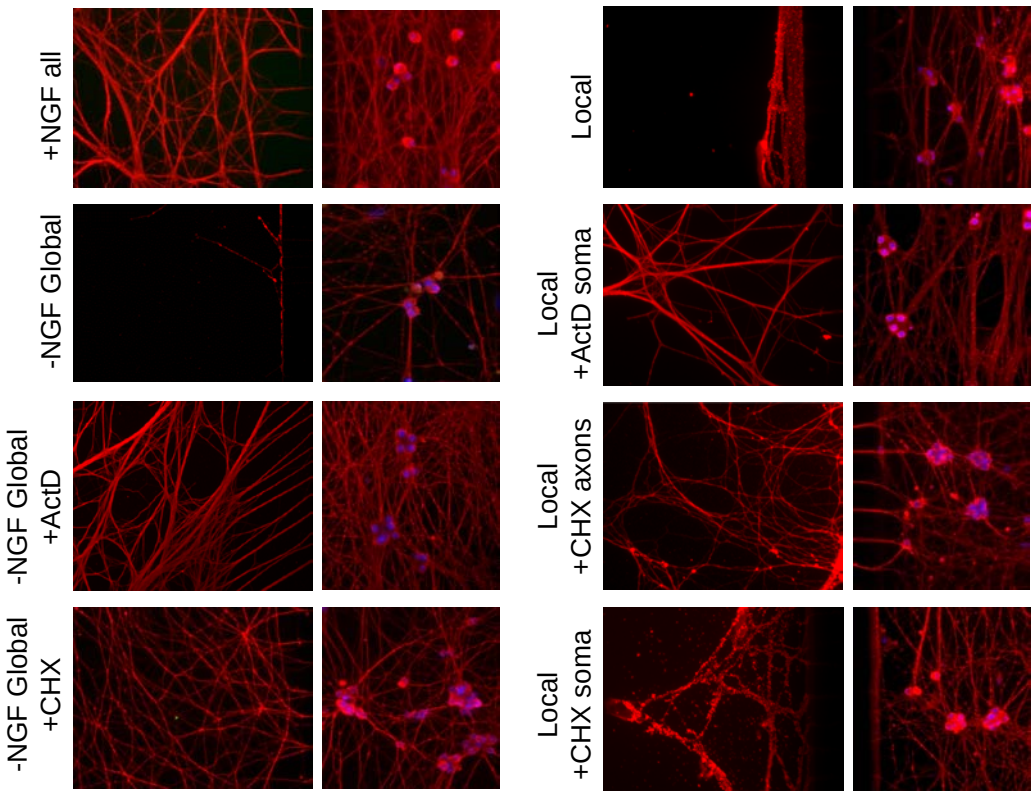

+NGF all
-NGF Global
-NGF Global +ActD
-NGF Global +CHX
Local
Local +ActD soma
Local +CHX axons
Local +CHX soma

Figure 4.2: Axon-specific translation is required for c-Jun phosphorylation during local deprivation.

Wildtype neurons (5 DIV) were globally or locally deprived in the presence of ActD and CHX, respectively, for 24 hours and immunostained for phosphorylated c-Jun (green) and Hoechst (blue). As expected, c-Jun was phosphorylated across all global deprivation scenarios and during local deprivation. Interestingly, inhibition of axon-specific translation blocked c-Jun phosphorylation during local deprivation while blocking transcription had no effect. This observation suggests that axon-specific translation takes place before the NGF-deprivation signal reaches the soma during local deprivation.

Figure 4.2

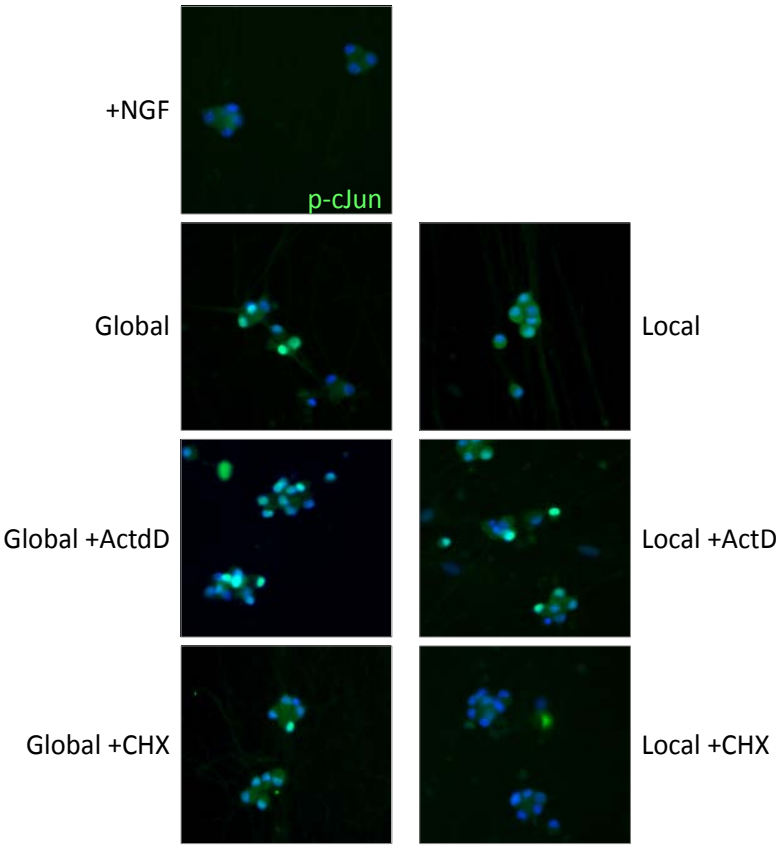

+NGF
p-cJun
Global
Local
Global +ActdD
Local +ActD
Global +CHX
Local +CHX

Figure 4.3: Axon-specific application of ABT-737 induces axon degeneration.

Wildtype neurons (4 DIV) were treated with ABT-737 (10 μM) on the axon compartment only and imaged every 24 hours for 2 days.

Figure 4.3

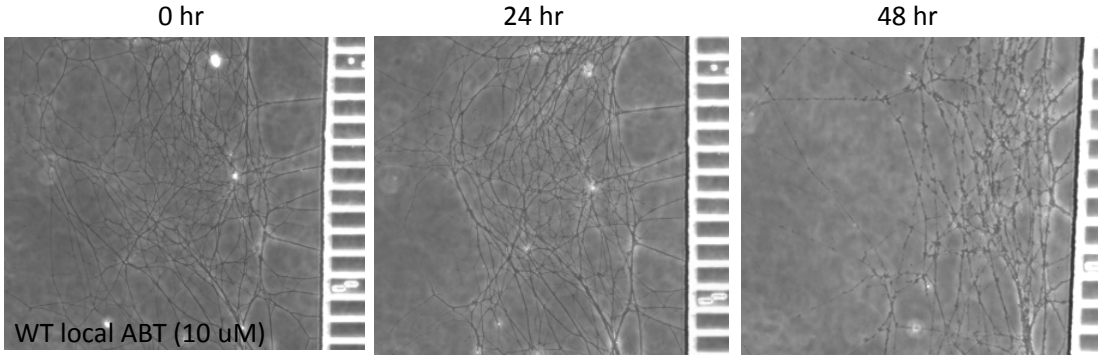


Figure 4.4: Levels of Bim mRNA increase in locally deprived axons.

Wildtype neurons (5 DIV) were maintained in NGF, globally deprived or locally deprived for 24 hours in the presence of pan-caspase inhibitor (25 μM QVD-OPH). Lysates from the soma and axon compartments, respectively, were collected in Trizol and pooled (three chambers per treatment). Following qRT-PCR, levels of Bim and Bmf mRNA were normalized to GAPDH. Whereas both BH3-only mRNAs are upregulated in the soma during global NGF deprivation, only Bim mRNA levels increase in an axon-specific manner during local deprivation.

Figure 4.4

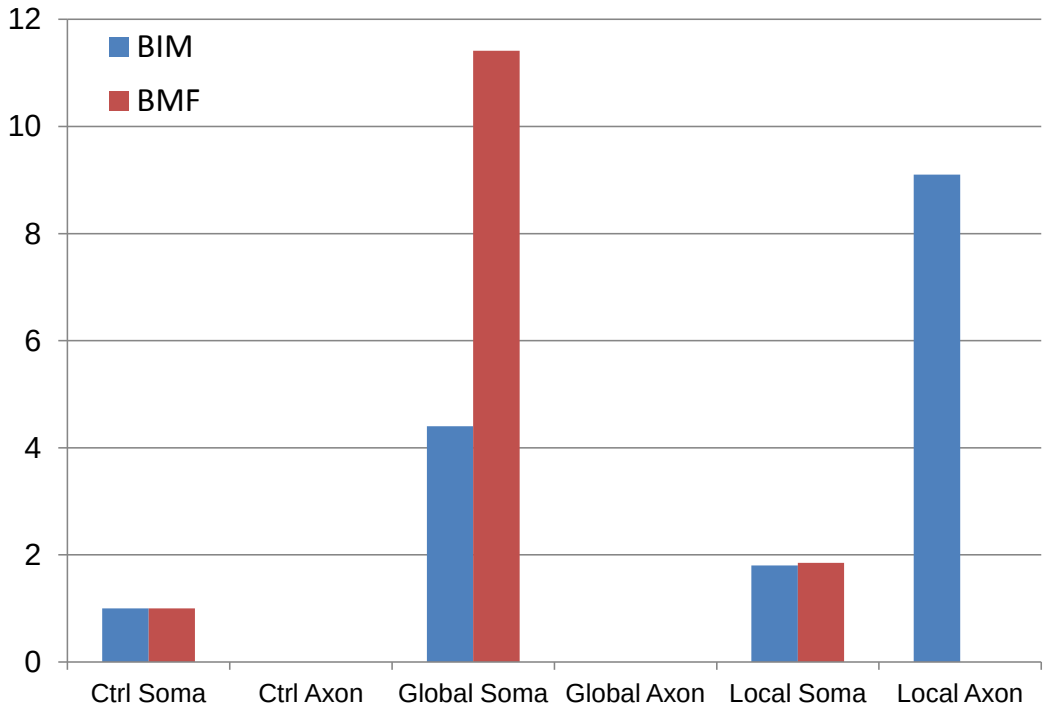

12
10
8
6
4
2
0
BIM
BMF
Ctrl Soma
Ctrl Axon
Global Soma
Global Axon
Local Soma
Local Axon

Figure 4.5: Bim -/- neurons degenerate their axons during local deprivation.

Bim -/- neurons (4 DIV) were locally deprived and imaged over the course of 52 hours (10X). Deprived axons degenerated while NGF-maintained cell bodies remained intact.

Figure 4.5

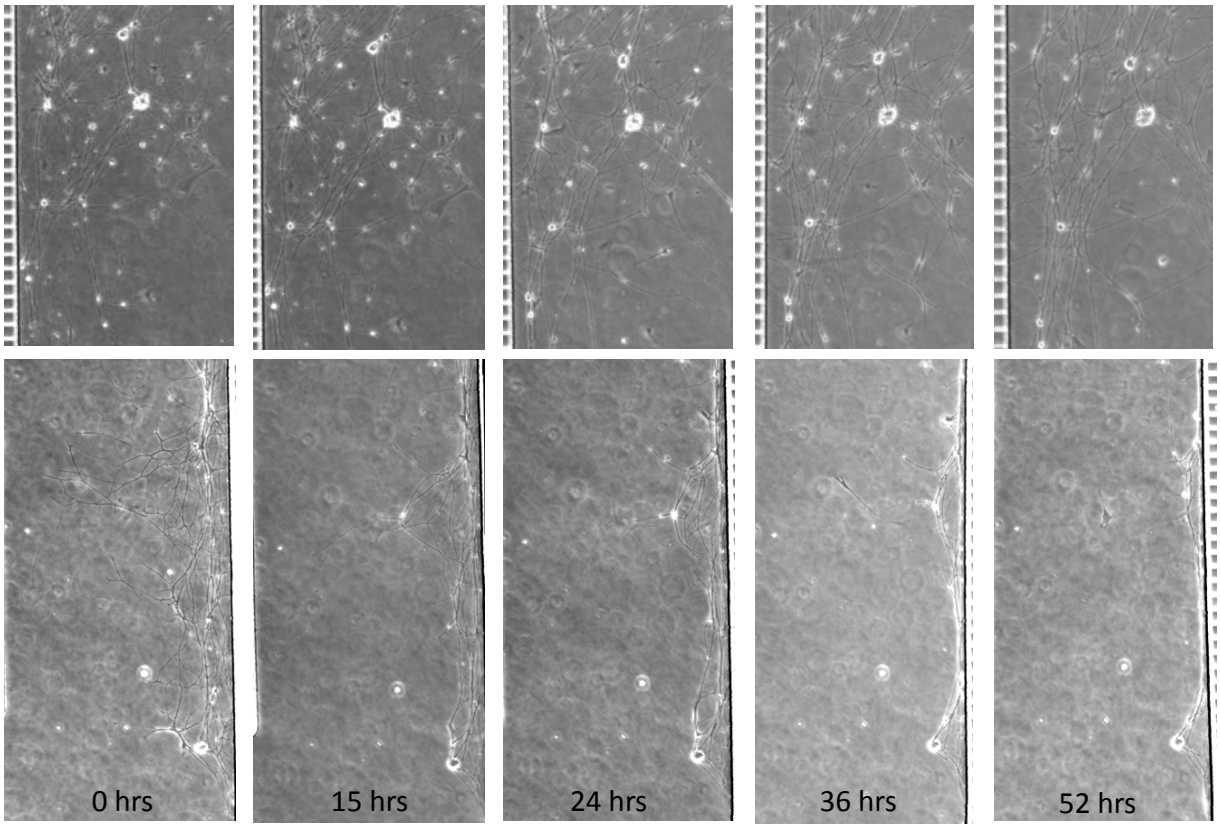


Figure 4.6: ABT-737 treatment enhances axon degeneration induced by local NGF deprivation.

Wildtype neurons (5 DIV) were maintained in NGF, globally deprived, or locally deprived with and without the drug ABT-737 (Abt; 1 μM) for 16 hours. While ABT-737 treatment (which inhibits Bcl-2, Bcl-XL, and Bcl-w) had no effect on NGF-maintained neurons, it greatly enhanced the degeneration induced by local and global NGF deprivation.

Figure 4.6

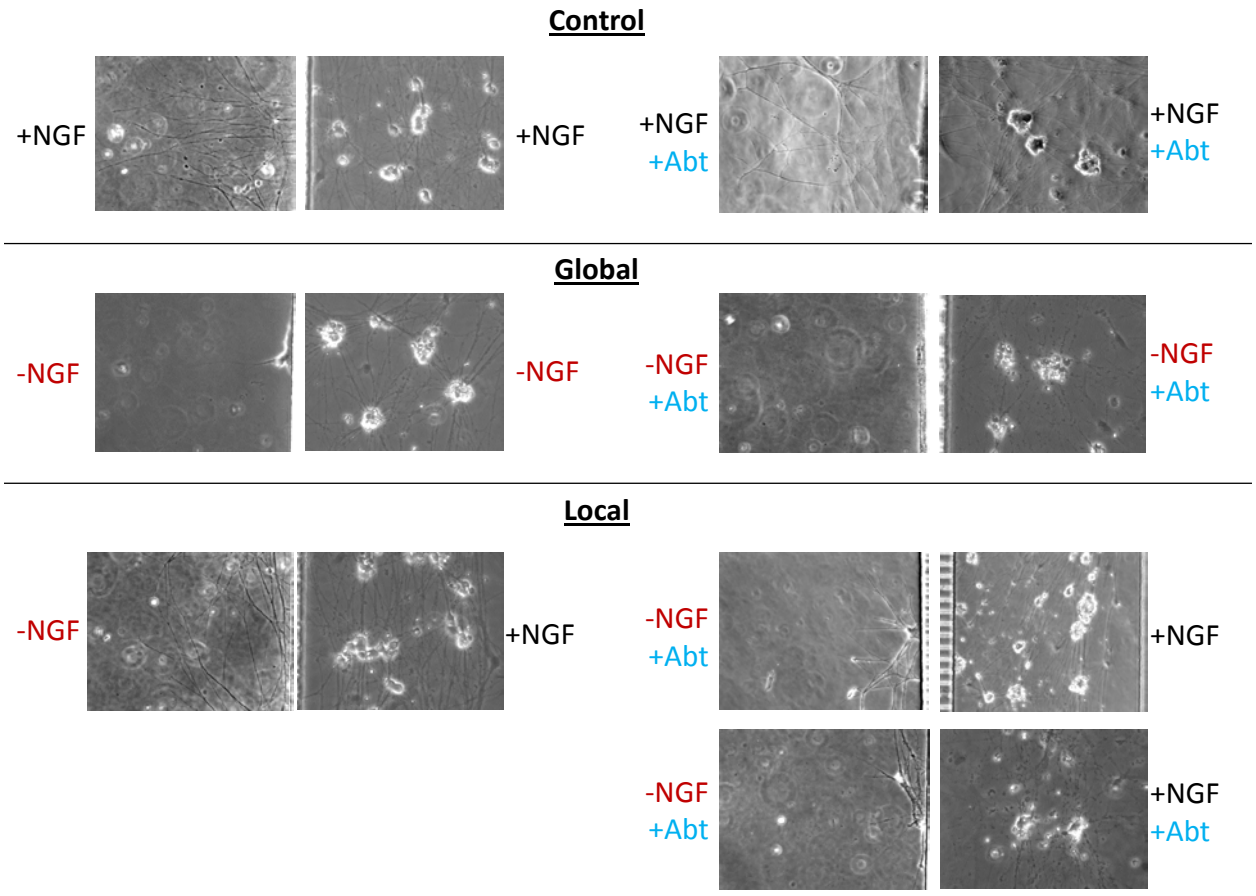
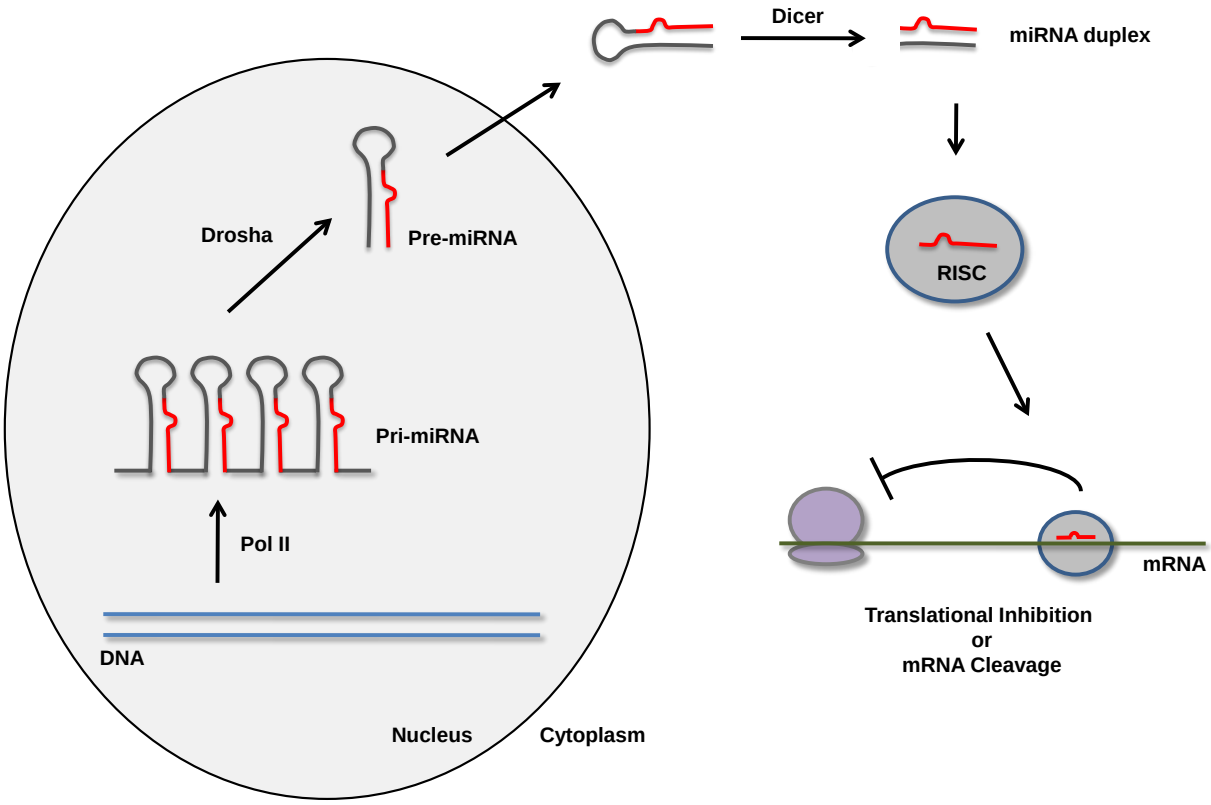

Control
+NGF
+NGF
+NGF
+Abt
+NGF
+Abt
Global
-NGF
-NGF
-NGF
+Abt
-NGF
+Abt
Local
-NGF
+NGF
-NGF
+Abt
+NGF
-NGF
+Abt
+NGF
+Abt

Figure 4.7: Overview of microRNA biogenesis.

Primary-microRNA (pri-miRNA) transcripts are transcribed and processed by Drosha in the nucleus. The cleavage product of Drosha (pre-miRNA) is transported into the cytoplasm where the nuclease Dicer processes the pre-miRNA hairpin into a dsRNA-miRNA duplex. This duplex is loaded into the RNA-induced silencing complex (RISC), which degrades target mRNAs or represses their translation based on the degree of complementarity.

Figure 4.7



Adapted from Dr. Adam J. Kole.

Figure 4.8: miR-29b levels drastically increase in the soma but not axons during neuronal maturation.

Explants of sympathetic ganglia were cultured on collagen for 5 days (P5) or 28 days (P28). Cell bodies and axons were collected separately and evaluated for miR-29b expression levels using RT-qPCR (performed by Dr. Vijay Swahari). Levels were normalized to U6, which is present in both cell bodies and axons. We observed the strikingly restricted upregulation of miR-29b in the somata of P28 neurons compared to axons or P5 neurons.

Figure 4.8

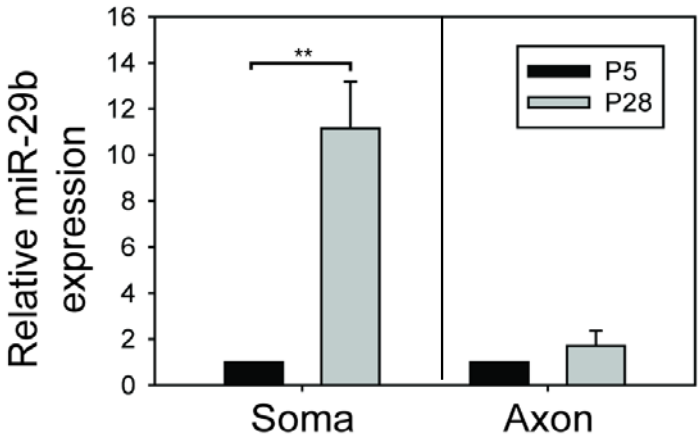


Figure provided by Dr. Vijay Swahari

4.5 Tables

Table 4.1: Primer sequences for BH3-only mRNAs.

qRT-PCR of BH3-only mRNAs		
Gene	**Forward**	**Reverse**
Bim	CAAGTCAACACAAACCCCAAGTC	GTCGTATGGAAGCCATTGCA
Bmf	CCAGAAAGCTTCAGTGTATTGCA	TCTCGGTTCTGCTGGTGTTG
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

CHAPTER FIVE: DISCUSSION

5.1 Summary of findings

The creation of a properly organized and connected nervous system requires the orchestration of a complex series of regulated cellular events, including early progressive processes of neural stem cell differentiation, neurite extension and synapse formation as well as the subsequent regressive processes of apoptosis and neurite pruning. Evolution has selected the mechanisms of apoptosis and pruning to carefully match developing neurons to their appropriate targets and ensure the establishment of precise neuronal circuitry. Although axons degenerate during both apoptosis (whole-cell destruction) and axon pruning (axon-specific degeneration), the fact that these pathways clearly share certain destructive components is only beginning to be appreciated.

As post-mitotic cells that need to survive the lifetime of the organism, neurons must maintain the ability to precisely eliminate axonal projections (e.g. to prune back misprojecting axons during development and to sustain synaptic plasticity throughout adulthood) without constantly putting the rest of the cell at risk for death. Thus, it is not surprising that young and mature neurons engage unique mechanisms to specifically facilitate axon pruning without triggering apoptosis. Here, I have investigated the molecular points of intersection and distinction between neuronal apoptosis and axon pruning. The main findings of this work are summarized below:

Establishing microfluidic chamber technology to isolate axon-specific degeneration from whole-cell degeneration:

1. A combined approach of deep reactive ion etching (DRIE) and standard photolithography can achieve extremely durable master molds that contain minute details, including perfectly smooth groove edges and an imprinted numbered system for easy axon identification over time.
2. Though relatively straightforward to prepare and assemble, I have also found microfluidic chambers to be sensitive to environmental changes and can fail for a variety of reasons, including the following: toxic or humid curing conditions, bad batches of polymer, old PDL, different lot numbers of serum (many contain nanobacteria), wear-and-tear of the master mold, and poorly humidified incubators, among others. Nevertheless, the microfluidic chambers have provided me with the capability to model axon pruning by selectively depriving NGF from the axon compartment

Distinct pathways mediate axon degeneration during apoptosis and axon-specific pruning:

1. Caspase-6 is selectively activated during NGF deprivation.
2. Caspase-6 is required for axon degeneration induced by local, but not global, NGF deprivation.
3. Axon degeneration induced by local NGF deprivation does not involve the extrinsic apoptotic pathway.
4. Axon degeneration is Apaf-1-independent but requires Caspase-9 and Caspase-3.

5. Proteasome activity restricts caspase (Caspase-6, Caspase-9, Caspase-3) activation during axon degeneration.
6. XIAP protects the soma during axon-specific NGF withdrawal.
7. Despite inhibiting apoptosis, mature neurons remain permissive for axon degeneration.

Exploring Bax activation in axons:

1. Transcription is required for axon pruning induced by local deprivation.
2. Local translation in axons (but not the soma) is required for NGF deprivation-induced pruning.
3. Inhibiting axonal translation during local deprivation prevents c-Jun phosphorylation in the soma.
4. Axon-specific addition of ABT-737 (an inhibitor of anti-apoptotic Bcl-2, Bcl-XL, Bcl-w, and Mcl-1) induces axon degeneration in NGF-maintained neurons and enhances axon degeneration during local deprivation.
5. Levels of the BH3-only mRNA, Bim, increase in locally deprived axons.
6. Bim-deficient neurons still undergo axon pruning during local deprivation, suggesting that other BH3-only proteins are required or can act redundantly for Bim in the axon pruning pathway.
7. Levels of the anti-apoptotic microRNA, miR-29, only increase in the soma during neuronal maturation.

5.2 Clinical relevance

Axon pruning in developmental disorders:

A. Autism:

Abnormal axon pruning has been implicated in the developmental disorder autism (Luo and O'Leary 2005). For example, Fragile X syndrome is caused by a mutation (an abnormally expanded CGG segment) that silences the FMR1 gene responsible for encoding fragile mental retardation 1 protein (FMRP). Loss of FMRP causes the structural over-elaboration of both dendritic and axonal projections in animal models (Tessier and Broadie 2008; De Rubeis, Fernandez et al. 2012), thus implicating dysfunctional pruning in this form of autism. Similarly, deficiencies in the timely pruning of axons to key cerebral cortical areas have been observed in a serotonin depletion-based animal model of autism (Boylan, Blue et al. 2007). Over-active pruning has also been linked to autism as children with infantile autism (IA) are thought to have prolonged pruning events in the medial frontal lobe system; the disconnect between this region and the supplementary motor area (SMA) leads to absent activity in the SMA, resulting in delayed response functions and other cognitive deficits (Saugstad 2008; Saugstad 2011).

B. Schizophrenia:

Substantial evidence indicates that abnormal nervous system development contributes to the pathophysiology of schizophrenia (Marenco and Weinberger 2000). Learning prompts significant axonal rearrangements in the adult brain (Holtmaat, Bonhoeffer et al. 2009), and these structural transformations in mature axons closely resemble axon loss in early circuitry wiring. Thus, axon-pruning processes (and

subsequent synaptic loss) occur in several stages throughout life to allow adult neurons to remain structurally plastic (Hoffman and Dobscha 1989; Bellon, Krebs et al. 2011). In studies of schizophrenic patients, positron emission tomography and cerebral blood flow measurements indicate diminished metabolism in frontal areas, a phenomenon called hypofrontality (Hoffman and Dobscha 1989). Synaptic reductions during adolescence preferentially involve the prefrontal cortex, and a pathological failure to turn off this developmental pruning process could result in hypofrontality. This process may explain why the onset of schizophrenia generally occurs in late adolescence and early adulthood - this is the time when the cumulative effects of excessive axonal pruning would first be fully realized. Correspondingly, neuroimaging studies demonstrate a relationship between grey matter loss and schizophrenia onset during adolescence (Thompson, Vidal et al. 2001; Vidal, Rapoport et al. 2006; Sun, Phillips et al. 2009; Sun, Stuart et al. 2009; Sun, Maller et al. 2009), and extensive pruning is correlated with auditory hallucinations in a computer-based model of neuronal networks (Hoffman and McGlashan 1997; McGlashan and Hoffman 2000).

Finding an explanation for excessive pruning in schizophrenic patients is an ongoing search. It may be that initial axonal formations and projections are abnormal in schizophrenia, as malformed axons are more prone to extensive removal (Bellon, Krebs et al. 2011). This hypothesis is supported by the observation that many schizophrenia patients demonstrate motor coordination anomalies and mildly reduced IQ prior to the onset psychosis (Marenco and Weinberger 2000). Alternatively, molecules involved in axonal maintenance (such as neurotrophins) may fail; this hypothesis is particularly interesting given the evidence that the expression of both neurotrophins and their

receptors undergo age-dependent changes during adulthood and aging. Thus, schizophrenia may be associated with the disabled ability of neurotrophins to maintain healthy, intact axon projections well after the initial stages of developmental axon formation are complete. Lastly, mutations in molecules involved in CNS pruning mechanisms may confer susceptibility to this illness as the plexin A2 gene (PLXNA2) and certain polymorphisms of semaphorin-3A (which was previously shown to be required for proper hippocampal pruning of the infrapyramidal axon bundle (Bagri, Cheng et al. 2003)) are reported to be associated with schizophrenia (Fujii, Uchiyama et al. 2011). Overall, the contributions of axon maintenance and pruning molecules only partially explain the structural deficiencies encountered in schizophrenia, but the idea that various stages of neurite formation, maintenance, and removal are affected highlights the vast complexity of this developmental illness.

Caspase-mediated axon pruning in neurodegenerative disease:

Highly relevant to the work presented here, Caspase-6 activity is intimately associated with the pathologies and early events that define AD, HD, and stroke (Graham, Ehrnhoefer et al. 2011). Active caspase-6 is abundant in neurofibrillary tangles, neuritic plaques and neuropil threads of human post-mortem AD brains, and its activity is also detected in pre-tangles as well as in subjects lacking any apoptotic brain morphology (Graham, Ehrnhoefer et al. 2011; LeBlanc 2013). These findings implicate caspase-6 activation in the early stages of tangle formation and the pathological lesions of AD. Caspase-6 is hypothesized to play a highly fundamental role in AD pathophysiology because its activity is observed across all stages of the disease in both sporadic and familial

forms of AD (LeBlanc 2013). In addition, caspase-6 is believed to be an important mediator of neuronal stress because it has been shown to do the following:

a.) Increase the production of the toxic amyloid beta (Aβ) peptide (LeBlanc 1995). Although the precursor of Aβ, amyloid precursor protein (APP), is a demonstrated substrate of both caspase-6 and caspase-3 (LeBlanc, Liu et al. 1999; Pellegrini, Passer et al. 1999; Weidemann, Paliga et al. 1999), mutating the caspase sites in APP does not prevent the overproduction of Aβ under stress (Tesco et al., 2003). However, caspase-3 cleavage of the Golgi-localized gamma ear-containing ADP ribosylation factor-binding protein GGA3 increases the stability of β-secretase β-site APP-cleaving enzyme (BACE), which leads to increased Aβ production (Tesco, Koh et al. 2003; Kang, Cameron et al. 2010; Walker, Kang et al. 2012). Even though only mild casaspe-3 activation is observed in AD brain, low GGA3 levels and high BACE levels are present (Selznick, Holtzman et al. 1999; Stadelmann, Deckwerth et al. 1999; Walker, Kang et al. 2012).

b.) Cleave the key cytoskeletal proteins tau and α-tubulin (Guo, Albrecht et al. 2004; Klaiman, Petzke et al. 2008).

c.) Disrupt the ubiquitin-proteasome-mediated degradation of misfolded proteins by cleaving valosin-containing protein (VCP)(Halawani, Tessier et al. 2010). Poly-ubiquitination of misfolded protein aggregates is a common pathology in various neurodegenerative diseases (Schwartz and Ciechanover 1999), and increased ubiquitination is strongly associated with dystrophic neurites, NFTs, paired helical filaments, and neuritic plaques (Mori, Kondo et al. 1987; Perry, Friedman et al. 1987; Perry, Mulvihill et al. 1989; Dickson, Wertkin et al. 1990; LeBlanc 2013). VCP/p97 is

a chaperone-like ATPase that facilitates the degradation of ubiquitinated proteins through the ubiquitin-proteasomal system (UPS)(Halawani and Latterich 2006; Jentsch and Rumpf 2007), and this protein was the highest-scored substrate in a proteomic analysis of caspase-6 substrates (Klaiman, Petzke et al. 2008). These results identify caspase-6 activation as a key mechanism of UPS impairment in AD.

d.) Cleave multiple post-synaptic density proteins that regulate actin dynamics, such as debrinin, which is known to decrease in AD (Harigaya, Shoji et al. 1996; Shim and Lubec 2002; Klaiman, Petzke et al. 2008).

It is important to note that caspase-3 is also capable of cleaving tau that is associated with NFTs, neuritic plaques, and neuropil threads (Chung, Song et al. 2001; Rohn, Rissman et al. 2002; Gamblin, Chen et al. 2003). Although caspase-3 substrates such as tau have been found in early AD, caspase-3 itself has only been detected at extremely low to non-existent levels (Selznick, Holtzman et al. 1999; Stadelmann, Deckwerth et al. 1999; Rohn, Rissman et al. 2002; Zhang, Zhang et al. 2009). Recently, very low, transient caspase-3 activity has been shown to be involved in synaptic plasticity (Li, Jo et al. 2010; D'Amelio, Sheng et al. 2012) without inducing apoptotic cell death, suggesting that caspase-3 may be rapidly turned over or present at low enough levels to cleave tau in AD brains (LeBlanc 2013).

Caspase-6 is also highly associated with HD and stroke. Activated caspase-6 is present in the brains of pre-symptomatic and early-stage HD patients and mouse models, and the levels of caspase-6 activity inversely correlate with the age of disease onset (Graham, Ehrnhoefer et al. 2011). In stroke models, caspase-6 is activated downstream of

caspase-9 in ischemia, and deleting caspase-6 or -9 significantly reduces stroke injury (Singh, Kaushal et al. 2002; Akpan, Serrano-Saiz et al. 2011). Active caspase-6 has also been found in brain tissue of post-mortem ischemic stroke patients (Friedlander and Yuan 1998), further implicating caspase-6 activation as a critical step in stroke pathogenesis.

Thus, caspase activation is hypothesized to initiate several parallel degeneration pathways that culminate in devastating pathologies. Numerous studies now implicate caspase-6 and/or caspase-3 or -9 in axon degeneration of both peripheral and central nervous system neurons, but I would like to follow the lead of Dr. Andrea LeBlanc in encouraging the use of caution when interpreting results related to extremely complex diseases such as AD (LeBlanc 2013). Several variables must be considered, such as the age of the neuron (Gill, Soriano et al. 2002), the type of neuron (sensory, hippocampal, and cortical neurons may respond differently to insults), the type and degree of insult used, and the discrepancies associated with caspase-deficient animals (specifically, that caspase-deficient neurons can elegantly illuminate caspase functions yet null animals have thus far failed to offer any predictive value in designing a drug against AD or other neurodegenerative diseases)(Geerts 2009). Another note of caution is encouraged for potentially using caspases as therapeutic targets for various neurodegenerative states, especially given the evidence of non-apoptotic role of caspase-3 in mediating synaptic plasticity (D'Amelio, Cavallucci et al. 2010; Li, Jo et al. 2010; D'Amelio, Sheng et al. 2012). It seems unwise to target a widely expressed protease such as caspase-6 in the brain, for example, when we still do not understand the basic mechanisms of its activation and deactivation, particularly in disease states.

5.3 Implications of caspase activation in non-apoptotic processes

Caspases in physiological neuronal functions:

It is becoming increasingly clear that caspases have non-apoptotic functions in the nervous system. For example, caspases have been shown to play roles in synaptic plasticity (Lu, Wang et al. 2006; Li, Jo et al. 2010), dendritic pruning in Drosophila (Kuo, Zhu et al. 2006; Williams, Kondo et al. 2006), chemotropic responses of retinal growth cones in Xenopus (Campbell and Holt 2003), neurite outgrowth (Westphal, Sytnyk et al. 2010), and olfactory sensory neuron development and maturation (Ohsawa, Hamada et al. 2010).

The nervous system uses synaptic plasticity, or the ability of synapses to adjust their strength, to respond to prior experiences and adapt to changes in the environment. Two major forms of long-lasting synaptic plasticity in the mammalian brain are NMDA receptor-dependent long-term potentiation (LTP) and long-term depression (LTD), and the movement of AMPA receptors in and out of the synapse is a key mechanism underlying synaptic efficacy in LTP and LTD (Li and Sheng 2012). Fascinatingly, inhibition of caspase-3 or caspase-9 blocks LTD in CA1 neurons, and active caspase-3 is required for AMPA receptor endocytosis during LTD (Li, Jo et al. 2010). These caspases appear to be activated through a BAD- and Bax-dependent mechanism as Bad -/- and Bax -/- mice are defective for LTD (Jiao and Li 2011), and LTD is also blocked by overexpression of anti-apoptotic Bcl-XL or XIAP (Li, Jo et al. 2010). In contrast, disrupting caspase-3 via genetic or pharmacologic means has no effect on LTP (Li, Jo et al. 2010; Jiao and Li 2011; Olsen and Sheng 2012). These findings implicate key components of the mitochondrial apoptotic pathway in LTD induction but at much lower and more transient levels of activation than observed during apoptosis (Jiao and Li 2011; Li and Sheng 2012). This low-level caspase

activity is restricted to the vicinity of stimulated synapses to prevent the spread of LTD, which mirrors the specific localization of caspase activity to NGF-deprived sections of axons in my own experiments. Indeed, I speculate that the local activation of the machinery that mediates synaptic pruning is likely very similar to those that mediate axon pruning. Based on this prediction, caspase-6, but not Apaf-1, may also mediate synaptic plasticity.

Apaf-1-independent pathways:

The finding that caspase-9 acts independently of Apaf-1 during NGF deprivation-induced axon pruning is one of the most surprising discoveries born from this work. As I often tell my undergraduates, Apaf-1 and caspase-9 are the "Batman and Robin" or "Frodo Baggins and Samwise Gamgee" of neuronal apoptosis – they are far more effective together than apart. Though extremely rare, a few instances of Apaf-1-independent, caspase-9-dependent death have been reported. These incidents are worth mentioning and include the following:

a.) Using S100 extracts from SAK-2 or 293T cell lysates and *in vitro* assays, Patrick Mehlen's lab demonstrated that DCC induces caspase-3 activation in the absence of cytochrome *c* and in Apaf-1 -/- cell lysates, establishing that DCC-induced caspase-3 activation is apoptosome-independent. In addition, DCC-induced caspase-3 activation was not accompanied by caspase-9 cleavage even though caspase-9 is clearly required as immunodepletion of caspase-9 inhibited DCC-induced caspase-3 activation (Forcet, Ye et al. 2001).

b.) The Zacksenhaus lab demonstrated that procaspase-9 is processed in Apaf-1 -/- myoblasts (but not fibroblasts) treated with cytotoxic drugs, and caspase-9 ablation

prevented drug-induced apoptosis in both cell types. These results suggest that the requirement and coupling of caspase-9 to Apaf-1 depend on the context (Ho, Li et al. 2004; Ho and Zacksenhaus 2004).

c.) The Kurata lab observed caspase-9 activation in Apaf-1 -/- spleen and bone marrow cells treated with staurosporine. This group also showed that procaspase-9 pre-exists in the mitochondria of these cells and can undergo auto-processing prior to the downstream cytosolic caspase cascade (Katoh, Sato et al. 2008).

d.) In another study using staurosporine, the Wesselborg lab demonstrated that this drug induces Apaf-1-independent caspase-9 activation in a variety of tumor cells overexpressing Bcl-2 and Bcl-XL as well as in murine embryonic fibroblasts, human lymphoma cells, and chicken DT40 cells (Manns, Daubrawa et al. 2011). Thus, staurosporine can induce caspase-9 activation and apoptosis independently of the apoptosome.

The mechanism of Apaf-1-independent caspase-9 activation has been best studied in the model of Patched (Ptc) dependence receptor-induced death. Dependence receptors can actively trigger cell degeneration in the absence of their respective ligands, and as previously described (see Introduction), dependence receptors such as p75NTR, DCC, Ptc, and TrkA are known to regulate neuronal death, axon guidance and axon pruning. Ptc receptors activate caspase-9 by forming a novel apoptosome-like complex called the dependosome (Mille, Thibert et al. 2009) that is comprised of the following members:

a.) TUCAN or NALP1, which (like Apaf-1) contain a CARD domain for interacting with caspase-9.

b.) DRAL, a LIM-domain adapter protein.

c.) Nedd4, an E3 ubiquitin ligase that polyubiqutinates caspase-9 to induce its activation (and not degradation) in this context (Fombonne et al., 2012).

This potential pathway will be exciting to explore because virtually nothing is known about how caspase-9 is activated during axon pruning. Future experiments should examine whether caspase-9 immunoprecipitates with any of the above proteins and test whether lentiviral knockdown of the above proteins inhibits axon pruning after local deprivation.

My results also indicate that cytochrome *c* (which binds to Apaf-1 during apoptosis) is released from mitochondria in deprived axons, but determining whether it is required for caspase-9 activation and axon-specific degeneration is heavily burdened by molecular and technical limitations: First, cytochrome *c* levels cannot be decreased without dire consequences due to this molecule's critical role in neuronal energetic processes (e.g. the electron transport chain). Second, determining whether cytochrome *c* is required and/or sufficient for axon pruning will require microinjections, which have proven to be exceptionally difficult in microfluidic chambers despite multiple efforts. Another informative research direction involves the essential features of caspase-9 that mediate its interaction with Apaf-1: the CARD domain (to bind Apaf-1), the active site (for protease activity), and the dimerization domain. Understanding which features of caspase-9 participate in axon pruning events will be critical for understanding how caspase-9 is

activated in axons without Apaf-1. Interestingly, caspase-9 cleavage is not required for caspase-9 activity during apoptosis (Riedl and Salvesen 2007), indicating that non-cleaved caspase-9 may be able to activate downstream caspase-3 and -6 during axon pruning. Caspase-9 activity can also be regulated via its phosphorylation by multiple kinase signaling pathways at multiple sites (Allan and Clarke 2009; Parrish, Freel et al. 2013). Thus, future attempts to fully understand Apaf-1-independent caspase-9 activation during axon pruning will need to examine a diversity of potential contributing factors.

Given the steadily growing evidence of non-apoptotic caspase functions in neurons, it is entirely plausible for these cells to possess alternative mechanisms that enable non-lethal caspases activation. When a neuron needs to die, it can do so beautifully: cytosolic cytochrome *c* binds the WD40 domain of Apaf-1 with a preference that far surpasses any other WD40 domain-containing protein, and caspase-9 activation on the apoptosome induces a rampant caspase cascade that leaves nothing behind. But what happens when a neuron needs to destroy a specific axon collateral without unleashing its mass mediators of destruction? Mature neurons, which shut down Apaf-1 expression, maintain the ability to degenerate their axons, and it makes sense for younger neurons (which are significantly more vulnerable to apoptosis) to take both Apaf-1 and full-fledged caspase-9 activation out of the equation to enable localized, caspase-mediated pruning without risking death.

Local translation and compartmentalization:

RNA-based mechanisms provide neurons with a highly adaptable link between extrinsic environmental signals and functional responses, especially in distal parts of the cell such as the axon. This feat is accomplished by localizing both protein-coding and non-

coding RNA in neurites followed by the regulated local translation of mRNA into protein. To achieve the spatially precise signaling required for neuronal function, neurons appear to create biological compartments that subdivide the cell into distinctly localized signaling domains (Hanus and Schuman 2013). For example, axons contain the functionally and anatomically distinct compartments of the growth cone, the axon initial segment, and the terminal arbor. Some compartments are less anatomical, such as plasma-membrane compartmentalization along the axon (Holt and Schuman 2013). Notably, the ability of cytochrome *c* to induce caspase-3 activation has been shown to depend on the cytosolic or membrane-bound localization of the protease (Krebs, Armstrong et al. 1999). This observation suggests that the highly spatialized nature of caspase activation during axon pruning may be due to their non-cytosolic compartmentalization in the axonal plasma membrane.

Such compartmentalized signaling and localized translation requires neurons to overcome the challenge of transporting and delivering specific molecules to their appropriate cellular destinations. Indeed, dysfunctional mRNA transport and delivery mediated by RNA-binding proteins (RBP; regulatory molecules that recognize specific RNA nucleotide sequences (Ray, Kazan et al. 2013)) have been implicated in Fragile X syndrome (due to mutations in the RPB FMRP) and spinal motor atrophy (Bear, Dolen et al. 2008; Liu-Yesucevitz, Bassell et al. 2011). Moreover, dysregulated local translation has recently been implicated as a key factor in autism (Gkogkas and Sonenberg 2013; Santini, Huynh et al. 2013). Defects in axonal protein synthesis likely contribute to the miswiring in neurodevelopmental disorders as regulated protein synthesis in the presynaptic compartment was recently shown to be critical for synapse formation (Taylor, Wu et al.

2013) and axon arborization (Hornberg and Holt 2013; Hornberg, Wollerton-van Horck et al. 2013; Kalous, Stake et al. 2013).

Additional regulation of axonal mRNAs is provided by non-coding RNAs, including miRNAs, which hold enormous potential for locally regulating mRNA stability and translation. In relation to axon pruning, our lab has made an intriguing observation about the differential localization of miR-29 in mature neurons and one of its targets, BACE (Hebert, Horre et al. 2008), which is the secretase that cleaves APP. The cleaved APP fragment generated by BACE has been shown to bind the death receptor DR6, leading to downstream caspase-6 activation and axon removal (Nikolaev, McLaughlin et al. 2009). Excitingly, we have found that miR-29 levels are upregulated only in the soma of mature neurons, therefore enabling this miRNA to maintain the survival of the cell body by inhibiting BH3-only protein synthesis while allowing the selective BACE- and caspase-6-mediated degeneration of axons. The somatic localization of miR-29 in mature neurons also means that any BH3-only mRNAs that are transported and translated in distal axons can actively participate in axon pruning mechanisms. Axon-targeted BH3-only mRNAs may escape the somatic repression of miR-29 due to masking by RBPs, which has been shown for other miRNA/mRNA target pairs (Kedde, Strasser et al. 2007) or possibly by being protectively chaperoned to axon transport machinery (Rage, Boulisfane et al. 2013).

Precise localization:

Throughout this project and even during earlier research experiences, I was always amazed by the ability of neurons to activate targeted, spatially restricted mechanisms that - in the midst of countless signaling factors and the need to integrate input from many

distinct projections and synapses - give rise to beautiful and precise circuitry. Neurons are clearly capable of discerning when to activate tightly regulated, specifically localized caspases in response to localized stimuli from when to robustly activate caspases simultaneously throughout the entire cell. Even in my own chamber experiments, any observed caspase activation and physical degeneration halts along the axon at the exact point where NGF becomes present again. The work presented here and by others demonstrates that axon pruning in response to axon-specific trophic factor deprivation is mediated by a complex, coordinated mechanism that involves kinase signaling, transport, transcription, translation, caspase activation, and safety brakes, as well as miR-29 in mature neurons. We are still trying to understand how a neuron determines, for example, where to deposit a newly transcribed mRNA along the axon for local translation that results in one branch's complete removal while its neighboring branch remains untouched. Is there a window of opportunity for the axon pruning mechanism to become reversed once initiated, and what components determine this potential saving period? Is there a threshold on caspase activation that, once tipped, ultimately results in death? Finding answers to these types of questions will promote the development of targeted therapeutics for patients suffering from developmental and neurodegenerative pruning disorders.

5.4 Concluding remarks

The work described here is the first to examine whether the axon degeneration that occurs during axon pruning is mediated by the same mechanism that degenerates axons during apoptosis. My results provide new insight into how neurons activate and regulate caspase-mediated destruction depending on the context of the insult. The finding that degenerating axons require classically apoptotic caspases but not the powerful caspase-activating protein Apaf-1 is complemented by the observation that mature neurons (which naturally lack Apaf-1 expression) remain permissive for axon pruning; removing the trigger for a lethal caspase cascade shows that neurons possess an alternative mechanism for caspase activation that promotes harmless axon removal. These data implicate critical components of the neuronal apoptosis pathway in axon-selective pruning and also identify key points of distinction that support life-long plasticity without a constant risk of death.

APPENDIX

Figure A.1: Caspase-6 is required downstream of c-Jun phosphorylation during local deprivation.

Caspase-6 -/- neurons (5 DIV) were maintained in NGF, globally deprived or locally deprived for 18 hours and probed for phosphorylated c-Jun (red) and cytochrome *c* (cyt *c*; green; staining control). Caspase-6 -/- neurons phosphorylate c-Jun in the soma during local deprivation just as reported in wildtype neurons (Mok, Lund et al. 2009). As expected, NGF-maintained cell bodies maintained their cytochrome *c* whereas NGF-deprived cell bodies lose their mitochondrial cytochrome *c*.

Figure A.1

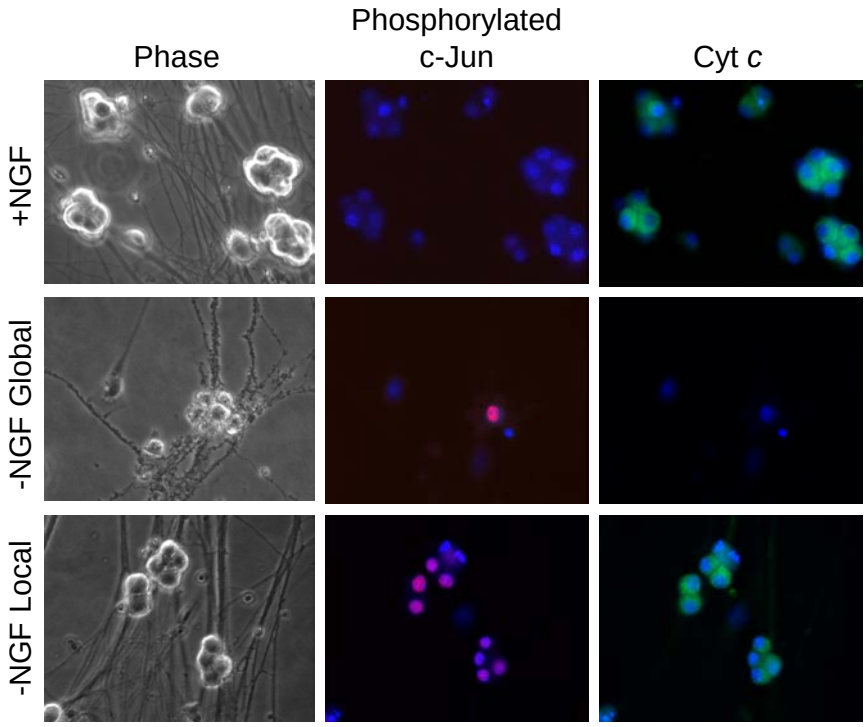


Figure A.2: Glutathione protects axons during local NGF deprivation.

Wildtype neurons (5 DIV) were globally or locally deprived with and without the reducing agent glutathione (10 mM) for 24 hours and probed for cleaved caspase-6 (green) and tubulin (red). Nuclei were labeled with Hoechst (blue), and NGF-maintained neurons served as controls. While glutathione addition failed to protect globally deprived neurons from apoptosis, it prevented axon degeneration induced by local deprivation. Glutathione can block the damaging effects of ROS and has also been shown to prevent cytochrome *c*-induced neuronal apoptosis in certain experimental contexts (Vaughn and Deshmukh 2008).

Figure A.2

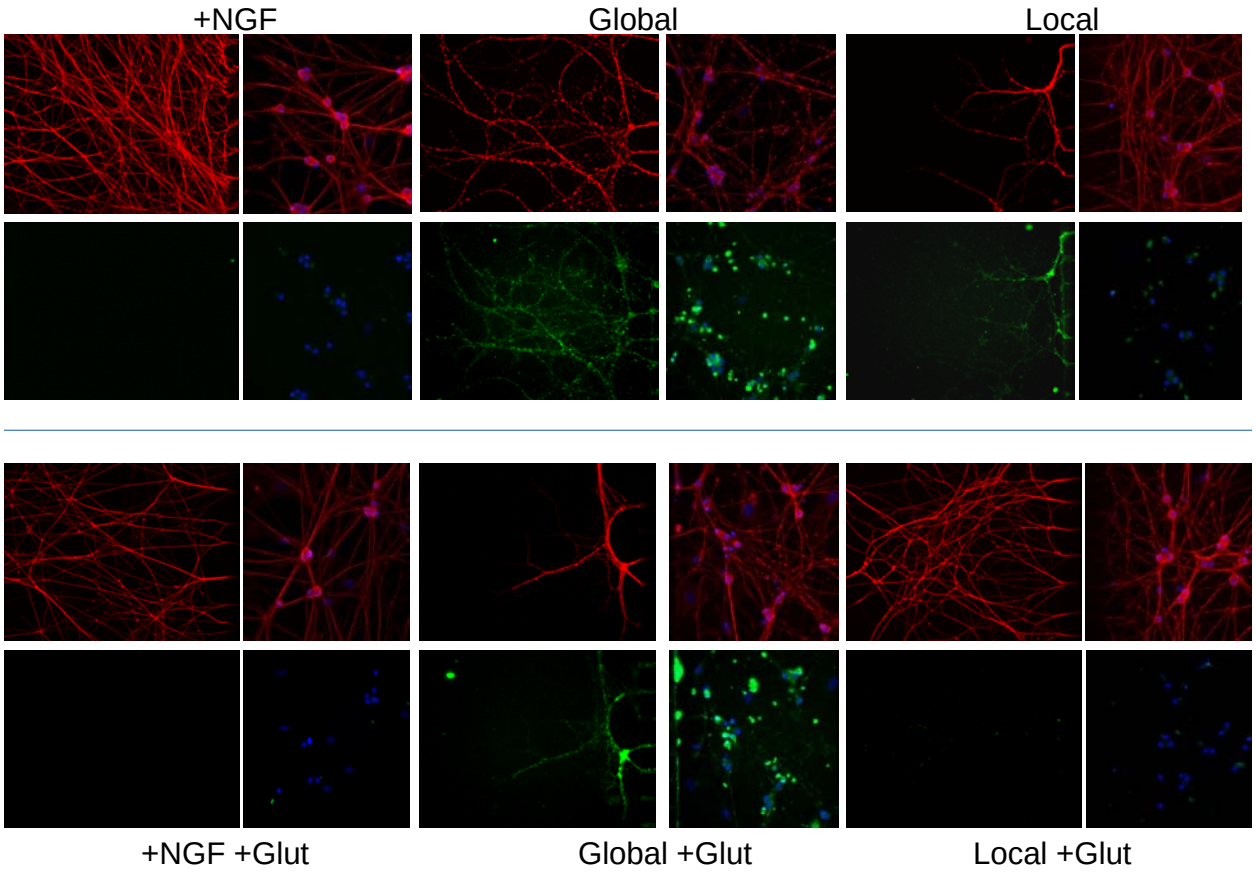
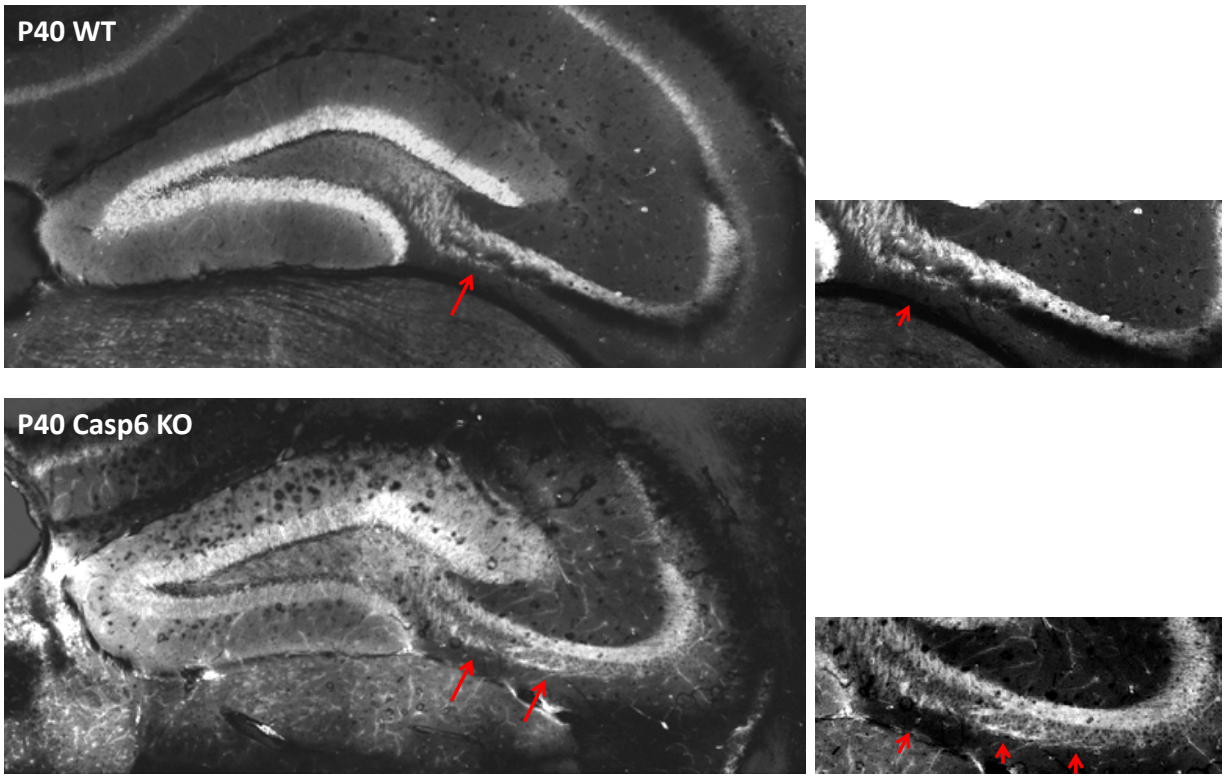


Figure A.3: Caspase-6 -/- mice demonstrate hippocampal pruning defects *in vivo*.

Brains of P40 wildtype and caspase-6 -/- mice were fixed, cut into coronal sections, and stained for calbindin. Images were acquired by mosaic imaging with an Olympus FV1000 confocal microscope. Whereas the infrapyramidal bundle (IPB) is typically pruned by P40 in wildtype mice (single red arrow), extensive IPB axon branches remain visible in the caspase-6 -/- hippocampus (multiple red arrows). This observation indicates the caspase-6 is also required for developmental axon pruning events in the CNS. Images were acquired with assistance from Dr. Vijay Swahari, Ayumi Nakamura and Dr. Vladimir Ghukasyan.

Figure A.3



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