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Molecular Underpinnings of Developmental Axon Degeneration

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Abstract

During nervous system development, an overabundance of neurons, axons, and synapses is selectively eliminated to establish functional circuits. Axons compete for trophic cues, with winners stabilizing and losers degenerating. This chapter explores molecular pathways involved in axon degeneration, focusing on progressive axon stabilization pathways (e.g., PI3K-AKT, MAPK/ERK, calpastatin, etc.) and regressive pro-degenerative pathways (e.g., death receptors, JNK, DLK, calcium, calpain, etc.). Additionally, distinct axonal death pathways include soma-derived pro- and anti-degenerative cues. A more comprehensive understanding of the molecular mechanisms behind axon degeneration, the transitions between early and late degeneration phases, and the role of cell death pathways will provide invaluable insights into not only the development of the nervous system but also related diseases.

5.1 Introduction

During development of the nervous system, neurons are initially overproduced and then selectively eliminated to sculpt circuits (Burek & Oppenheim 1996). This process is mediated by the access to limited supply of trophic factors, molecules required for neuronal survival, which are produced by innervation targets (Levi-Montalcini 1987). Neurons then compete for these

survival cues, and winners induce death in their neighbors via paracrine signaling to leave behind appropriately wired systems (Deppmann et al. 2008; Luo & O'Leary 2005).

Elimination of axons is a common feature in nervous system development, trauma, and neurodegenerative diseases. During nervous system development, an overabundance of neurons is born and many more axon branches are created than are ultimately required. At later stages of development, these excess neurons and axon branches are removed, leaving what is remaining to participate in the functional circuit. It is believed that the pathways involved in this process of developmental degeneration may also play a role in certain pathological conditions, where excessive pruning/degeneration occurs. As such, both pathological and developmental degeneration share morphological characteristics including beading and blebbing of axonal membranes as well as fragmentation and breakdown of the cytoskeleton (Martin et al. 1988; Raff et al. 2002; Wang et al. 2012). Consistent with this idea, neurodegenerative diseases such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and multiple sclerosis (MS) display axon loss morphologically indistinguishable from developmental degeneration (Coleman & Perry 2002; Luo & O'Leary 2005). Preservation of axonal structure has become an important therapeutic target for these disorders (Coleman & Perry 2002; De Vos et al. 2008; Luo & O'Leary 2005; Wang et al. 2012). While there are several excellent reviews on axon degeneration in pathologies like injury (Neukomm & Freeman 2014; Vargas & Barres 2007; Yong et al. 2021), there has been less emphasis of late on the emerging themes in developmental axon degeneration, which will be focused on herein. This chapter primarily focuses on developmental degeneration in vertebrate systems, but there are several excellent reviews on the seminal work performed in invertebrates (Furusawa & Emoto 2021; Lu & Mizumoto 2019; Williams & Truman 2005).

5.2 Models of Developmental Degeneration

There are two main categories of developmental axonal regressive events: retraction-like pruning and degeneration-like pruning (Riccomagno & Kolodkin 2015). Retraction occurs when axons draw back without shedding axonal material and can be observed during axon remodeling of the hippocampal infrapyramidal tract (IPT) (Bagri et al. 2003; Riccomagno et al. 2012). While retraction is a developmental regressive event, it is not generally considered a degenerative event. In contrast, developmental pruning eliminates circuitry by breakdown and elimination of the axonal material. It has hallmarks similar to non-developmental degeneration (e.g., injury-induced

Wallerian degeneration (WD)) including membrane blebbing/beading, formation of spheroids, loss of cytoskeletal integrity, and engulfment of debris by phagocytes (Nazareth et al. 2021; Van Broeckhoven et al. 2021; Yong et al. 2021). Pruning occurs in virtually all vertebrate neuron types; however, the molecular mechanism underlying stabilization versus degeneration is not as well characterized in the central nervous system (CNS) as it is in the peripheral nervous system (PNS). In the PNS, the decision to stabilize or degenerate is dictated by a competition for limiting amounts of target derived trophic factors like the neurotrophin family of ligands (Deppmann et al. 2008; Martin et al. 1992; Wang et al. 2012). Those that receive sufficient quantities of these trophic cues persist and those that do not, degenerate. Significantly, the mechanisms regulating degeneration induced by local and global triggers (such as trophic withdrawal on distal axons versus mass culture) are overlapping but distinct from caspase-dependent cell death pathways (Cusack et al. 2013; Geden et al. 2019). This may be important when an axon collateral is being degenerated while sparing the rest of the axonal arbor and/or entire neuron (Geden & Deshmukh 2016; Riccomagno & Kolodkin 2015).

Although all of the necessary components for developmental degeneration can be found within the axon, it is important to consider how non-neuronal cells influence degeneration. Such cells include ensheathing glia and phagocytic responders. Because of these roles for non-neuronal cells, a comprehensive understanding of degeneration requires its study in an *in vivo* context. In mammals like mice and rats, investigators have examined pruning in response to axonal interactions with repulsive cues like myelin, which is produced by non-neuronal Schwann cells and oligodendrocytes. Such an interaction occurs between septal neurons and the white matter of the corpus callosum (Park et al. 2010). Branch elimination is studied *in vivo* using whole mount organ staining and retrograde injection into final innervation targets (Glebova & Ginty 2004; Singh et al. 2008). While these approaches are advantageous because they are a reasonable approximation of general mammalian physiology (i.e., humans), conclusions drawn from them about axon degeneration are all based on inference because we can only examine developmental snap shots. Additionally, while genetic manipulations are both possible and common, mammals' slower gestation and maturation times, gamete inaccessibility, and smaller litter sizes can make the process of establishing a new transgenic line slow, difficult, and costly.

To overcome the disadvantages of mammalian *in vivo* studies, there are several other model systems well suited to study degeneration. Models such as zebrafish (*Danio rerio*), fruit flies (*Drosophila melanogaster*), and roundworms (*Caenorhabditis elegans*) are transparent, which allows for

real-time imaging of axon degeneration *in vivo*. These live imaging studies frequently require cell labeling with an endogenously produced fluorescent marker to visualize processes of interest, which is made possible by the insertion of a transgene. Conveniently, transgenic zebrafish, fruit flies, and *C. elegans* are all easily generated through established methods such as injecting transgene-carrying plasmids into a fertilized egg. Such methods are used in tandem with live imaging to study developmental degeneration signaling pathways in *C. elegans* and in *Drosophila* (Lu & Mizumoto 2019; Williams & Truman 2005).

In vitro models have also been useful to interrogate the molecular pathways underlying developmental axon degeneration. By and large, sympathetic or sensory neurons from mice or rats are used to examine degeneration pathways. This is due to their relative ease of isolation and culture. These neurons are also exquisitely dependent on neurotrophic factors for both soma and axon survival (Levi-Montalcini 1965; Martin et al. 1992). When sympathetic or sensory neurons are grown in culture and deprived of nerve growth factor (NGF), the axons degenerate at (18–24 h later) and the cell bodies apoptose shortly thereafter (Deckwerth & Johnson 1993). This timing is typical of a global deprivation paradigm whereby both the cell body and axons lack access to trophic factor. While this is a convenient method to study axon degeneration, it is a bit different from the *in vivo* situation, where trophic factors responsible for promoting cell body and axon survival are derived from target tissues, setting up a scenario whereby axon collaterals from the same neuron might be receiving different amounts of target-derived trophic factor. Compartmentalized culture systems like Campenot chambers and microfluidic devices have been very useful toward modeling local trophic deprivation paradigms (Yong et al. 2020c). For example, a three chambered device where cell bodies are in the middle and axon branches can project into one of two chambers have been instrumental in uncovering the notion that branches may compete with each other (Singh et al. 2008). Another model of local trophic deprivation comes from two chambered Campenot chambers and microfluidic devices. In this paradigm, cell bodies are established in NGF and allowed to project to the other side of the device, which also contains a trophic factor. Once axons have projected, trophic factor is withdrawn from the axonal, but not cell body, side of the chamber. In this scenario, the cell body survives, but axons degenerate within 36–48 h (Cusack et al. 2013; MacInnis & Campenot 2005; Yong et al. 2019). It is important to note that the timing of degeneration appears to be sensitive to the volume of media that axons are bathed in, suggesting the role of autocrine/paracrine degenerative factors, which will be discussed more in the next section. Interestingly, the mechanism by which

this degeneration occurs in local trophic deprivation appears to be distinct from the mechanism triggered in response to global deprivation. This axonal deprivation also triggers long-distance regressive signaling, which we have previously reviewed (Pathak et al. 2021).

5.3 Stages of Axon Degeneration

Axon degeneration is an evolutionary conserved process that can be activated by different stimuli, including trophic factor deprivation, mechanical damage, oxidative stress, and toxic drug incubation. After a degenerative trigger, axons undergo three distinct stages of degeneration: latent, transition, and catastrophic.

1. The latent phase varies in length of time depending on the trigger (typically 18–20 h after global trophic withdrawal in MFDs). In developmental degeneration, several factors are known to influence the length of this latency phase including transcription, anterograde transport of degenerative factors, activation of death receptors, and activation of caspases (Deckwerth & Johnson 1993; Deshmukh & Johnson 1997; Gamage et al. 2017a; Maor-Nof et al. 2016; Simon et al. 2016; Singh et al. 2008; Yong et al. 2019). In WD scenarios, there is a very short latent phase (roughly an hour after enucleation in MFDs) (Yong et al. 2020a). The molecular pathways regulating the duration of the latent phase in injury contexts is less well characterized; however, it does not appear to rely on de novo synthesis or death receptor activation (Yong et al. 2020a).

2. We have previously defined the onset of the transition phase with the formation of spheroids (Yong et al. 2019). These structures were first observed in spinal cord, distal to a site of injury by Ramon y Cajal, who indicated that "the voluminous balls along the nerves were the seat of destructive process," as they appeared earlier than nerve fragmentation (Ramón y Cajal 1928). Similar to injury in the CNS, crushed peripheral nerve fibers swell, causing a beaded spherical morphology (Gershenbaum & Roisen 1978). Axonal spheroids are also observed in the aging brain (Bridge et al. 2009), traumatic brain injury (TBI) (Newell et al. 1999), and many neurodegenerative diseases including Alzheimer's disease (AD) (Stokin et al. 2005), Parkinson's disease (PD) (Galvin et al. 1999), and amyotrophic lateral sclerosis (ALS) (Carpenter 1968). We recently found that spheroids arise at the transition between latency and catastrophic degeneration in both trophic

withdrawal and injury (Yong et al. 2019, 2020a). The function of axonal spheroids in degeneration has been largely overlooked. However, we have suggested that the rupture of growing spheroids corresponds to the release of a pro-degenerative molecule that helps a field of susceptible axons coordinately enter catastrophic degeneration. This transition phase lasts 20–40 min (Yong et al. 2019).

3. The final stage of degeneration is a rapid and near synchronous "catastrophic"/execution phase, in which the cytoskeleton disintegrates (Beirowski et al. 2005; Rosenberg et al. 2012; Wang et al. 2012). In the catastrophic phase, molecules related to cytoskeleton breakdown, such as calpain, drive the final stage of axon disintegration (Ma et al. 2013; Salvadores et al. 2017). The degradation of calpastatin and activation of calpain are implicated in the common downstream pathways shared by both developmental degeneration and WD (Yang et al. 2013). Importantly, re-supplementation of NGF in previously trophically deprived axons in the early latent phase, but not in the late catastrophic phase, is able to reverse the degeneration progress in a manner dependent on *de novo* synthesis, suggesting the existence of "point of no return" in axon and neuronal death (Deckwerth & Johnson 1993).

5.4 Role of Cell Death Pathways in Developmental Axon Degeneration

Neuronal axons can be 1000 times larger in surface area and volume than their cell bodies (Friede 1963), giving rise to demanding axonal transport needs that make them susceptible to rupture and "supply chain issues." The intense metabolic demands of these transport processes combined with those of neuronal ion pumps result in extreme sensitivity to decreases in ATP availability and, subsequently, vulnerability to degeneration (Fricker et al. 2018). Axons typically degenerate before the neuronal soma must choose how it will die, a phenomenon sometimes termed "dying back" for its retrograde progression (Conforti et al. 2014; Raff et al. 2002). The interplay between axonal and neuronal death programs is complex and frequently involves multiple exchanges between the two compartments of the neuron.

A classic model for developmental pruning is trophic withdrawal. In sympathetic and some sensory neurons, this is accomplished by removing NGF either from the entire neuron or locally on axons using compartmentalized culture approaches. The mechanism by which NGF-TrkA signaling prevents apoptosis and degeneration is through activation of the pro-survival

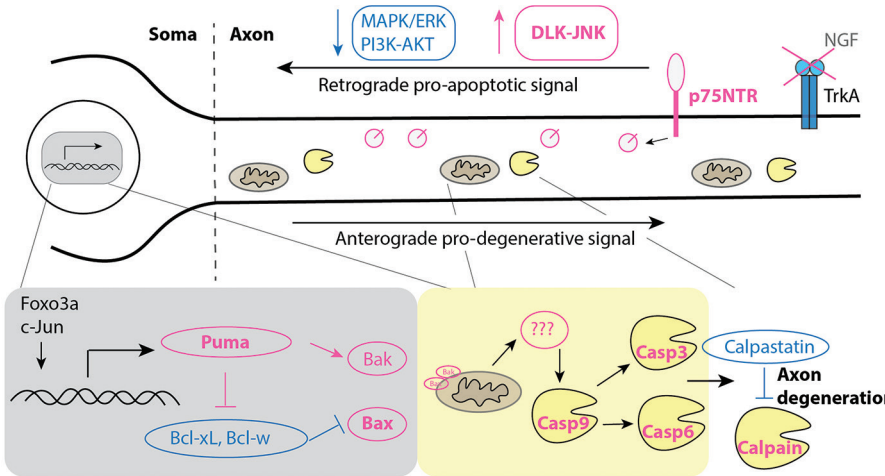

Figure 5.1 Developmental axon degeneration pathways. When neurons are deprived of NGF, the retrograde pro-apoptotic pathway initiated from p75NTR is activated, with decreased ERK and AKT signaling and increased DLK/JNK activity. The upregulation of transcriptional factors leads to increased expression of the pro-death BH3-only gene, Puma and inhibition of Bcl-2 family genes (Bcl-xL, Bcl-w) in the cell body. Puma induces anterograde pro-degenerative signaling by activating Bax. APAF-1 is dispensible for trophic withdrawal induced pruning so that the mechanism by which Bax transloaction induces caspase activation remains unclear. Activation of downstream caspase cascade leads to degredation of calpastatin and catastrophic axon degeneration.

PI3K-AKT (phosphoinositide 3-kinase-AKT/protein kinase B) and MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathways.

The molecular pathways underlying local axon degeneration in development are well documented (Figure 5.1). Loss of these trophic signaling pathways initiates a retrograde activation of prodegenerative JNK and DLK signaling (Ghosh et al. 2011). Tessier-Lavigne and colleagues as well as Yaron and colleagues observed that locally depriving distal axons of NGF increased somal expression of the pro-apoptotic protein Puma (Maor-Nof et al. 2016; Simon et al. 2016). Puma overcomes the inhibition by pro-survival factors Bcl-xL and Bcl-w and initiates axon degeneration, indicating an important role for the cell body in developmental axon degeneration (Courchesne et al. 2011; Maor-Nof et al. 2016; Pease-Raissi et al. 2017; Pease & Segal 2014; Simon et al. 2016, 2021). Puma activation then triggers a change in anterograde trafficking of an as-yet unknown degeneration-inducing protein along the axon, which could be prevented by transection from the soma. Curiously, this is the opposite of a speculative, anterogradely

trafficked anti-degeneration cue hypothesized to be lost during WD (Lubińska 1982). The Coleman group has definitively shown that the NAD+ producing enzyme, and NMNAT2 is likely to be this anterograde survival cue (Coleman & Freeman 2010; Coleman & Höke 2020; Gilley et al. 2013; Milde et al. 2013). This suggests at least two distinct pathways for axonal death: one for delivery of a soma-derived pro-degenerative cue, and one for transection-like deaths that could also involve a soma-derived anti-degenerative cue. However, it is likely that there are more trafficked degeneration regulators to be elucidated (Pathak et al. 2021).

It is important to note that there are differences in trophic withdrawal-induced cell death versus axon degeneration pathways. While Bax is involved in both axon degeneration and cell death, Apaf-1 appears to be dispensable for axonal pruning induced by local NGF deprivation (Geden et al. 2019). Apaf-1 is a crucial component of the apoptosome, along with Caspase 9 and cytochrome C, which raises questions about how Caspase-9 is activated during local deprivation in the absence of Apaf-1. This also casts doubt on whether the release of cytochrome C into the axoplasm is necessary to initiate the activation of Caspase 9 and the downstream effector caspases 3 and 6 following local trophic deprivation (Cusack et al. 2013; Simon et al. 2012). Interestingly, aging can also play a role in neuronal susceptibility to trophic withdrawal. Sympathetic neurons aged 28 days *in vitro* do not degenerate in response to global trophic withdrawal but are still susceptible to degeneration triggered by local withdrawal (Cusack et al. 2013). Deshmukh and colleagues have proposed that the expression of XIAP may limit axon-selective degeneration and may be the mechanism underlying the age-related difference in susceptibility to global versus local trophic deprivation (Cusack et al. 2013).

Much of the work surrounding axon degeneration has centered around components of the apoptotic machinery. More recent evidence suggests that engagement of necroptotic signaling proteins may also play a role in mediating axon demise following injury and other pathological triggers. Necroptosis is a form of regulated necrosis traditionally orchestrated by the receptor interacting protein kinases 1 and 3 (RIPK1 and RIPK3) and the pseudokinase MLKL (Degterev et al. 2005, 2008; Linkermann & Green 2014). Pharmacologic inhibition of RIPK1 or genetic knockdown of either RIPK3 or MLKL offered protection against injury-induced degeneration in both CNS and PNS axons (Arrázola et al. 2019; Ko et al. 2020). Given the frequent overlap in axonal death mechanisms across contexts, whether or not necroptosis also occurs in developmental degeneration merits further consideration.

SARMoptosis, a type of non-caspase-dependent cell death, has primarily been associated with injury-induced degeneration and cell death after viral infection (DiAntonio 2019; Leak & Dixon 2023; Panneerselvam & Ding 2015; Sankar et al. 2023). Surprisingly, loss of *SARM1* appears to accelerate the spread of axon degeneration in response to trophic deprivation (Gamage et al. 2017b), which contrasts with the dramatic protection observed in injured *Sarm1*$^{-/-}$ axons (Henninger et al. 2016; Osterloh et al. 2012).

5.5 Role of Death Receptors in Developmental Axon Degeneration

Death receptors (DRs) are members of the tumor necrosis factor receptor (TNFR) superfamily (TNFRSF) whose cytoplasmic tail contains a conserved death domain (DD). In spite of their name, DRs can trigger either cell death or survival. During development, DRs and their ligands regulate neuronal expansion, growth, differentiation, and regional pattern formation, processes critical for brain development (Twohig et al. 2011). In neurodegenerative diseases, DR expression and signaling mediate neuronal death, axon degeneration, excitotoxicity, and inflammation (Haase et al. 2008). Adapter proteins, along with other binding partners, are recruited by means of DD-interaction to transduce DR signals. For example, Fas, DR4, and DR5 primarily bind Fas-associated DD (FADD), while tumor necrosis factor receptor 1 (TNFR1), DR3, and DR6 primarily bind TNFR-associated DD (TRADD) to promote downstream signaling events (Mc Guire et al. 2011; Walczak 2013).

Previous studies describe that developmental axon pruning involves the death receptor p75 neurotrophin receptor (p75NTR) and one of its ligands, brain-derived neurotrophic factor (BDNF) (Park et al. 2010; Singh et al. 2008). Developmental cell death and axonal loss appear to be driven by competition between antagonistic trophic and "punishment" signaling pathways, which promote axon stability or degeneration, respectively (Deppmann et al. 2008; Singh et al. 2008). Interestingly, the punishment pathways appear to rely on autocrine/paracrine mechanisms allowing "strong" axons to expedite the elimination of weaker neighbors. The highly related TNFRSF members, p75NTR, TNFR1α, and DR6 are implicated in this extrinsic degeneration program in the peripheral nervous system (PNS) (Olsen et al. 2014; Park et al. 2010; Singh et al. 2008; Vilar et al. 2009; Wheeler et al. 2014). For example, mice deficient in p75NTR or its ligand, BDNF, display hyperinnervation of sympathetic target organs suggesting that p75NTR and BDNF are essential for axonal pruning *in vitr*o and *in vivo* (Lee et al. 1994; Singh

et al. 2008). Similarly, DR6 promotes axon degeneration in developmental paradigms (Olsen et al. 2014).

Axon degeneration induced by death receptors is also impacted by trophic signaling. When trophic signaling (e.g. NGF-TrkA) is high, TNFR family members are unable to induce degeneration. The molecular basis of this cross-talk remains unknown. Lack of NGF-TrkA signaling permits the prodegenerative cues originating from death receptors, including TNFR1a, p75NTR, and DR6, which have been implicated in developmental axon degeneration in the PNS (Barker et al. 2001; Olsen et al. 2014; Park et al. 2010; Wheeler et al. 2014). p75NTR and DR6 will be explored further below.

p75NTR:

p75NTR is widely expressed in the developing nervous system, including sympathetic, sensory, and motor neurons, as well as glial cells. The expression of p75NTR is diminished in adulthood but can be upregulated or re-expressed in response to injury or degenerative insult (Ibáñez & Simi 2012; Zeng et al. 2011). Similar to other DRs, the extracellular structure of p75NTR is highlighted by conserved cysteine-rich domains (CRDs), responsible for binding to all the neurotrophins (NGF, BDNF, NT-3, and NT-4/5) and their pro-forms with similar affinities (Chao 1994; Teng et al. 2010). Unlike other death receptors, p75NTR does not signal through the recruitment of adaptor proteins by its DD. Depending on the ligand and co-receptor, p75NTR can initiate pro-survival and pro-apoptotic signals and regulate cell cycle entry, axonal elongation, and synaptic transmission (Dechant & Barde 2002; Meeker & Williams 2015; Yamashita et al. 2005).

In the presence of NGF, p75NTR interacts with the TrkA receptor to promote sympathetic neuron survival through activation of phosphoinositide 3-kinase (PI3K)-AKT, Ras-mitogen-activated protein kinase (MAPK), and nuclear factor kappa light chain enhancer of activated B-cells (NF-κB) pathways (Gentry et al. 2004). However, when sympathetic neurons are maintained with low quantities of NGF or KCl, BDNF can activate the p75NTR and c-Jun N-terminal kinase (JNK) pathway to cause neuronal apoptosis (Bamji et al. 1998). During developmental sympathetic axon competition, BDNF secreted from active axons drives the activation of p75NTR on "losing" axons to cause degeneration (Singh et al. 2008). Moreover, p75NTR also modulates neural connectivity in the intact mature nervous system. Specifically, myelin triggers local axon degeneration by p75NTR-dependent sequestration of Rho guanine nucleotide dissociation inhibitor (Rho-GDI) and downstream activation of Rho and caspase-6 (Park et al. 2010). Importantly, p75NTR forms disulfide-linked dimers through the highly conserved Cys^{257}

in its transmembrane domain in both the liganded and unliganded condition. This suggests that the p75NTR is activated via conformational change rather than oligomerization. Upon neurotrophin binding, the close association between dimeric p75NTR DDs is transiently disrupted by a conformational change to allow binding of the caspase recruitment domain of RIP2 kinase for activation of the NF-kB pathway, competing with the binding of Rho-GDI for Rho activation (Lin et al. 2015; Tanaka et al. 2016; Vilar et al. 2009). Additionally, proteolysis of p75NTR in axons by the metalloprotease TNFα-converting enzyme (also known as a disintegrin and metalloprotease (ADAM)17) and γ-secretase are required for generation of the intracellular domain of p75NTR for retrograde degenerative signaling in response to trophic deprivation or oxidative stress (Kraemer et al. 2014; Pathak et al. 2018). Recent studies demonstrate that p75NTR mediates retrograde pro-apoptotic signaling in response to trophic deprivation or BDNF binding to p75NTR (Pathak et al. 2018). Whether and how p75NTR and other death receptors interact with retrograde and anterograde pro-degenerative signals in response to local NGF deprivation require further investigation.

It is widely appreciated that p75NTR is important for axon degeneration in sympathetic and basal forebrain cholinergic neurons (BFCNs) in response to trophic withdrawal; however, it has long been unclear what phase of axon degeneration p75NTR participates in (Park et al. 2010; Singh & Miller 2005; Singh et al. 2008). We recently reported that p75NTR acts during the latent phase of axon degeneration but not during the catastrophic phase (Yong et al. 2019, 2021). We also found that p75NTR is upstream of prodegenerative transcription, Rho and caspase activity, all of which are required to promote spheroid formation, thereby gating entry into the catastrophic phase of degeneration. It is possible that this pathway governs the length of the latency window. In the context of injury, p75NTR does not seem to impact the kinetics of degeneration; however, it is an integral protein for Schwann cell response to injury (Boerboom et al. 2017; Song et al. 2006; Yong et al. 2020a).

DR6:

DR6 is a single-pass transmembrane receptor that possesses four extracellular TNFR-like CRDs, a transmembrane domain, and a cytoplasmic DD related to those of all known DRs (Pan et al. 1998). DR6 is expressed in most human tissues, and abundant transcripts are detected in heart, brain, placenta, pancreas, thymus, lymph node, and several non-lymphoid cancer cell lines (Pan et al. 1998). Studies show that DR6 associates with TRADD but not FADD to couple to downstream caspases and activation of NF-κB and JNK (Kasof et al. 2001; Pan et al. 1998).

DR6 did not receive much attention in the field of degeneration until the identification of amyloid precursor protein (APP) as a potential ligand for DR6 in axonal pruning. It was reported that trophic factor deprivation could trigger β-secretase (BACE)-dependent shedding of surface APP, which binds DR6 and activates caspase 6 to promote axon degeneration (Nikolaev et al. 2009). However, further genetic and biochemical analyses revealed that the *n*-terminal portion of APP is not a *bona fide* ligand of DR6 (Kuester et al. 2011; Olsen et al. 2014). However, it has been suggested that DR6 and APP may act as co-receptors (Xu et al. 2015).

We previously described a role for DR6 in mediating degeneration in response to both trophic withdrawal and injury (Yong et al. 2019, 2020a). Interestingly, we found that DR6 is not required for the formation of spheroids. Instead, it is important for responding to the prodegenerative factors released by ruptured axons, placing its participation at the beginning of the catastrophic phase of degeneration (Yong et al. 2019). DR6 has also been shown to mediate spinal cord axonal degeneration induced by prion peptide (Wang et al. 2015). It is important to note that DR6 is not universally required for axon degeneration. For example, DR6 is not required for axonal degeneration in injured retinal ganglion cells (Fernandes et al. 2018; Vohra et al. 2010).

Interestingly, in addition to the cell autonomous function of DR6 in axon degeneration, a recent study has revealed a non-cell autonomous function of DR6 in Schwann cell proliferation (Colombo et al. 2018). As a novel substrate for ADAM10 and γ-secretase, neuronal DR6 was found to be cleaved and act *in trans* on Schwann cells to mediate their proliferation and myelination during development, independently of its cytoplasmic DD (Colombo et al. 2018).

Cultured sympathetic neurons deprived of NGF experience significant axon degeneration, with about 50% of neurites disintegrating within approximately 18 h (Deckwerth & Johnson 1993). However, this process can be delayed to roughly 24 h in the absence of *p75NTR* or *DR6* (Gamage et al. 2017b). By 36 h of trophic withdrawal in sensory neurons, *DR6*$^{-/-}$ *axons* displayed a very modest protection compared to wild-type controls (Olsen et al. 2014).

5.6 Calcium Signaling in Axon Degeneration

It is widely appreciated that axoplasmic calcium concentrations increase as axons degenerate (Wang et al. 2012; Yong et al. 2019). Such calcium transients act as temporal and spatial cues that trigger pruning and can be

compartmentalized to branches targeted for destruction (Adalbert et al. 2012; Kanamori et al. 2013; Vargas et al. 2015). Even within such a compartment, however, calcium is not evenly distributed. Axonal spheroids concentrate up to 10 times more calcium compared to surrounding axonal regions (Barsukova et al. 2012). These spheroids can then rupture, expelling calcium into the surrounding areas *in vitro* (Yong et al. 2019), the effects of which are unknown. The calcium that remains within the axon continues to increase, eventually activating the protease calpain and another set of destructive machinery, the ubiquitin proteasome system, which culminates in breakdown of the axon (Yang et al. 2013; Watts et al. 2003). During the latency phase, there are robust axoplasmic calcium transients, but calpain-induced degeneration is prevented. According to Tessier-Lavigne and colleagues, this is due to the gradual degradation of the calpain inhibitor, Calpastatin, following injury or trophic withdrawal. This degradation can be reversed in the absence of Sarm1 or Caspase-3, respectively, indicating an important mechanism for controlling the transition from latent to catastrophic degeneration (Yang et al., 2013).

Where does rising axoplasmic calcium come from? It has been observed that chelating extracellular calcium delays fragmentation in response to trophic deprivation (Johnstone et al. 2019; Vargas et al. 2015). This extracellular calcium passes through membrane channels in a variety of degenerative contexts; blocking calcium-specific channels or their subunits such as voltage-gated calcium channels (VGCCs) and sodium-calcium exchanger (NCX) can delay axonal degeneration in response to injury or oxidative stress (Barsukova et al. 2012; Dombert et al. 2017; LoPachin & Lehning 1997; Ribas et al. 2017; Tabata et al. 2018; Tedeschi et al. 2016). This suggests that calcium influx from the extracellular space via calcium channels and NCX reversal contribute to axon degeneration. However, the subtypes of calcium channels involved in the degeneration process may be dependent on neuronal types and the degenerative trigger. It is also possible that extracellular calcium enters non-selectively across the axonal plasma membrane via nanoscale ruptures, as observed in a multiple sclerosis model of axon degeneration (Witte et al. 2019).

Intracellular stores within the endoplasmic reticulum (ER) and mitochondria also contribute to the axonal calcium rise observed in degeneration. Preventing calcium release through pharmacological depletion of ER calcium stores, blockage of ryanodine and IP_3 receptors, disruption of the mitochondrial permeability transition pore (mPTP), or inhibiting store-operated calcium entry (SOCE) inhibit cytoskeletal degradation and secondary axonal degeneration (Orem et al. 2017, 2020; Staal et al. 2010; Tian et al. 2020; Villegas et al. 2014). As such, both extracellular calcium and

intracellular stores contribute to axon degeneration in various degenerative etiologies, though their exact mechanisms may vary depending on developmental context.

5.7 Contrasting Developmental Degeneration with Wallerian Degeneration

One of the best studied models of degeneration is injury-induced or WD. Similar to developmental degeneration, WD is characterized by break up of endoplasmic reticulum (ER), swelling, and local accumulation of mitochondria and fragmentation of neurofilaments (Vial 1958; Webster 1962). WD also has three stages of degeneration; however, in contrast to developmental degeneration, WD has a very short lag phase preceded by a rapid burst of calcium influx immediately after injury. The difference in latent phase duration between developmental pruning and injury paradigms suggests distinct pathways.

One of the most surprising differences between trophic withdrawal and injury is the role of Caspases. As outlined in Section 5.4, Caspases-3, -6, and -9, as well as Bax translocation have been implicated in trophic deprivation-mediated pruning (Cusack et al. 2013; Schoenmann et al. 2010; Simon et al. 2012). Notably, Deshmukh and colleagues make a compelling argument that degeneration induced by trophic degeneration of the entire neuron versus local axon trophic deprivation use distinct mechanisms, with global trophic deprivation more closely resembling conventional apoptotic pathways (Geden & Deshmukh 2016). In contrast, Caspase 3 and Bax are not essential for injury-induced degeneration (Simon et al. 2012; Whitmore et al. 2003).

In the past, we and others have found that pharmacologically inhibiting transcription blocks axon degeneration after trophic withdrawal (Deckwerth & Johnson 1993; Maor-Nof et al. 2016; Simon et al. 2016; Yong et al. 2019). However, this is not the case for injury-induced degeneration where axons lose their connection to the cell body and thus lose their ability to receive new transcripts. Collectively, these findings suggest that during the latent phase of trophic withdrawal-induced degeneration, *de novo* synthesis and caspase activation are required, whereas Wallerian degeneration (WD) does not require these processes.

It is known that injury-induced degeneration is regulated by the levels of nicotinamide adenine dinucleotide (NAD+) (Figure 5.2). Wallerian degeneration-slow (Wld^S) mutant mice contain an in-frame fusion protein composed of full-length nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) and N-terminal 70 amino acids of E4-type ubiquitin ligase

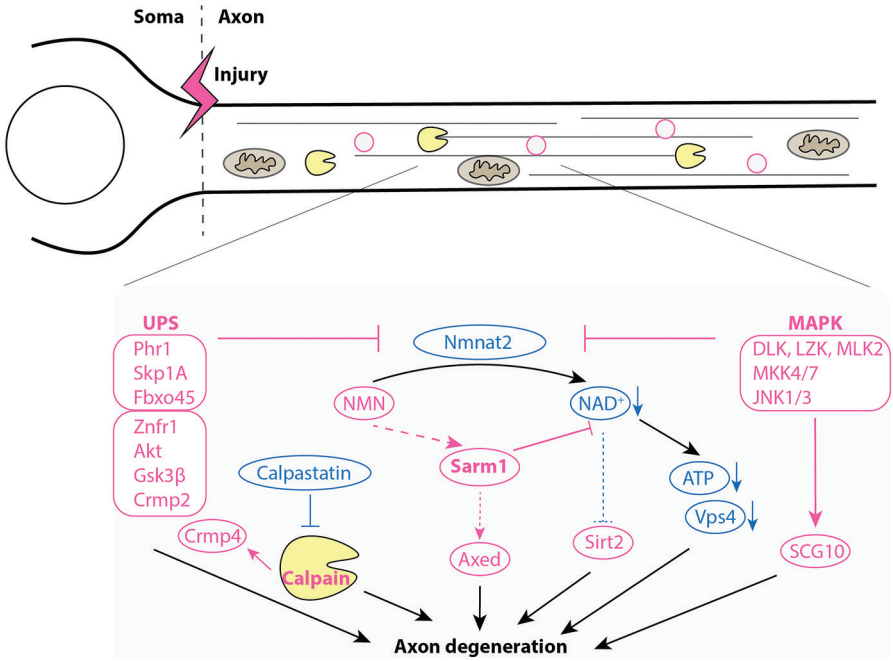

Figure 5.2 Wallerian degeneration pathways. In response to injury, activation of MAPK signaling cascade and UPS lead to NMNAT2 degradation, increase of NMN/NAD^+ ratio, and subsequent Sarm1 activation. Energy depletion along the axons and the cytoskeleton breakdown mediated by calpain, Axed, Sirt2, SCG10, and CRMP2/4 promote catastrophic axon degeneration.

(Ube4b) (Coleman & Freeman 2010; Conforti et al. 2000; Mack et al. 2001). This allows for increased biosynthesis of NAD^+, which prevents injured axons from degenerating. Likewise, axonal protection after injury can be provided by overexpressing the NMNAT enzyme (Magni et al. 2004). Three NMNAT mammalian isoforms have different subcellular localizations: Nmnat1 is localized to the nucleus; Nmnat2 is found in the cytoplasm and trafficked anterogradely in the axoplasm; and Nmnat3 is mostly localized to mitochondria, allowing compartmentalization of NAD^+ metabolic pools for diverse cellular activities (Di Stefano & Conforti 2013). When NAD+ catabolism is inhibited through loss of the NADase, Sterile alpha, and TIR motif containing 1 (Sarm1), injury-induced axon degeneration is also inhibited (Gerdts et al. 2013, 2015; Osterloh et al. 2012).

Do NAD+ levels also influence degeneration induced by trophic withdrawal? Neurons cultured from mice harboring *Wld*^{S} or overexpressing of

NMNAT display delayed degeneration in response to trophic withdrawal; however, the phenotype does not appear as dramatic as observed in injury indicating parallel and perhaps redundant pathways that are present in developmentally degenerating but not WD axons (Vohra et al. 2010; Gamage et al. 2017b). Interestingly, neurons lacking *Sarm1* remain susceptible to degeneration induced by trophic withdrawal (Gamage et al. 2017b; Osterloh et al. 2012). These findings suggest that the rate of NAD+ depletion determines the length of the latent phase in injured axons, but this does not apply to trophic deprivation.

Because of the morphological similarity between all forms of axon degeneration, it is tempting to speculate that disparate triggers funnel toward a common catastrophic degeneration pathway. Regardless of the degenerative trigger, the latent and catastrophic phases appear to be demarcated by the appearance of spheroids (Yong et al. 2021). Therefore, the regulators of latency in trophic deprivation or Wallerian degeneration are also upstream regulators of spheroid formation and rupture, within their respective contexts (Yong et al. 2019, 2020a).

Axon degeneration induced by trophic withdrawal and WD converge on the Calpain/calpastatin axis described above to enter the catastrophic phase of degeneration (Yang et al. 2013). We have previously found that spheroid formation and rupture are upstream of calpain activation after trophic deprivation (Yong et al. 2019). Whether and how spheroid formation and rupture leads to activation of degradation of calpastatin and activation of Calpain remains to be determined.

5.8 Conclusions and Future Directions

Developmental degeneration is an essential process in sculpting an efficient and functional nervous system. This type of pruning has long been studied apart from pathological degenerative triggers like Alzheimer's disease or injury. We now appreciate that although axonal degeneration can have many different triggers, the catastrophic phase of degeneration may be mediated by pathways common to different etiologies. While degeneration is described in many different developmental and pathological contexts, it remains to be seen whether these different "flavors" of degeneration are more like trophic withdrawal or more like injury (WD). Because many neurodegenerative disorders display impaired retrograde trafficking, which would mimic trophic withdrawal, it stands to reason that disorders like ALS or Parkinson's would be more similar to developmental degeneration. On the other hand, chemotherapeutic neuropathy, which is often the result of damaged neuronal

cytoskeleton, may be similar to WD. Determining whether different etiologies of degeneration can be binned between developmental or WD types will be a profitable avenue of future investigation.

Beyond understanding the differences in molecular mechanisms between different etiologies of degeneration, there remain several open questions:

1. Because we now appreciate that degeneration occurs in different phases, it will be important to place previously identified molecular players in this degeneration timeline. This will be critical as we seek to understand how transitions from latent to catastrophic phases occur.
2. Determining how cell death pathways, particularly non-apoptotic pathways, are repurposed to promote axon degeneration.
3. Understanding the functionally antagonistic relationship between trophic signaling and regressive signaling.
4. Examining the role of phagocytosis in mediating degeneration.

A better mechanistic appreciation of degeneration will not only inform a general logic for neural development but will also help us rationalize anti-degeneration therapies.

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