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Mechanisms of Pathological Axonal Degeneration

Ying Cao and Jing Yang

IDG/McGovern Institute for Brain Research, Center for Life Sciences, School of Life Sciences, Peking University, China
Corresponding Email: jing.yang@pku.edu.cn

Abstract

Pathological axonal degeneration is one of the common features of neurodegenerative diseases, which can occur independently from neuronal cell death. By interrupting the functional or structural connectivity of neurocircuits, such axonal pathology can directly contribute to the onset and progression of clinical symptoms in patients. Therefore, delay or prevention of axonal damage is indispensable for effectively treating neurodegenerative diseases. Notably, pathological axonal degeneration is often distinct from several known types of programmed cell death, for example, apoptosis, necroptosis, or pyroptosis. Instead, this destructive process is intrinsically linked to energy metabolism, particularly the co-enzyme nicotinamide adenine dinucleotide (NAD^+), within damaged axons. This book chapter reviews the history of the research field and highlights the landmark works elucidating the molecular mechanisms of pathological axonal degeneration. Also, critical questions that still await future investigations are discussed.

As one of the unique structures of neurons, axons act as the bridge between neurons and their innervating targets. By transducing action potentials, axonal connections form the foundation for neural development and functions. As a result, the structural and functional integrity of axons is essential for various neurophysiological processes, for example, sense, motility, memory, and cognition. Conversely, damage or destruction of axons causes the abnormality or breakdown of corresponding neural circuits, leading to

severe neuropathological defects such as numbness, pain, blindness, paralysis, ataxia, dementia, and even death.

Through decades of research, pathological loss of axons has been documented as a hallmark feature in almost all types of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (also known as Lou Gehrig's disease), multiple sclerosis, Guillain–Barré syndrome, Charcot–Marie–Tooth disease, glaucoma, traumatic neural injuries, and other types of central or peripheral neuropathy (Coleman, 2005; Coleman and Hoke, 2020; DiAntonio, 2019; Neukomm and Freeman, 2014; Wang et al., 2012). Further, it has become increasingly recognized that pathological axonal degeneration contributes to the onset and progression of clinical symptoms by directly interfering with normal neural functions. Therefore, molecular mechanisms underlying pathological axonal degeneration are integral to neurodegeneration, and preserving axonal structures inflicted by neurodegenerative insults should be indispensable for effective strategies to prevent, delay, or revert disease symptoms.

Research in the past decades has achieved some of the most important and exciting breakthroughs in unraveling pathological axonal degeneration. Such advances have opened up a new dimension to our understanding of this unique axonal pathology and neurodegenerative diseases in general. This book chapter aims to review the updated knowledge of pathological axonal degeneration, highlighting entry points that may eventually lead to conquering those currently incurable human diseases.

6.1 Overview of Pathological Axonal Degeneration

Pathological axonal degeneration is the programmed destruction of axons. Such axonal pathology can precede the demise of corresponding neuronal cells in neurodegenerative conditions, implicating the spatial and temporal separation of pathological axonal degeneration from neuronal cell death. In addition, pathological axonal degeneration may occur independently of neuronal death, that is, a neuron may survive after losing its axon. Therefore, although the loss of neurons and their axons are observed simultaneously in many neurodegenerative diseases, it is essential to distinguish pathological axonal degeneration as a self-standing process.

Pathological axonal degeneration in different disease scenarios shares similar morphological and functional features. This axonal pathology can be characterized in a simplified model by several temporally distinct but related

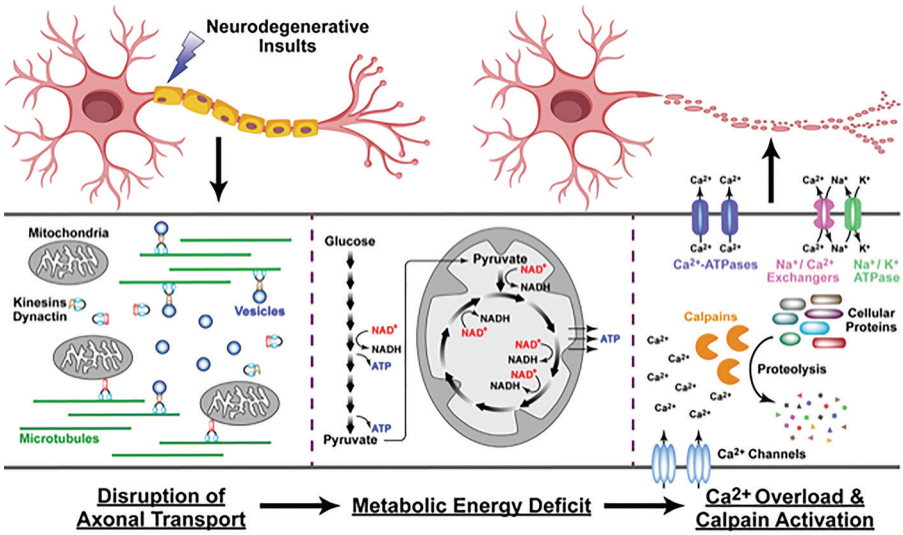

Figure 6.1 An overview of pathological axonal degeneration. Upon neurodegenerative insults, the concomitant or sequential occurrence of several key steps, including disruption of axonal transport, metabolic energy deficit, Ca^{2+} overload, and calpain activation, designates the process of pathological axonal degeneration.

steps (Figure 6.1). Disruption of axonal transport is often the first detectable sign of axonal damage. It has been well known that axonal transport moves a variety of cargoes, for example, mitochondria, vesicles, mRNAs, cytoskeletons, and other proteins, in both anterograde and retrograde directions within axons (De Vos et al., 2008; Maday et al., 2014; Millecamps and Julien, 2013). As a result, the disrupted axonal transport causes the focal accumulation of such cellular components, producing axonal swelling. Those swellings, ranging from several to tens of micrometers, may be randomly distributed distal to a site of neurodegenerative damage. The specialized motor proteins, that is, Kinesins and Dynactin, enact axonal transport (Goldstein and Yang, 2000; Maday et al., 2014; Schroer, 2004). Because all these motor proteins require ATP as the energy source to migrate on microtubule bundles, their malfunction can result from an inadequate energy supply in damaged axons.

Indeed, in concomitant to or following the disruption of axonal transport, the next step of pathological axonal degeneration is the profound defect of energy homeostasis. This metabolic event involves the pathological depletion of the co-enzyme nicotinamide adenine dinucleotide (NAD^+) and the

vital energy source ATP. Importantly, this loss of axonal energy disables all essential biological processes such as axonal transport, protein synthesis or degradation, and transduction of action potentials. Such a systematic shutdown collectively results in the functional failure of axons even before any further structural destruction. This step of pathological axonal degeneration is sufficient to interfere with the normal function of neural circuits and likely correlate with the onset of disease symptoms.

The following step of pathological axonal degeneration is the massive Ca^{2+} overload. Intracellular Ca^{2+} levels in healthy axons are typically maintained within the range of submicromolar concentrations by the combinatorial action of Ca^{2+}-ATPases and Na^{+}/Ca^{2+} exchangers. Of importance, Ca^{2+}-ATPases depends on the energy input from ATP. Although Na^{+}/Ca^{2+} exchangers do not directly consume ATP, their function relies on the Na^{+} gradient across the plasma membrane established by Na^{+}/K^{+}-ATPase that requires ATP as the energy source. Therefore, the metabolic energy deficit occurring in the previous step of pathological axonal degeneration leads to the inevitable buildup of Ca^{2+} influx via various Ca^{2+} channels. Such Ca^{2+} overload then triggers a specific group of Ca^{2+}-activated proteases, that is, calpains, which rapidly proteolyze axonal cytoskeletons and other cellular proteins (Goll et al., 2003; Liu et al., 2004). Also, blebbing and fragmentation of the plasma membrane of axons happen, morphologically resembling that observed in programmed cell death. This breakdown of structural integrity is the terminal, irreversible step of pathological axonal degeneration.

Notably, these steps of pathological axonal degeneration may proceed with drastically variable timelines under different neurodegenerative conditions, ranging from days to months or even years. Despite such temporal divergence, research has elucidated that pathological axonal degeneration shares specific molecular mechanisms (Coleman, 2005; Coleman and Hoke, 2020; DiAntonio, 2019). We will discuss several signaling pathways extensively investigated in this process of axonal pathology.

6.2 Apoptotic Pathway

As described above, pathological axonal degeneration shares certain aspects of morphological similarity to programmed cell death, for example, blebbing and fragmentation of the plasma membrane. Therefore, research efforts have been made to determine whether the signaling pathways involved in programmed cell death may also participate in such axonal pathology. The classic apoptotic pathway is one of the most prominent types of programmed cell death (Budihardjo et al., 1999; Elmore, 2007). In the intrinsic mechanism

of apoptosis, releasing cytochrome c from mitochondria via BAX or BAK proteins is the primary trigger of cell death. The released cytochrome c binds to the apoptotic protease-activating factor 1 (APAF1) protein in the cytosol, forming a complex known as the apoptosome. This complex then recruits and activates caspase-9. The activated caspase-9 further cleaves to activate the effector caspases, that is, caspase-3, caspase-6, and caspase-7. These active caspases degrade many essential cellular proteins, thus destroying biological processes critical for cell survival. This intrinsic apoptotic mechanism can be initiated under various conditions, including loss of trophic signals, DNA damage, and other cellular stresses. In addition to such intrinsic signals, extrinsic stimuli also induce apoptotic cell death. In particular, the engagement of Fas ligand (FasL) or tumor necrosis factor-alpha (TNF-alpha) to their specific receptors, that is, Fas or tumor necrosis factor receptor 1 (TNFR1), respectively, leads to activation of the downstream caspase-8. The active caspase-8 then directly cleaves to activate the effector caspases, such as caspase-3, to induce apoptotic cell death. Alternatively, the active caspase-8 cleaves the BID protein into tBID. tBID then promotes the activation of BAX or BAK that causes the release of cytochrome c from mitochondria, thus establishing the crosstalk with the intrinsic mechanism. This combinatory action of the intrinsic and extrinsic mechanisms consists of the apoptotic pathway.

Apoptosis is broadly involved in neurodevelopment. In particular, studies have shown that axonal pruning, also known as developmental axonal degeneration, often depends on the apoptotic pathway (Geden and Deshmukh, 2016; Schuldiner and Yaron, 2015). Molecular mechanisms governing developmental axonal degeneration have been discussed in a previous chapter of this book. In contrast, evidence supporting the involvement of the apoptotic pathway in pathological axonal degeneration is relatively limited. For example, the activation of caspase-3 and caspase-6 was detected in the neuronal cells inflicted by Alzheimer's disease (Albrecht et al., 2009; Selznick et al., 1999). Although the overexpression of the anti-apoptotic BCL-2 protein could reduce the accumulation of amyloid plaques and neurofibrillary tangles in the mouse model of Alzheimer's disease (Rohn et al., 2008), the effect of such apoptosis inhibition on pathological axonal degeneration was unclear. Also, the caspase-9 activation was observed in the motor neurons damaged in amyotrophic lateral sclerosis (Inoue et al., 2003). The genetic deletion of BAX blocked the cell death of motor neurons in the mouse disease model induced by the overexpression of mutant human superoxide dismutase 1 (SOD1) protein but did not affect the loss of motor axons (Gould et al., 2006). Similarly, the BAX deletion preserved the survival of cerebellar granule neurons but not their axonal structures in the mouse model of prion disease (Chiesa et al.,

2005). Further, in the traumatic injury of optic nerves or the glaucoma condition, the genetic deletion of BAX or BAK effectively inhibited the death of retinal ganglion cells while failing to stop the pathological degeneration of optic axons (Libby et al., 2005; Whitmore et al., 2003). Therefore, a general theme has been proposed that neuronal cell death and pathological axonal degeneration are regulated separately, with the apoptotic pathway only controlling the demise of neuronal cells in many neurodegenerative conditions.

A few examples currently suggest the role of apoptosis in pathological axonal degeneration. For instance, the caspase-3 activation occurred in the dying dopaminergic neurons in Parkinson's disease (Hartmann et al., 2000). While the BAX deletion fully protected the dopaminergic neurons in the mouse disease models induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine, this genetic blockage of apoptosis also partially mitigated the destruction of their axonal projections (Kim et al., 2011; Vila et al., 2001). In addition, there was the activation of caspase-3 and caspase-8 in the brain regions damaged by traumatic injuries (Clark et al., 1999; Zhang et al., 2003). The genetic deletion of caspase-8 was reported to lessen the death of cortical neurons and their axons in the mouse disease model (Krajewska et al., 2011). However, the involvement of the apoptotic pathway in this particular context might be indirect. At least in the in vitro cultured neurons, the caspase activation could not be detected in axons upon traumatic neural injuries, and the genetic deletion of BAX or caspases failed to prolong the survival of traumatically injured axons (Simon et al., 2012). It is plausible that while the apoptotic pathway has essential roles in neurodevelopment, its participation in pathological axonal degeneration is restricted.

6.3 Necroptotic Pathway

Necroptosis is another critical type of programmed cell death under pathological conditions (Christofferson and Yuan, 2010; Sun and Wang, 2014; Xu et al., 2021). The engagement of TNFR1 or Fas receptors represents a main trigger of the necroptotic pathway. Rather than inducing the downstream apoptotic signals described above, a kinase cascade involving RIPK1, RIPK3, and MLKL can initiate necroptotic cell death. Although RIPK1 also controls the caspase-8 activation, RIPK3 and MLKL are involved explicitly in necroptosis but not apoptosis. MLKL oligomerizes upon its phosphorylation by RIPK3 and then inserts into the plasma membrane to form the pore structure. This MLKL-mediated membrane permeabilization causes the rapid flux of intracellular and extracellular components such as proteins and ions.

Such destruction of the plasma membrane integrity leads to necrosis-like cell death.

Research efforts have begun to investigate the potential roles of necroptosis in neurodegenerative diseases. For instance, the phosphorylation of RIPK3 and MLKL was detected in the spinal cord tissues of patients with amyotrophic lateral sclerosis or multiple sclerosis (Ito et al., 2016; Ofengeim et al., 2015). The genetic deletion of RIPK3 delayed the disease symptoms, including the degeneration of motor axons, in the mouse models of amyotrophic lateral sclerosis induced by the mutant human SOD1 or the loss of Optineurin protein (Ito et al., 2016). Also, the RIPK3 deletion modestly protected the axonal structures inflicted by the cuprizone-induced demyelination in the mouse model of multiple sclerosis (Ofengeim et al., 2015). In addition, the RIPK3 deletion lessened the neuronal loss and the behavioral defects in the mouse models of traumatic brain injury or spinal cord injury (Fan et al., 2016; Liu et al., 2018), although the effect of such necroptosis inhibition on pathological axonal degeneration was not examined. Further, the genetic deletion of MLKL mitigated the axonal damage in the mouse experimental autoimmune encephalomyelitis that recapitulates the condition of multiple sclerosis (Zhang et al., 2019). However, recent studies have reported that the expression of MLKL protein appears undetectable in the central nervous system, and the MLKL deletion fails to exert any protection in the mouse model of SOD1-induced amyotrophic lateral sclerosis (Wang et al., 2020). Therefore, the definitive function of the necroptotic pathway in pathological axonal degeneration awaits clarification. While the genetic deletion or the pharmacological inhibition of RIPK1 has been reported to confer beneficial effects under various neurodegenerative conditions (Mifflin et al., 2020; Yuan et al., 2019), for example, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and traumatic neural injuries, it remains undetermined whether RIPK1 may directly instruct pathological axonal degeneration via the necroptotic pathway in such disease scenarios.

6.4 Pyroptotic Pathway and Other Cell Death Pathways

In addition to apoptosis and necroptosis, other pathways of programmed cell death exist in disease conditions. For instance, pyroptosis has been recently uncovered to control the death of immune cells such as macrophages (Broz and Dixit, 2016; Kovacs and Miao, 2017; Shi et al., 2017). In response to various immune stimuli, for example, intracellular pathogenic infection, activating caspase-1, caspase-11, or other caspases leads to the cleavage of gasdermin proteins. The cleaved gasdermins, for example, gasdermin D

(GSDMD), then oligomerize and insert into the plasma membrane to form the pore structure. Like the MLKL-mediated necroptosis described above, gasdermin-triggered membrane permeabilization causes rapid leakage of proteins and ions, leading to pyroptotic cell death. Although the GSDMD cleavage and pyroptosis of retinal neurons or enteric neurons were reported under specific disease conditions in mice (Huang et al., 2021; Ye et al., 2020), whether the pyroptotic pathway might directly participate in pathological axonal degeneration has been untested. Also, certain types of neuronal cells, for example, cortical neurons, exhibit the minor expression of gasdermins (Tsuchiya et al., 2019), likely precluding the involvement of the pyroptotic pathway in the pathological loss of their axons.

Besides pyroptosis, several additional types of programmed cell death, for example, ferroptosis, parthanatos, and NETosis, have garnered attention for their potential roles in disease scenarios. Ferroptosis is an iron-dependent death pathway characterized by significant oxidative stress and excessive oxidation of lipids within cells (Lei et al., 2019; Xie et al., 2016). Accordingly, ferroptotic cell death can be alleviated by antioxidant molecules. Parthanatos is induced when cells suffer extensive DNA damage resulting in the overactivation of poly(ADP-ribose) polymerases (PARPs) proteins (David et al., 2009; Fatokun et al., 2014). The overactive PARPs produce a significant amount of poly(ADP-ribose) in cells. This poly(ADP-ribose) accumulation induces the release of apoptosis-inducing factor (AIF) and endonuclease G proteins from mitochondria, which then causes nuclear condensation and cell death. NETosis is a unique type of cell death occurring in neutrophils (Remijsen et al., 2011; Thiam et al., 2020). Neutrophils shed their genomic DNA to entrap invading pathogens through the NETosis process. Although these different pathways have been indicated in various pathological conditions, their potential roles in neurodegenerative diseases remain mostly uncharted. Moreover, it is worth noting that novel pathways of programmed cell death are still being discovered and might act in specific contexts of pathological axonal degeneration.

6.5 NAD^+-dependent Pathway

Significant research advances have revealed that pathological axonal degeneration can intrinsically link to energy metabolism, particularly NAD^+, within axons. This research direction is traced back to 1850 when the British physiologist Augustus Waller reported that the traumatic injury of peripheral nerves in frogs could lead to the stereotyped dismantling of axonal structures in the following days or weeks (Waller, 1850). In recognition of this historical

discovery, pathological axonal degeneration is also known as Wallerian degeneration. As stated in his original publication, "*...it is particularly with reference to nervous diseases that it will be most desirable to extend these researches....*" Augustus Waller predicted the potential significance of pathological axonal degeneration in human diseases. However, the relevance of Wallerian degeneration to neurodegenerative conditions had been long overlooked. In particular, it has been a common belief, even till now, that an axon cannot maintain its own survival independently of the neuronal cell body. Indeed, as described above, neuronal cell bodies provide many essential cellular components via axonal transport, for example, mRNAs, ribosomes, proteins, vesicles, and mitochondria. Therefore, a traumatic injury to axons disconnects such axonal transport and, as a result, deprives the critical trophic support of neuronal cell bodies. In light of such a view, Wallerian degeneration occurring in the context of traumatic neural injuries had been regarded as merely a passive, necrotic process of axonal destruction, denying any involvement of molecular mechanisms.

6.5.1 WLD^s Mutant Protein

Modern research into Wallerian degeneration was re-ignited in the 1980s when British neuroscientists challenged the long-held view of Wallerian degeneration as a passive death event. They serendipitously identified a mutant mouse line, now called *Wallerian degeneration slow* (*Wld*^{s}), in which the breakdown of traumatically injured axons in sciatic nerves was delayed for weeks (Lunn et al., 1989). Moreover, such axons completely disconnected from neuronal cell bodies in the *Wld*^{s} mice could still sustain the ability to transduce action potentials after injury. This observation indicated for the first time that Wallerian degeneration would be an active process of axonal death instructed by some unknown signaling mechanisms. With this discovery of the *Wld*^{s} mutant mouse, the research field has embarked on the endeavor to unravel the signaling pathway of Wallerian degeneration in the following decades.

Studies then showed that the *Wld*^{s} mutant gene functions in a neuron-intrinsic manner (Perry et al., 1990a) and is autosomal dominant (Perry et al., 1990b). The decade-long research of map-based cloning eventually identified *Wld*^{s} as the triplication mutation of an 85-kb region on chromosome 4 of the mouse genome (Coleman et al., 1998; Conforti et al., 2000). This 85-kb region originally contains the entire coding sequences of nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) and retinol binding protein 7 and also the partial 5'-coding sequence of ubiquitin conjugation factor E4B (UBE4b).

Notably, a new mutant protein is generated on the boundary between every two triplicated regions by the in-frame fusion of the N-terminal 70 amino acids of UBE4b with the full-length protein of NMNAT1. This UBE4b/NMNAT1 fusion protein has been proven as the causative factor conferring axonal protection against neurodegenerative insults, thereby named the WLD^s mutant protein (Mack et al., 2001). However, studies have later demonstrated that the axonal protection afforded by the WLD^s protein does not directly involve the ubiquitin system as implicated by UBE4b. On the other hand, NMNAT1 is one of the enzymes catalyzing the last step of NAD^+ synthesis from nicotinamide mononucleotide (NMN) in mammalian cells, including neurons. Notably, this enzymatic activity of NMNAT1 is indispensable for the axonal protective effect of WLD^s (Araki et al., 2004). Moreover, the overexpression of an NMNAT protein derived from the archaebacterium *Methanococcus jannaschii*, which exhibits almost no sequence homology to mammalian NMNATs, could still be sufficient to delay the pathological axonal degeneration upon traumatic neural injuries (Sasaki et al., 2009). This observation is in accordance with the view that the enzymatic activity is solely responsible for the axonal protection by the WLD^s mutant protein. Further, it has been unequivocally proven that the WLD^s protein exerts its action explicitly in axons but not in neuronal cell bodies (Sasaki and Milbrandt, 2010). These results have suggested that Wallerian degeneration is linked to the NAD^+-related signaling mechanism.

With such insight into the potential link between Wallerian degeneration and NAD^+ metabolism, it was then noted that the intracellular NAD^+ levels in axons decrease significantly upon traumatic neural injuries (Wang et al., 2005). Such NAD^+ depletion further triggers the blockage of energy production, particularly by glycolysis and the tricarboxylic acid cycle, resulting in the consequential decrease of axonal ATP levels. Conversely, manipulations that sustained the axonal NAD^+ or ATP levels in traumatically injured axons effectively delay Wallerian degeneration (Wang et al., 2005; Yang et al., 2015). This evidence has demonstrated that the metabolic energy deficit underlies pathological axonal degeneration.

Understandably, the research field has been prompted to examine the axonal protective effect of WLD^s in the context of various neurodegenerative diseases in addition to traumatic neural injuries. Indeed, the axonal structures of dopaminergic neurons were protected by WLD^s in the mouse models of Parkinson's disease (Cheng and Burke, 2010; Sajadi et al., 2004). Also, the WLD^s protein inhibited the axonal damage in the mouse model of experimental autoimmune encephalomyelitis (Kaneko et al., 2006). Moreover, WLD^s can prevent axonal pathology in other rodent models of

pathological axonal degeneration, for example, gracile axonal dystrophy (Mi et al., 2005), Charcot–Marie–Tooth disease (Meyer zu Horste et al., 2011), chemotherapy-induced periphery neuropathy (Wang et al., 2002), progressive motor neuronopathy (Ferri et al., 2003), and glaucoma (Beirowski et al., 2008; Howell et al., 2007). Till now, only a few exceptions have been reported in which the WLD^s mutant protein fails to confer axonal protection, for example, the mouse model of amyotrophic lateral sclerosis induced by the mutant human SOD1 protein (Fischer et al., 2005; Vande Velde et al., 2004) or the mouse model of spinal muscular atrophy (Kariya et al., 2009). These accumulating research efforts have given rise to a consensus that pathological axonal losses under most disease scenarios share a common signaling pathway regulated by the WLD^s protein.

6.5.2 NMNAT2 Protein

Given that the WLD^s mutant protein functions exclusively in axons, it was suggested that WLD^s might act to substitute an endogenous NMNAT protein. There are three isoforms of NMNATs in mammalian cells, that is, NMNAT1, NMNAT2, and NMNAT3. These three isoforms have distinct subcellular localizations (Belenky et al., 2007; Cambronne and Kraus, 2020). NMNAT1 is primarily concentrated in the nucleus. NMNAT2 is present in the cytosol and may be associated with membrane-bound vesicles via protein palmitoylation (Lau et al., 2010). NMNAT3 was reported to reside in mitochondria, though its precise localization has been debated. It was then found that while NMNAT2 and NMNAT3 are both detectable in axons, the protein level of NMNAT2 rapidly decreased upon traumatic injuries (Gilley and Coleman, 2010). This phenomenon is due to proteosome-mediated degradation as the specific proteasomal inhibitors suppress the loss of NMNAT2 protein in traumatically injured axons. Significantly, such inhibition of the proteasomal activity also delayed pathological axonal degeneration (Gilley and Coleman, 2010; Zhai et al., 2003), supporting its functional relevance in this process. Furthermore, at least in the in vitro cultured neurons, the NMNAT2 protein is initially synthesized in cell bodies and then continuously transported into distal axons (Gilley and Coleman, 2010). Once arriving in axons, NMNAT2 exhibits a short half-life time of several hours because of the proteasomal degradation primed by the PHR1-SKP1-FBXO45 ubiquitin E3 ligase complex. Therefore, traumatic injuries cut off NMNAT2 transported from neuronal cell bodies, leading to its depletion within axons. Accordingly, the genetic deletion of PHR1, SKP1, or FBXO45 could all delay the degeneration of traumatically injured axons (Babetto et al., 2013; Yamagishi and

Tessier-Lavigne, 2016). The protein stability of NMNAT2 within axons may also be modulated by the mitogen-activated protein kinase (MAPK) signal (Walker et al., 2017).

As additional evidence substantiating the essential role of NMNAT2 in axonal survival, recent studies have identified the rare loss-of-function mutations of NMNAT2 associated with severe neural defects resulting in embryonic lethality or childhood polyneuropathy in humans (Huppke et al., 2019; Lukacs et al., 2019). Similarly, the genetic deficiency of NMNAT2 in mice causes widespread defects of axonal projections within the central and peripheral nervous systems at the embryonic stage (Gilley et al., 2013). Of particular importance, the WLD^s mutant protein rescues most of the neural defects associated with the NMNAT2 deficiency (Gilley et al., 2013), confirming that WLD^s can functionally replace the endogenous NMNAT2 protein in maintaining axonal survival. These research findings have highlighted that NAD^+ metabolism is crucial for designating pathological axonal degeneration.

6.5.3 SARM1 Protein

Several research groups conducted genetic screenings in the 2010s in the hope of revealing new molecular components in pathological axonal degeneration. Mutagenesis screening in flies revealed that the genetic deletion of *Drosophila* sterile alpha and Armadillo motif (dSARM) or its mouse ortholog sterile alpha and Toll/interleukin-1 receptor motif-containing protein 1 (SARM1) could significantly prolong the survival of traumatically injured axons (Osterloh et al., 2012). In parallel, gene knockdown screening in cultured mouse neurons identified SARM1 as a central regulator of pathological axonal degeneration induced by traumatic injuries (Gerdts et al., 2013). SARM1 or dSARM has an ortholog in Caenorhabditis *elegans*, that is, *tir-1*, originally identified as an essential gene controlling asymmetric neuronal differentiation (Chuang and Bargmann, 2005). The mammalian SARM1 protein belongs to the Toll/interleukin-1 receptor (TIR) domain-containing adaptor family (O'Neill and Bowie, 2007). This protein family also contains MYD88, TRIF, TRAM, and TIRAP. These other four family members have been extensively studied in the context of various immune responses. For instance, MYD88 and TRIF are central for transducing the innate immune response mediated by Toll-like receptors (TLRs) upon pathogenic infections. Although SARM1 or its ortholog *tir-1* was reported to modulate innate immune signals (Carty et al., 2006; Couillault et al., 2004), the precise function of SARM1 in immunity has remained controversial.

Studies have demonstrated that the SARM1 expression is highly enriched in all types of neurons but limited in immune cells (Kim et al., 2007). SARM1 is present in the cytosol of neurons and axons and may be associated with mitochondria through its N-terminal mitochondria-targeting sequence, though its precise subcellular localization remains to be unequivocally determined. Importantly, the genetic deletion of SARM1 delays the pathological axonal degeneration in traumatically injured mouse nerves, and the protective effect is comparable to, if not more robust than, that observed in the *Wld*^{s} mutant mice (Gerdts et al., 2013; Osterloh et al., 2012). Also, the SARM1 deletion can effectively mitigate chemotherapy-induced peripheral neuropathy triggered by vincristine or paclitaxel (Geisler et al., 2016; Wang et al., 2019). Similarly, the axonal structures of dopaminergic neurons in the mouse model of Parkinson's disease induced by 6-hydroxydopamine are protected by the SARM1 deletion (Peters et al., 2021). In addition, the SARM1 deletion suppresses the pathological axonal degeneration in several other common models of neurodegenerative diseases, for example, amyotrophic lateral sclerosis induced by the mutant human TAR DNA-binding protein 43 kDa (TDP-43) protein (White et al., 2019), experimental autoimmune encephalomyelitis (Viar et al., 2020), and diabetic peripheral neuropathy (Turkiew et al., 2017). Moreover, recent studies have reported that SARM1 participates in some previously-unrecognized events of pathological axonal degeneration, for example, the sympathetic neuropathy in the liver under metabolic stress (Liu et al., 2021) or the loss of catecholaminergic axons in the enteric nervous system in acute inflammation (Sun et al., 2021). The disease spectrum of axonal protective effects by the SARM1 deletion factually mirrors that observed with the WLD^{s} mutant protein, suggesting that SARM1 and WLD^{s} may act with the related molecular mechanism. Till now, among all the examined models of neurodegeneration, there are two exceptions in which the SARM1 deletion cannot confer axonal protection, that is, amyotrophic lateral sclerosis induced by the mutant human SOD1 (Peters et al., 2018) and Parkinson's disease caused by the overexpression of human alpha-Synuclein protein (Peters et al., 2021). SARM1 appears dispensable for normal neurodevelopment in mice, particularly for developmental axonal degeneration (Kim et al., 2007; Osterloh et al., 2012). Similarly, the neuron-specific deletion of dSARM in *Drosophila* does not affect the developmental loss of neurons or axons (Osterloh et al., 2012). At the same time, the WLD^{s} mutant protein also does not affect developmental axonal degeneration (Hoopfer et al., 2006). This functional similarity shared by SARM1 and WLD^{s} further supports their convergence into the same signaling pathway dedicated to pathological axonal degeneration.

Surprisingly, the SARM1 deletion reverts the aforementioned severe neural defects observed with the NMNAT2 deficiency in mice (Gilley et al., 2015). This finding implicated for the first time the involvement of SARM1 in NAD^+ metabolism. Studies then revealed that the recombinant TIR domain of SARM1 from different species, including fruit flies, fish, mice, and humans, can catalyze the in vitro degradation of NAD^+ (Essuman et al., 2017). Such NAD^+ breakdown produces nicotinamide and adenosine diphosphate ribose (ADPR) or cyclic ADPR. In contrast, the recombinant TIR domain derived from other TIR domain-containing proteins in mammals, such as MYD88 and TLR4, does not possess such NAD^+-degrading ability. On the other hand, many TIR domain-containing proteins from bacteria, archaebacteria, and plants exhibit a similar NADase activity (Essuman et al., 2018; Wan et al., 2019), which may represent a conserved, ancient family of NAD^+-consuming enzymes. Of importance, the NADase activity of mammalian SARM1 proteins critically depends on a glutamate residue of the TIR domain, that is, E642 residue in the mouse or human SARM1. The E642A mutation abolishes the NADase activity of SARM1 and its ability to prime the degeneration of traumatically injured axons (Essuman et al., 2017). This research breakthrough has identified the critical mechanistic link of SARM1 to the NAD^+ metabolism during pathological axonal degeneration.

The SARM1 protein is consistently present inside axons, but the NAD^+ depletion only occurs in response to neurodegenerative insults. This fact clearly implies that SARM1 must be relatively inactive under healthy, undamaged conditions. Several recent studies have achieved the high-resolution protein structure of SARM1 (Bratkowski et al., 2020; Figley et al., 2021; Horsefield et al., 2019; Jiang et al., 2020; Shen et al., 2021; Sporny et al., 2020), showing that it is organized as an octamer (i.e., eight protein molecules) via the interaction of its sterile alpha motif (SAM) domain (Figure 6.2). Moreover, the NADase activity of the TIR domain is inhibited through its binding to the N-terminal Armadillo motif (ARM) domain within the octamer complex. In particular, the TIR domain resides on a hydrophobic interface with the ARM domain. Such interactions result in the physical blockage of the enzymatic pocket of the TIR domain, thus precluding the NAD^+ access to the catalytic site. The mutations of several hydrophobic residues within this interface could disrupt the interaction between TIR and ARM domains and cause the enhanced NADase activity of SARM1 that is sufficient to trigger the spontaneous axonal degeneration in the absence of any neurodegenerative insult (Jiang et al., 2020). It has been implicated that the TIR domain needs to dimerize or oligomerize to become activated, though research efforts are ongoing to pursue the high-resolution SARM1 structure in an active status (Shi et al., 2022).

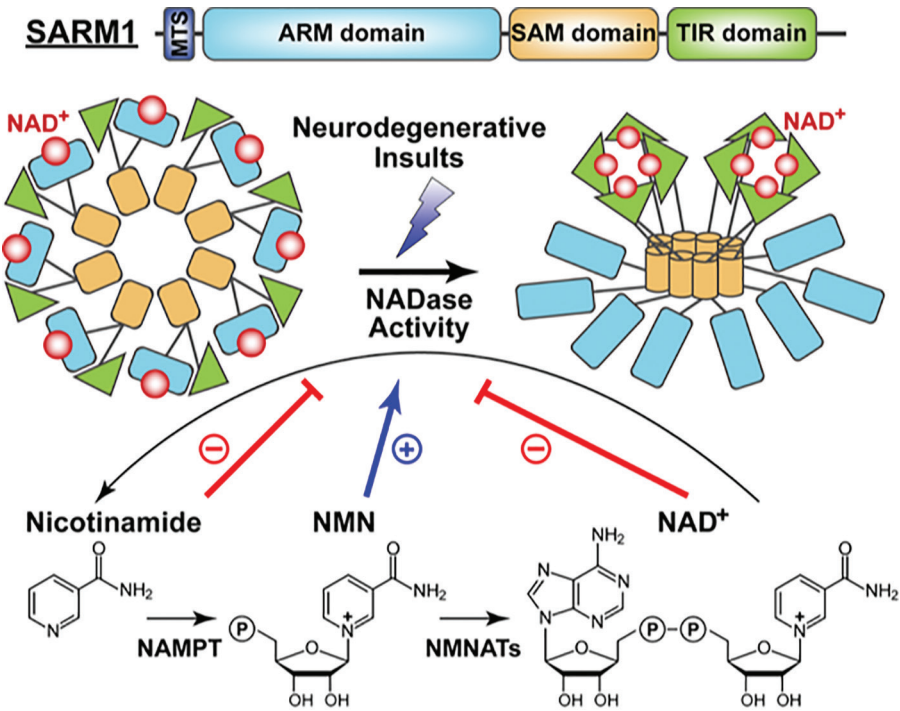

Figure 6.2 Functional link of SARM1 to NAD⁺ metabolism. SARM1 is organized as an octamer and maintained inactive through the NAD⁺ binding to the ARM domain. Upon neurodegenerative insults, the NADase activity of the TIR domain is triggered to convert NAD⁺ back into its metabolic precursor, nicotinamide. Notably, nicotinamide inhibits, but the NAD⁺ synthesis intermediate nicotinamide mononucleotide (NMN) stimulates, the NADase activity of SARM1. MTS, mitochondria-targeting sequence; NAMPT, nicotinamide phosphoribosyltransferase; NMNATs, nicotinamide mononucleotide adenylyltransferases.

Complexity has been realized in the functional link between SARM1 and axonal NAD⁺ metabolism. In the structural analyses of the SARM1 protein, it was observed unexpectedly that the ARM domain contains a new site of NAD⁺ binding (Jiang et al., 2020; Sporny et al., 2020). The mutations of critical residues involved in such NAD⁺ binding significantly boost the NADase activity of SARM1 and initiate the degeneration of uninjured axons. This surprising discovery has revealed that NAD⁺ is not only an enzymatic substrate of SARM1 but also acts as an inhibitor of the protein via allosteric regulation. In support of this view, the detailed biochemical tests showed that the NADase activity of SARM1 was suppressed by high concentrations of NAD⁺ (e.g., over 0.5 mM) but would increase when the NAD⁺ concentration dropped (e.g., below 0.1 mM). Notably, intracellular NAD⁺ levels are

reported to be approximately 0.5 mM (Yang et al., 2007; Yang et al., 2019). Therefore, it becomes conceivable that such high levels of NAD^+ within healthy axons suffice to maintain the SARM1 protein in the inactive status. On the other hand, following traumatic axonal injuries or other neurodegenerative insults, an initial decrease of local NAD^+ levels due to the membrane breakage or the NMNAT2 depletion interferes with this NAD^+-mediated inhibition of SARM1. The active SARM1 then degrades more NAD^+ molecules, establishing a positive feedforward cascade along the entire length of damaged axons. Conversely, the WLD^S mutant protein or other NMNATs can effectively replenish axonal NAD^+ levels and prevent such a cascade. This delicate NAD^+-mediated regulation of SARM1 has formed the central mechanism controlling pathological axonal degeneration.

Moreover, the link between SARM1 and axonal NAD^+ metabolism extends beyond the NAD^+ molecule itself. Accumulating evidence has demonstrated that the NADase activity of SARM1 is also influenced by the NAD^+ metabolic precursors, that is, nicotinamide and NMN. Nicotinamide can inhibit the NADase activity of the TIR domain, though such inhibition requires high concentrations of nicotinamide (Essuman et al., 2017). At the same time, the administration of high dosages of nicotinamide decreased neural damage in the mouse model of traumatic brain injury (Goffus et al., 2010). Also, it preserved the axonal structures inflicted in the mouse model of experimental autoimmune encephalomyelitis (Kaneko et al., 2006). Notably, nicotinamide is catalyzed into NMN by the enzyme nicotinamide phosphoribosyltransferase (NAMPT) in mammalian cells, including neurons. Under normal conditions, NMN is further synthesized into NAD^+ by the NMNAT proteins. However, the NMNAT2 depletion occurring in damaged axons abolishes the conversion of NMN to NAD^+. Indeed, at least in the in vitro cultured neurons, axonal NMN levels increase significantly after traumatic injuries (Di Stefano et al., 2015). Intriguingly, recent studies have shown that NMN is an activator of the NADase activity of SARM1 (Angeletti et al., 2022; Loreto et al., 2015; Zhao et al., 2019). As a result, the NMN accumulation in damaged axons likely contributes to the SARM1 activation triggered by NAD^+ depletion. In addition, certain neurotoxins such as Vacor (a rodenticide) or 3-acetylpyridine can be metabolized into the NMN mimetics capable of activating SARM1 to cause pathological axonal degeneration (Loreto et al., 2021; Wu et al., 2021). Although the detailed mechanism underlying this NMN-mediated SARM1 activation remains to be determined, a paradigm has emerged that the NAD^+ metabolic pathway regulates SARM1 and the onset of pathological axonal degeneration.

6.5.4 Ca^{2+} and Calpains

NAD^+ has a central role in energy-producing metabolic processes, particularly in the catalytic steps by glyceraldehyde-3-phosphate dehydrogenase in glycolysis, or pyruvate dehydrogenase, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, and malate dehydrogenase in the tricarboxylic acid cycle. Therefore, the NAD^+ depletion in damaged axons inevitably causes the blockage of such essential processes, resulting in the continuous decrease of axonal ATP levels. As expected, this axonal energy deficit is prevented by maintaining NAD^+ levels via the WLD^s mutant protein or the SARM1 deletion (Wang et al., 2005; Yang et al., 2015). Otherwise, the lasting energy deficit severely interferes with many cellular events, for example, axonal transport, protein synthesis or degradation, and ion gradients across the plasma membrane. Among them, the significant accumulation of Ca^{2+} within axons has been documented as a common hallmark under neurodegenerative conditions, often occurring immediately before the final dismantling of axonal structures. As discussed above, such Ca^{2+} overload can be attributed to the failure of Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers to clear out Ca^{2+} influx into axons through various channels.

It has already been discovered in the 1970s that high levels (e.g., tens of micromolar concentrations) of intracellular Ca^{2+} are sufficient to induce the destruction of axonal structures (Schlaepfer, 1971; Schlaepfer, 1974). This process is executed by a group of Ca^{2+}-activated proteases known as calpains (Goll et al., 2003; Liu et al., 2004). Calpains are broadly expressed in mammalian cells, and the predominant forms in the cytosol of neurons and axons include calpain-1 and calpain-2. Under low Ca^{2+} concentrations (i.e., submicromolar concentrations), calpain-1 and calpain-2 remain mostly inactive. When Ca^{2+} levels increase, the two calpains are triggered into action. Accordingly, removing extracellular Ca^{2+} or chelating intracellular Ca^{2+} effectively prevents the calpain activation and preserves the structural integrity of traumatically injured axons (George et al., 1995; Schlaepfer, 1974). However, such manipulations could not rescue the depletion of ATP levels in damaged axons (Yang et al., 2015), consistent with the metabolic energy deficit being the upstream causative event.

Because of their promiscuous specificity for protein substrates, the active calpains cleave many essential proteins in axons, such as neurofilaments, microtubules, and motor proteins. Calpain-mediated proteolysis is the final stage of pathological axonal degeneration, reminiscent of apoptotic cell death executed by active effector caspases. Conversely, the specific small-molecule inhibitors of calpains preserve the structural integrity of

damaged axons while not reverting the metabolic energy defect (Yang et al., 2015). Notably, the neurofilament proteins proteolyzed by calpains may be released from degenerating neurons or axons into the cerebrospinal fluid or the blood circulation. Such neuron-derived protein fragments, for example, serum neurofilament light chain (sNf-L), have been exploited as biomarkers to diagnose certain neurodegenerative diseases (Kapoor et al., 2020; Khalil et al., 2020; Rohrer et al., 2016).

Importantly, due to this irreversible calpain activation, the safety mechanism exists in axons. Calpastatin is an endogenous specific protein inhibitor of calpains and is abundantly distributed within neurons and axons (Goll et al., 2003). By directly binding to calpains, calpastatin prevents the inadvertent activation of the proteases by physiological fluctuations of intracellular Ca^{2+} levels, for example, that occurring in action potentials. Interestingly, calpastatin is a calpain substrate and can be degraded by prolonged calpain activity. As a result, the Ca^{2+} overload caused by the metabolic energy deficits in damaged axons continuously activates calpains and thus overrides this safety mechanism enforced by calpastatin. Conversely, calpastatin overexpression suppresses the calpain activity and the structural destruction of axons under neurodegenerative insults (Yang et al., 2013).

In retrospect, the original discovery of the WLD^s mutant protein has served as the prelude to the characterization of the NAD^+-dependent pathway, composed of several sequential events, that is, NMNAT2 depletion, SARM1 activation, metabolic energy deficit, Ca^{2+} overload, and calpain activation. This central mechanism, distinct from all the known types of programmed cell death, represents a common signal instructing pathological axonal degeneration in many neurodegenerative conditions.

6.6 Future Perspectives

We have summarized the current knowledge of the molecular mechanisms underlying pathological axonal degeneration. However, it is essential to emphasize that this unique axonal pathology represents the stereotyped self-destruction of axons under different neurodegenerative insults. Importantly, it can occur independently of or even preceding neuronal cell death in many circumstances. Therefore, in-depth investigations into pathological axonal degeneration are crucial for effectively treating neurodegenerative diseases. Indeed, we have witnessed some of the tremendous advances built upon the decades of accumulating efforts in the field. Meanwhile, we would like to highlight several critical questions that still await to be addressed in future research:

1. Whether unknown signaling pathways may exist to control pathological axonal degeneration demands more examinations. For instance, at least in the mouse model of amyotrophic lateral sclerosis induced by the mutant human SOD1, the genetic deletion of the central apoptotic component BAX did not prevent the death of motor axons (Gould et al., 2006). At the same time, the WLD^s mutant protein (Vande Velde et al., 2004) or the SARM1 deletion (Peters et al., 2018) failed to delay this SOD1-induced axonal pathology. In addition, the RIPK3 deletion only modestly preserved the motor axons in this disease model (Ito et al., 2016). Therefore, unknown mechanisms other than the apoptotic, necroptotic, and NAD^+-dependent pathways may be involved in this particular type of pathological axonal degeneration. Detailed studies need to focus on molecular or biochemical alterations beyond those known signaling pathways in the motor axons inflicted by amyotrophic lateral sclerosis, which could pave the way to the mechanistic dissection of this axonal degeneration process.

2. It remains an open question whether some common neurodegenerative diseases, particularly Alzheimer's disease, may involve the currently known pathways. The challenge related to this question is that most of the available rodent disease models are based on the genetic overexpression or knock-in of the mutant forms of human amyloid precursor protein (APP) or gamma-secretase. However, although such disease models recapitulate several neuropathological features in vivo, for example, amyloid plaques, neurofibrillary tangles of TAU proteins, and synaptic defects, they cannot reproduce the massive loss of neurons and axons in the cortex or the hippocampus as that occurring in patients. As a result, the genetic evidence either proving or precluding the involvement of apoptosis, necroptosis, or the NAD^+-dependent pathway in the axonal degeneration of Alzheimer's disease is still lacking. Developing more efficient animal models in other species, such as nonhuman primates, will be instructive in answering this vital question (Capitanio and Emborg, 2008; Verdier et al., 2015).

3. Although it has been demonstrated that the NAD^+-dependent pathway is essential for pathological axonal degeneration in many disease scenarios, how this mechanism is triggered remains incompletely understood. For instance, SARM1-dependent axonal degeneration occurs upon exposure to neurotoxins such as 6-hydroxydopamine or rotenone (Peters et al., 2021; Sur et al., 2018). However, how such neurotoxins would alter the NAD^+ metabolism and activate SARM1 is unclear. Those neurotoxins

disrupt the electron transport chain, leading to mitochondrial damage. Meanwhile, the SARM1 protein may be anchored to mitochondria via its N-terminal mitochondria-targeting sequence. Whether this unique subcellular localization of SARM1 would be linked to mitochondrial damage becomes an attractive question. Also, though SARM1 has an essential role in chemotherapy-induced peripheral neuropathy (Geisler et al., 2016; Wang et al., 2019), how axonal NAD^+ metabolism is affected by chemotherapeutic agents such as vincristine or paclitaxel is unknown. The interference of microtubules by those chemotherapeutic agents may cause the reduced transport of NMNAT2 protein from neuronal cell bodies, resulting in the disrupted NAD^+ metabolism in axons. More in-depth research on the engagement of the NAD^+-dependent pathway under specific disease conditions is warranted.

4. It has been intriguing whether different signaling pathways may be synergetically involved in a particular neurodegenerative condition. For example, the apoptotic pathway partially controls the degeneration of axonal projections of dopaminergic neurons in mouse models of Parkinson's disease (Kim et al., 2011; Vila et al., 2001). The WLD^s mutant protein (Cheng and Burke, 2010; Sajadi et al., 2004) or the SARM1 deletion (Peters et al., 2021) could also mitigate the pathological axonal degeneration in such disease models. Therefore, how the apoptotic pathway and the NAD^+-dependent pathway crosstalk with each other need to be explored. Similarly, recent studies have reported that the TNF-alpha-induced pathological degeneration of mouse optic, sensory, or sympathetic axons depends on the SARM1 signal (Ko et al., 2020; Liu et al., 2021). Meanwhile, the pharmacological induction of MLKL oligomerization, which is the core step of necroptosis, is sufficient to trigger the SARM1 activation (Ko et al., 2020). Such results have implicated the potential intertwining between necroptosis and the NAD^+-dependent pathway. If the crosstalk of signaling pathways exists in the context of specific diseases, it will emphasize the necessity of simultaneously targeting individual pathways for more efficient therapeutic strategies.

5. While the current focus has been mainly on the intrinsic molecular mechanisms of pathological axonal degeneration, extensive evidence has demonstrated that non-neuronal cells, especially glial cells, and immune cells, also exert critical roles in neurodegenerative diseases. For instance, myelinating oligodendrocytes and Schwann cells shuttle essential metabolic intermediates to axons (Boucanova and Chrast,

2020; Simons and Nave, 2015). As a result, the myelin destruction by aberrant autoimmunity occurring in multiple sclerosis or Guillain–Barré syndrome leads to the death of demyelinated axons. Accordingly, targeting such autoimmune responses has been proven effective in mitigating the progression of those diseases (Dobson and Giovannoni, 2019; van den Berg et al., 2014). How the functions of non-neuronal cells, for example, metabolic shuttling, inflammation, and phagocytosis, may modulate pathological axonal degeneration is still incompletely understood and is becoming a new frontier in the research field.

6. Although the specific mechanisms of pathological axonal degeneration have been extensively explored for decades, it remains daunting that most neurodegenerative diseases are still incurable. This reality could indicate our incomplete understanding of such signaling pathways and reflect the challenge of translating basic research into therapeutic applications. However, with recent research advances, especially the breakthrough revelation of the NAD^+-dependent pathway, novel entry points for combatting pathological axonal degeneration have emerged. In particular, different approaches such as small-molecule inhibitors, macromolecules, or genetic manipulations to target axonal NAD^+ metabolism could hold promise and have been pursued.

In our great hope, the combination of enduring basic research and pioneering translational endeavor could eventually conquer those dreadful, debilitating neurodegenerative diseases in years to come.

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