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The Drama of Wallerian Degeneration: The Cast, Crew, and Script

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Abstract

Significant advances have been made in recent years in identifying the genetic components of Wallerian degeneration, the process that brings the progressive destruction and removal of injured axons. It has now been accepted that Wallerian degeneration is an active and dynamic cellular process that is well regulated at molecular and cellular levels. In this review, we describe our current understanding of Wallerian degeneration, focusing on the molecular players and mechanisms that mediate the injury response, activate the degenerative program, transduce the death signal, execute the destruction order, and finally, clear away the debris. By highlighting the starring roles and sketching out the molecular script of Wallerian degeneration, we hope to provide a useful framework to understand Wallerian and Wallerian-like degeneration and to lay a foundation for developing new therapeutic strategies to treat axon degeneration in neural injury as well as in neurodegenerative disease.

INTRODUCTION

WD: Wallerian degeneration

***Wallerian degeneration slow* (WLD^S):** a spontaneous genetic mutation in mice that potently delays injury-induced axon degeneration

SCI: spinal cord injury

Axons are specialized processes that project from neuronal cell bodies (i.e., somas) to connecting neurons or nonneural cells for signal transmission. While most axons are only about one to a few micrometers in length, the longest axons of the human neurons, with their cell bodies in the dorsal root ganglia of the spinal cord, can extend over one meter in distance (11, 49). In contrast, a soma is usually in the range of several to a few dozen micrometers in diameter. Thus, axons can be ten to hundreds of thousands times longer than the dimensions of their soma, which makes them especially vulnerable to physical injury.

After an axonal injury, the axon trunk distal to the site of the injury degenerates in a well-regulated, self-destructive process, which is characterized by beading, fragmentation, and disintegration of injured axons, followed by the clearance of debris by glia and immune cells. This process is termed Wallerian degeneration (WD), named after Dr. Augustus Waller, who first characterized the pathology of injury-induced axon degeneration in frogs more than 170 years ago (150).

Why study WD? Are injured axons (which lack supplies of energy and nutrients) doomed to die? And why bother to preserve an axon that is already severed from the soma? Questions like these are often brought to us, especially by interested students who are new to this field. The following are some of our thoughts about why it is important to study WD:

1. Since the discovery of *Wallerian degeneration slow* (WLD^S) mice (83), research over the past 30 or more years has demonstrated that the degeneration of injured axons is not merely a passive process caused by disconnection from the soma. Rather, WD is an active process that is tightly regulated at the molecular and cellular levels (28, 29, 37, 44, 56), and intervention in injury-induced axon degeneration is possible (29, 65, 77).
2. Many patients with spinal cord injury (SCI), traumatic brain injury, or traumatic axon injury only have a small portion of axons that are completely transected. Axons that are crushed or stretched but are still connected to the soma after the injury, as well as axons within the nerve bundle that are undamaged in the initial insult, eventually degenerate anyway, along with others that are damaged. So understanding the mechanisms that control the self-destruction process of axons can help us protect these axons and preserve the physiological function of the injured nerve as much as possible.
3. Severed axons are not completely dead axons. For example, transected mouse axons can propagate action potentials for up to 24 h (83), and axonal transport is maintained to some extent after injury (83, 96, 130). In invertebrates such as *Caenorhabditis elegans*, before axons start to break apart, the proximal end can quickly reconnect with the distal end or the lower branch of injured axons (160). Thus, preserving the distal trunk of an injured nerve may provide a chance for axons to reconnect, which is a lot less challenging than rebuilding the entire nerve projection by axon regeneration (see the paragraph below).
4. Regeneration of an axon in the adult central nervous system is difficult (25, 142). It requires not only switching on the regrowth program in neurons but also reinnervation (and re-myelination in some cases) of newborn axons. In other words, a regenerating axon would face a monumental, if not impossible, task: to regrow over a long distance on the right track, to exit the nerve bundle at the right level, and to connect with the right target after development is long over. It would involve comprehensive and well-coordinated regulation at the molecular, cellular, and systemic levels to ensure accurate pathfinding, projection, and innervation. Of note, the long axons of the human spinal neurons are connected to their targets in muscles before the nerves elongate with the growth of the body during development. Therefore, to preserve maximum neuronal connection, it is conceptually less challenging

if we can preserve the distal trunk of an injured nerve rather than inducing de novo nerve regeneration in mature nervous systems in adults.

5. The study of WD may shed light on dying-back axon degeneration (Wallerian-like degeneration) in neurological disorders such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (18, 27, 29).

NAD$^+$: nicotinamide adenine dinucleotide

NMNAT: nicotinamide mononucleotide adenylyltransferase

In this review, we first introduce the key genetic and molecular players that are involved in the self-destruction program of injured axons, both on the stage and behind the scenes (the cast and crew). Next, we summarize the major cellular events in WD, both intrinsically within axons and extrinsically with glia and immune cells, with an effort to put a timescale on the process to envision what would occur after axon injury (the script). Finally, we discuss the main challenges in the field and conclude with future perspectives on the study of axon injury and WD.

THE CAST AND CREW OF WALLERIAN DEGENERATION: MAJOR GENETIC AND MOLECULAR PLAYERS

WLD^S and NMNAT

The earliest evidence that WD is an active cellular process came from the fortuitous observation of the extended survival of injured nerves in the *WLD^S* mice (60, 83). In these mice, axons in the transected sciatic nerve remained morphologically continuous for over two weeks. In wild-type mice, it usually takes less than two days for injured axons to fragment and degenerate. The *WLD^S* mutant allele arose from a spontaneous chromosomal triplication and recombination, resulting in a chimeric transcript encoding a fusion protein composed of (*a*) the N-terminal 70 amino acids of the ubiquitination factor E4B (UBE4B) protein but without the enzyme activity domain, (*b*) the full length of the nicotinamide adenine dinucleotide (NAD$^+$)-synthesizing enzyme nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), and (*c*) a unique linker of 18 amino acids from the 5′ untranslated region (UTR) of NMNAT1 between (*a*) and (*b*) (87).

Although the basal NAD$^+$ levels are not altered in *WLD^S* mice (124), the fusion protein of WLD^S likely provides a sustained NMNAT activity in the axoplasm after injury (6, 31, 32, 121). Studies in various animal models have demonstrated that overexpression of NMNAT is sufficient to protect injured axons in animals from fruit flies (*Drosophila melanogaster*) to zebrafish (*Danio rerio*) to mammals (5, 6, 7, 32, 45, 48, 88, 121, 123, 124, 158). Furthermore, loss-of-function (LOF) studies indicate that NMNAT is required for maintaining the morphological and functional integrity of axons in animal models in vivo (45, 58, 155), while mutations in the human *NMNAT1* gene can cause Leber congenital amaurosis, an early-onset inherited retinal dystrophy with severe vision loss (26, 35, 43, 76).

Mammals, including humans, have three *NMNAT* genes: *NMNAT1*, *NMNAT2*, and *NMNAT3*. Each one shows a preferential subcellular localization: NMNAT1 is predominantly in the nucleus, NMNAT2 is mostly in the cytoplasm and the Golgi apparatus, and NMNAT3 is mainly in mitochondria (14, 21). Nevertheless, all NMNATs participate in NAD$^+$ biosynthesis, and the NAD$^+$-synthase activity is required for NMNATs to be axoprotective (6, 32, 45). In addition, studies have shown that NMNAT possesses chaperone activity (86, 165) and can combat oxidative stress to keep mitochondria healthy (20, 112).

In mammals, NMNAT2 is believed to be the major isoform that controls axonal survival because depletion of NMNAT2 but not NMNAT1 or NMNAT3 triggers spontaneous axon degeneration in cultured mouse neurites in vitro (58). NMNAT2 protein has a half-life of less than 4 h, and it is degraded within several hours after injury, which is prior to axon degeneration (58). In flies, the E3 ubiquitin ligase *Highwire* (*Hiw*) regulates the turnover of *Drosophila* Nmnat

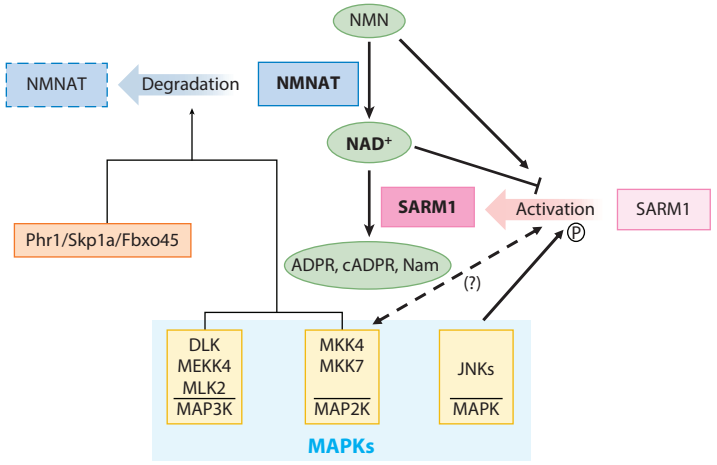


Figure 1

A central molecular paradigm of NMNAT-NAD^+-SARM1 in WD. NAD^+, NMNAT (the enzyme that catalyzes the biosynthesis of NAD^+ from NMN), and SARM1 (the enzyme that hydrolyzes NAD^+ to cADPR and Nam) play a central role in injury-induced axon degeneration. The MAPK signaling pathway is extensively involved in WD by regulating the turnover of NMNAT and the activation of SARM1. Researchers have also proposed that SARM1 activation may be upstream of MAPK signaling and that NMN promotes whereas NAD^+ inhibits SARM1 activation. In addition, the Phr1/Skp1a/Fbxo45 complex also participates in the regulation of NMNAT turnover through the ubiquitination of NMNAT and by targeting it for proteasome-mediated degradation. The dotted arrow with the question mark indicates contradictory observations. Abbreviations: ADPR, adenosine diphosphate ribose; cADPR, cyclic ADPR; DLK, dual leucine zipper-bearing kinase; Fbxo45, F-box only protein 45; JNKs, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; MAP2K, MAPK kinase; MAP3K, MAPK kinase kinase; MEKK4, MAPK kinase kinase 4; MKK, MAPK kinase; MLK2, mixed lineage kinase 2; NAD^+, nicotinamide adenine dinucleotide; Nam, nicotinamide; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; P, phosphorylation; Phr1, Pam/highwire/rpm-1 protein; SARM1, sterile alpha and TIR motif-containing protein 1; Skp1a, S-phase kinase-associated protein 1a; TIR, Toll/interleukin-1 receptor domain; WD, Wallerian degeneration.

(dNmnat) protein (156). Consistently, depletion of *PAM/Highwire/RPM-1* (*Phr1*), the mammalian homolog of *Hiw*, increases NMNAT2 protein levels in axons and promotes the survival of injured nerves in mice (8). In addition, researchers have proposed that the Phr1/S-phase kinase-associated protein 1a (Skp1a)/F-box only protein 45 (Fbxo45) ubiquitin ligase complex degrades nonpalmitoylated NMNAT2, whereas palmitoylated NMNAT2 is degraded by mitogen-activated protein kinase (MAPK)-dependent proteolysis involving dual leucine zipper-bearing kinase (DLK). Inhibition of either pathway leads to the accumulation of NMNAT2 in axons, which protects injured axons from degeneration (133, 159) (**Figure 1**).

MAPK: mitogen-activated protein kinase

DLK: dual leucine zipper-bearing kinase

TIR: the Toll/interleukin-1 receptor domain

SARM1: sterile alpha and TIR motif-containing protein 1

SARM1

From 2012 to 2013, two research teams reported the axonal function of sterile alpha and Toll/interleukin receptor (TIR) motif-containing protein 1 (SARM1): One isolated the *Drosophila* Sarm (*dSarm*) gene (previously known as *Ect4*) in a fly genetic screen (107), and the other identified *Sarm1* in a genome-wide RNA interference (RNAi) screen using mouse primary neurons (57). Later on, LOF of SARM1 was shown to manifest potent axoprotection in various in vitro and in vivo models (36, 55, 57, 107), which indicated an essential role of SARM1 in promoting injury-induced axon degeneration. Before it emerged as a major player in WD, SARM1 and its

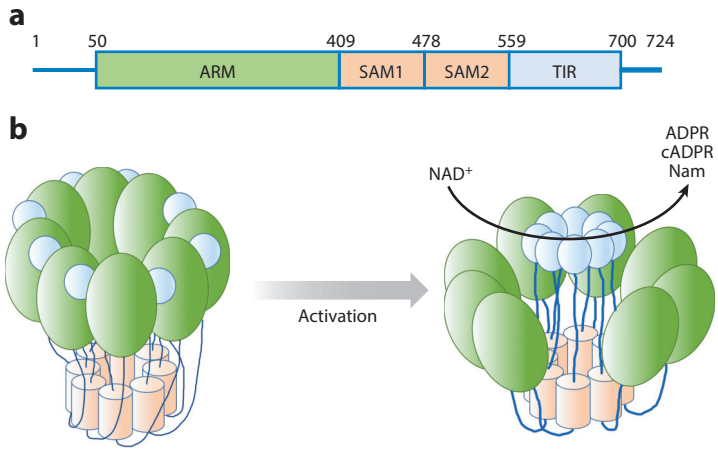


Figure 2

The major domains and autoinhibition of SARM1. (*a*) The major functional domains of the SARM1 protein. (*b*) The octamer ring formed by the SAM domains. The ARM domain inhibits the dimerization of the TIR domains of the adjacent two SARM1 molecules, thereby suppressing the activation of the NADase activity of SARM1. Abbreviations: ADPR, adenosine diphosphate ribose; ARM, armadillo repeat motif; cADPR, cyclic adenosine diphosphate ribose; NADase, NAD^+ glycohydrolase; Nam, nicotinamide; SAM, sterile alpha motif; SARM1, sterile alpha and TIR motif-containing protein 1; TIR, Toll/interleukin-1 receptor.

ortholog *tir-1* in *C. elegans* were studied mainly for their functions in stress responses, cell death, and immunity (15, 23, 33, 72, 79, 97, 108).

SARM1 is a multidomain protein containing an N-terminal armadillo repeat motif (ARM), two tandem sterile alpha motif (SAM) domains, and a C-terminal TIR domain (**Figure 2*a***). The SAM domain mediates the protein–protein interaction between SARM1 molecules (55, 66, 132), and the TIR domain possesses the NAD^+ glycohydrolase (NADase) activity, hydrolyzing NAD^+ to adenosine diphosphate ribose (ADPR), cyclic ADPR (cADPR), and nicotinamide (Nam) (41, 42, 66, 151). The intrinsic NADase activity of the TIR monomer is minimal but is substantially boosted when dimerized or multimerized by the SAM domain (120, 151), which is required to promote NAD^+ depletion and axon degeneration after injury (41). The ARM domain self-inhibits the prodegenerative activity of SAM-TIR so that SARM1 is maintained in the inactive conformation in axons under normal conditions (55, 57, 126, 131).

The recent advances in cryogenic electron microscopy (cryo-EM) have provided valuable structural insights into the regulation of SARM1 (16, 68, 126, 131). They have shown that the tandem SAM domains form an inner ring that assembles a homo-octamer of SARM1, whereas the ARM domains form an outer ring so that two ARM domains from the adjacent SARM1 proteins decorate the catalytic TIR domain of a third SARM1 molecule and prevent its dimerization and activation (**Figure 2*b***). Interestingly, binding of SARM1 to its own substrate NAD^+ in an allosteric location in the ARM domain appears to stabilize the inactive conformation, and mutation in the interface breaks the lock and activates the NADase activity of the TIR domain, leading to axon degeneration (68, 131).

ADPR: adenosine diphosphate ribose

cADPR: cyclic adenosine diphosphate ribose

NAD^+ and NMN

As the function and mechanism of NMNAT (an NAD^+ biosynthesis enzyme) and SARM1 (an NAD^+ hydrolysase) in axons become increasingly understood, NAD^+ has taken center stage in

the investigation of WD. NAD^+, an important coenzyme and abundant metabolite in cells, plays an essential role in many metabolic processes and is required for the maintenance of cellular homeostasis (80, 144). NAD^+ is rapidly depleted in axons after injury (55, 153). Increasing NAD^+ levels by providing NAD^+ or its precursors, such as nicotinamide riboside (NR) and nicotinic acid riboside (NAR), in the culture medium can delay axon degeneration induced by injury or the chemotherapy drug vincristine (81, 122, 134).

NMN: nicotinamide mononucleotide

JNK: c-Jun N-terminal kinase

Interestingly, LOF of SARM1 rescues the development and survival of NMNAT2-deficient axons (59), whereas NMNAT1 blocks SARM1-mediated NAD^+ depletion and axon degeneration (122). Moreover, the MAPK pathway appears to regulate NMNAT2 levels upstream of SARM1 (149). Thus, it is unclear whether NMNAT and SARM1 simply act synergistically to regulate NAD^+ levels or are in a linear molecular pathway. Adding to the complexity, in an in vitro enzyme activity assay, 2 mM of NAD^+ almost completely inhibits the NADase activity of SARM1 (68, 131), suggesting a feed-forward regulatory mechanism for injury-induced NAD^+ depletion. Together, these studies suggest that rapid turnover of NMNAT2 in injured axons leads to decreases in NAD^+ levels, which in turn relieve the autoinhibition of the NADase activity of SARM1 and further deplete NAD^+ in injured axons.

In addition to the decrease in NAD^+ levels, the accumulation of nicotinamide mononucleotide (NMN) in injured axons because of the rapid turnover of NMNAT2 may also contribute to WD. Studies have shown that injury-induced axon degeneration can be suppressed by pharmacological or genetic reduction of NMN levels (36, 82). In addition, NMN may be an endogenous activator of SARM1, as NMN and a cell-permeant mimetic of NMN can activate SARM1, causing cADPR production, NAD^+ depletion, and nonapoptotic cell death (16, 167). While these studies support the notion that maintaining NAD^+ levels while eliminating NMN accumulation can protect axons from injury-induced degeneration, other reports show that elevating NMN by itself is not sufficient to trigger axon degeneration (81) and even alleviates brain lesions in a cryoinjury model in mice (166).

MAPKs

The MAPK pathway participates in the transduction of the injury signal to axon deconstruction in WD. *Wallenda* (*Wnd*)—the *Drosophila* homolog of *DLK*, a MAPK kinase kinase (MAP3K)—is the first MAPK family member discovered to play a role in axon injury and degeneration. Deletion of fly *Wnd* or mouse *DLK* delays degeneration of injured axons in vivo and in vitro (92, 128, 157, 162). Yang and colleagues (162) tested a panel of MAPK family kinases by RNAi and showed that a triple knockdown of *DLK* with two other MAP3Ks, *MAPK kinase kinase 4* (*MEKK4*) and *mixed lineage kinase 2* (*MLK2*), significantly suppressed degeneration of injured axons.

The MAPK pathway is almost immediately activated upon axonal injury, which is evident from the phosphorylation of MAPK kinase 4 (MKK4) as early as 5 min after injury (162). And, as mentioned above, the activated MAPK pathway promotes the degradation of NMNAT2 by DLK- and MKK4/MKK7-mediated proteolysis (128, 148, 133). The regulation of NMNAT2 by MAPK signaling appears to be independent of the function of SARM1 (148). SARM1 activation may also involve the MAPK signaling pathway, as SARM1 can be phosphorylated in the SAM domain by the downstream MAPK protein c-Jun N-terminal kinase (JNK), which enhances the NADase activity of SARM1 (98).

It should be noted that, although the recent progress on NMNAT, SARM1, MAPK, and their genetic interactions has assigned NAD^+ to center stage in the molecular mechanisms of WD (see **Figure 1**), not all injury-induced axon degeneration happens according to the NAD^+ paradigm. For example, in *C. elegans*, overexpression of WLD^S or *NMNAT* or knockout of *tir-1* (the *C. elegans* homolog of *SARM1*) failed to protect injured axons of both sensory and motor neurons after

axotomy (104), indicating that other important factors and mechanisms may also participate in the process and regulation of WD.

Autophagy

Autophagy is a lysosome-dependent self-eating process required for the bulk clearance of cytoplasmic contents and maintenance of cellular homeostasis (71, 94, 163). Dysfunction of autophagy is associated with neurodegenerative diseases such as AD and Huntington's disease (91, 95). The association of autophagy with injury-induced axon degeneration was first noted several decades ago, when increased autophagic vacuoles in the soma and injured proximal axons were observed by electron microscopy (EM) (38, 89). After that, many studies have shown that autophagic markers such as microtubule-associated proteins 1A/1B-light chain 3 (LC3) and vacuoles that morphologically resemble autophagosomes accumulate in injured or degenerating axons, especially at the early stage (75, 110, 115, 152, 154, 164). Interestingly, injury-induced increase of autophagy occurs independently of the NMNAT-NAD^+-SARM1 mechanism (**Figure 3**), as overexpression of the axoprotective *dNmnat* in the fly nerve cannot suppress injury-induced accumulation of axonal autophagosomes (152).

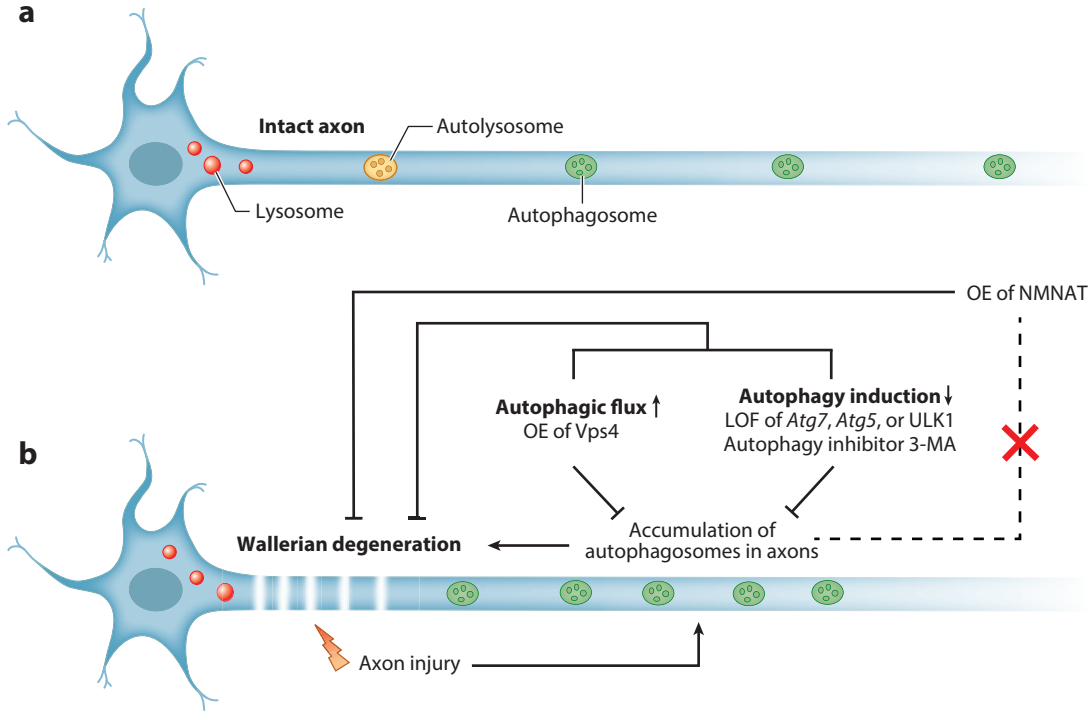


Figure 3

Autophagy in axon degeneration. (*a*) Autophagy plays a critical role in maintaining the cellular homeostasis in healthy axons. (*b*) Injury induces an increase in the axonal accumulation of autophagosomes, which is caused by disrupted autophagic flux due to rapid turnover of the ESCRT protein Vps4 and upregulation of the *Atgs* that promote autophagy induction. OE of Vps4 or inhibition of autophagy induction attenuates the degeneration of injured axons. In contrast, OE of the neuroprotective protein NMNAT cannot suppress injury-induced autophagy, indicating an NAD^+-independent mechanism. Abbreviations: 3-MA, 3-methyladenine; *Atg*, autophagy related gene; ESCRT, endosomal sorting complexes required for transport; LOF, loss-of-function; NAD^+, nicotinamide adenine dinucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; OE, overexpression; ULK1, Unc-51 like autophagy activating kinase 1; Vps4, vacuolar protein sorting 4.

Atg: *autophagy related gene*

ULK1: *Unc-51 like autophagy activating kinase 1*

ESCRT: endosomal sorting complexes required for transport

Vps4: vacuolar protein sorting 4

Injury-induced increases in axonal autophagosomes may result from increased induction of autophagy by the upregulation of autophagy genes. For example, the protein levels of *autophagy related gene 5* (*Atg5*), *Atg*7, and *Unc-51 like autophagy activating kinase 1* (*ULK1*) are upregulated within hours after injury in a rat model of SCI (115). And the inhibition of autophagy by genetic ablation of *Atg5* or *Atg*7, by using an autophagy inhibitor 3-methyladenine (3-MA), or by suppressing the function of the autophagic protein ULK1 can attenuate axon degeneration caused by physical injury or neurotoxin (24, 75, 141, 148, 164). Additionally, zinc and ring finger 1 (ZNRF1), an E3 ubiquitin ligase, promotes WD by targeting protein kinase B (PKB, also known as AKT) to proteasome-dependent degradation, which activates glycogen synthase kinase 3β (GSK3β) to promote the phosphorylation of collapsin response mediator protein 2 (CRMP2) (147). Studies have shown that inhibition of CRMP2 phosphorylation can suppress the degeneration of injured axons (73). In the meantime, activated GSK3β increases the phosphorylation of myeloid cell leukaemia-1 (Mcl-1), which subsequently activates another key autophagic protein, Beclin-1, inducing autophagy in injured axons and promoting axon degeneration (148).

Impaired autophagic flux and/or disrupted axonal transport can also lead to the accumulation of autophagosomes in injured axons. The endosomal sorting complexes required for transport (ESCRT) complex plays an essential role in the biogenesis of multivesicular bodies (MVBs) and the endolysosomal-mediated autophagy pathway (116, 137). Researchers have recently discovered that a major ESCRT component, vacuolar protein sorting 4 (Vps4), is rapidly depleted in mouse dorsal root ganglion (DRG) neurites after axotomy, which impedes the autophagic flux and causes the accumulation of axonal autophagosomes. Upregulation of *Vps4* significantly reduces the accumulation of axonal autophagosomes and delays the degeneration of the transected fly wing nerve. Moreover, overexpression of the human homolog *Vps4B* in mouse neurons exhibits remarkable neuroprotection in both in vitro and in vivo models (152), suggesting an evolutionarily conserved role for Vps4 in WD and the possibility of ameliorating axon degeneration by promoting autophagic clearance (**Figure 3**).

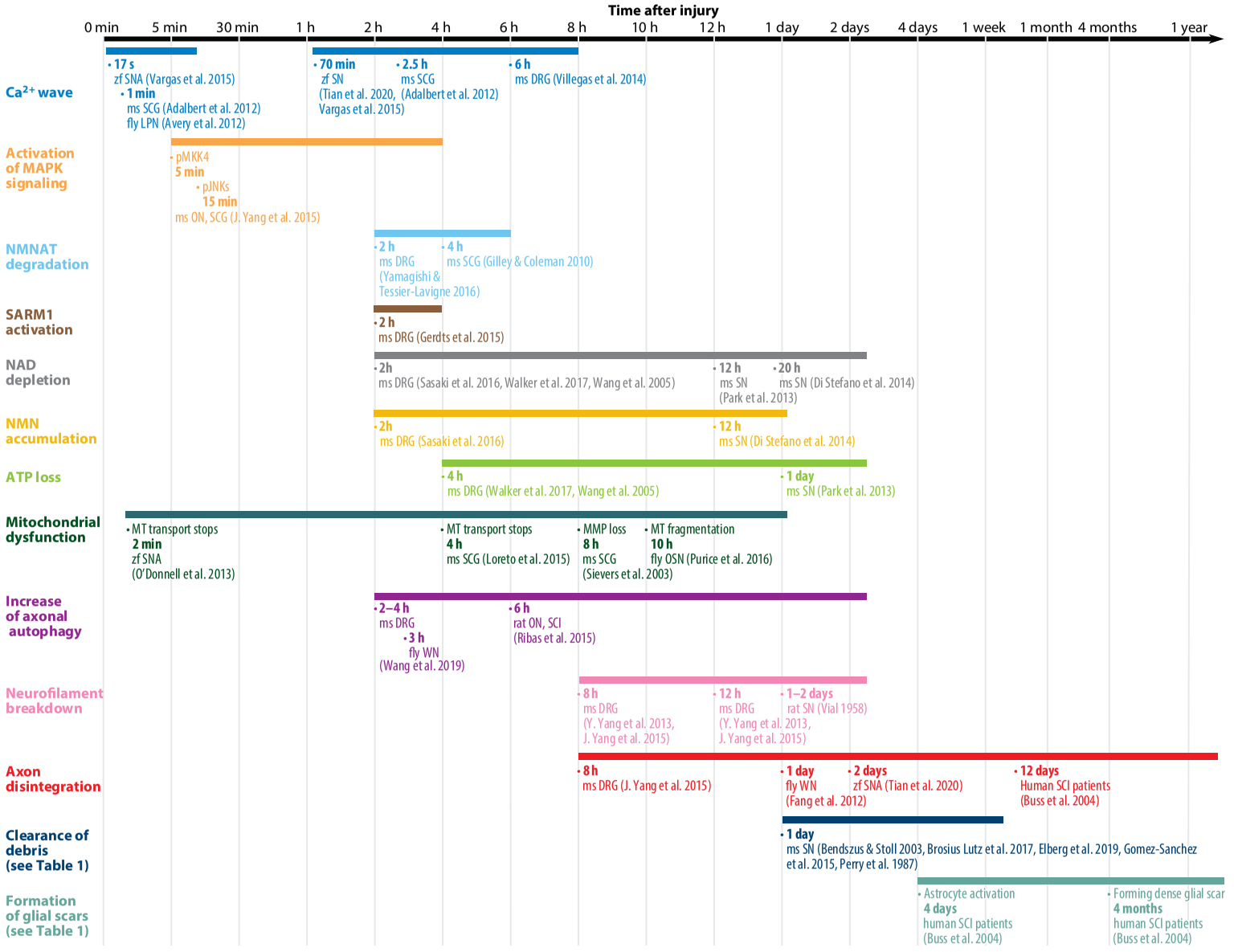
Other Factors

Other molecular factors and cellular players such as Ca^{2+} (140), Ca^{2+}-dependent calpains (30), the ubiquitin-proteasome system (UPS) (84), mitochondria (74), axonal transport (69), microtubule dynamics (117), and others also play an important role in WD and have been reviewed elsewhere. In addition, an increasing number of new WD-modifying genes have recently been identified (46, 103). Further investigation is warranted to understand the molecular mechanisms of these genes in WD and whether they have a conserved function in mammalian nervous systems. In addition to the intrinsic factors within the injured axons, glia and immune cells also respond to nerve injury and take an active role in the process of WD and the subsequent axon regeneration (2, 34, 119).

THE SCRIPT OF WALLERIAN DEGENERATION: MAJOR CELLULAR EVENTS AND THEIR TIMING

Axons undergoing WD exhibit stereotypical morphological changes, and the process is classified into four pathological phases: the acute response, latency, degeneration, and clearance phases. However, the pathological phases do not always correspond to what occurs at the molecular and cellular levels. For example, although little axonal destruction takes place during the latency phase, several key genetic players and molecular pathways are activated in this phase that transduce the injury signal to the downstream cascades and effectors that initiate axon degeneration.

Here, we aim to elaborate the time course of major events in WD at the cellular and molecular levels based on the current literature available (**Figure 4**). Each major event is plotted in



(Caption appears on following page)

Figure 4 (*Figure appears on preceding page*)

The major cellular and molecular events in WD plotted according to their time sequence. The onset or earliest time point at which an event is observed is noted, and the width of each block represents the approximate duration of the event. The specific animal and injury models as well as the reference(s) are shown. Of note, the time axis is symbolic and not proportional. Abbreviations: ATP, adenosine triphosphate; DRG, dorsal root ganglion; LPN, larval peripheral nerve; MAPK, mitogen-activated protein kinase; MMP, mitochondrial membrane potential; ms, mouse; MT, mitochondria; NAD, nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; ON, optical nerve; OSN, olfactory sensory nerve; SARM1, sterile alpha and TIR motif-containing protein 1; SCG, superior cervical ganglion; SCI, spinal cord injury; SN, sciatic nerve; SNA, sensory neuron axons; pJNKs, phosphorylation of c-Jun N-terminal kinases; pMKK4, phosphorylation of MAPK kinase 4; WD, Wallerian degeneration; WN, wing nerve; zf, zebrafish.

the timetable in **Figure 4** according to its sequence and duration to the best of our knowledge. However, this timetable is not meant to be a dogmatic set of rules for injury-induced axonal responses because the exact timing of a specific event varies depending on the animal model (mouse, zebrafish, fly, or worm), experimental setting (in vivo versus in vitro), neuronal subtype (sensory neurons versus motor neurons), injury type (transection versus crush), age of the injured animals, and other conditions being examined.

Prologue: The First Hour

Upon injury, a rapid Ca^{2+} surge can be detected within the first minute in mouse neurites in vitro as well as in fly and zebrafish nerves in vivo (1, 5, 143). This fast, transient Ca^{2+} wave originates at the site of injury and is caused by an influx of extracellular Ca^{2+}. Depletion of the extracellular Ca^{2+} by adding ethylene glycol tetraacetic acid (EGTA) or by inhibiting the voltage-gated Ca^{2+} channels can significantly delay injury-induced axon degeneration (53, 93). The mitochondria near the injury site are impaired by the initial Ca^{2+} influx, which cannot be suppressed by the neuroprotective WLD^S protein (143). And as early as 2 min after axotomy, disrupted axonal transport of mitochondria is observed in zebrafish in vivo (106). In addition, the MAPK pathway is also activated rapidly by axon injury. Phosphorylation of mouse MKK4 at Ser257/Thr261, indicative of its activation, can be detected as early as 5 min after injury and peaks at 15–30 min, along with phosphorylation of the downstream JNKs (162).

Main Show: All in One Day

The second Ca^{2+} wave starts at about 1 h after injury in zebrafish (143), 2.5 h in cultured mouse superior cervical ganglia (SCG) neurites (1), and 6 h in cultured mouse DRG neurites (146). This second wave is caused mostly by Ca^{2+} released from the endoplasmic reticulum and mitochondria in injured axons (146). Researchers report that the second Ca^{2+} wave occurs right before the onset of axonal fragmentation (82, 135, 143, 146), and expression of the axoprotective WLD^S or knockout of the prodegenerative SARM1 can suppress the second Ca^{2+} wave and protect injured axons (1, 135, 143). These findings indicate that the rise of Ca^{2+} in injured axons, especially during the second wave, serves as a key signal to initiate axon degeneration. Indeed, Ca^{2+} overload can impair mitochondria, accumulate reactive oxygen species (ROS), and activate the protease calpain to break down neurofilaments (10, 60, 85, 125, 146, 161).

The protein levels of axonal NMNAT2 decrease rapidly, starting from about 2 h after injury in in vitro cultured mouse DRG neurites (159) or 4 h in neurites derived from mouse SCG (58). Meanwhile, the NADase activity of SARM1 is activated about 2–4 h after injury in DRG neurites (55). As the biosynthesis of NAD^+ is reduced due to NMNAT turnover and the hydrolysis of NAD^+ is boosted by the activation of SARM1, the NAD^+ levels begin to drop at 2 h and become almost undetectable by 6 h after injury in mouse DRG axons in vitro (122, 148, 153). The timeline

of NAD^+ drop is extended in in vivo models. A significant decrease of NAD^+ level is observed at 12 h (109) or 20 h (36) in the sciatic nerve in mice after injury. Meanwhile, the accumulation of NMN is detected at 2 h in severed mouse DRG axons (122) or 12 h in the injured sciatic nerve in mice (36). As NAD^+ is an important coenzyme for energy and metabolic homeostasis, its depletion leads to decreased axoplasmic adenosine triphosphate (ATP) levels. In in vitro models, ATP loss can be detected 4 h after injury in mouse DRG neurites (148), and ATP levels become undetectable by 12 h after injury in the same model (153, 162). In an in vivo setting, a significant ATP loss is observed ~24 h after injury in a mouse model of sciatic nerve injury (109).

Mitochondrial dysfunction also contributes to energy failure in injured axons. As mentioned above, in zebrafish, mitochondrial transport close to the injury site of the distal axon is completely stopped 2 min after axotomy, and the accumulation of mitochondrial ROS spikes at about 1.5–2 h (106). In mammalian models such as cultured SCG neurites, mitochondrial motility decreases ~4 h after injury (82). Loss of mitochondrial membrane potential is evident 8 h after axotomy in DRG neurites (129). In the fly olfactory sensory nerve, mitochondria become fragmented ~10 h after axon injury (113). Healthy mitochondria not only are required for maintaining normal energy levels in cells but also participate in ROS production, Ca^{2+} buffering, and protease activation, all of which are involved in axon degeneration (52, 74, 127).

Injury-induced axonal autophagy occurs within several hours both in vitro and in vivo. Significant accumulation of axonal autophagosomes is observed within 2–4 h in mouse DRG explants and 3 h after injury in the fly wing nerve (152). In rats, a significant increase of LC3-positive puncta in the optic nerve is observed 6 h after a crush injury (75). At the molecular level, the ESCRT protein Vps4, which is required for autophagic flux, is degraded in about 2 h in injured mouse DRG neurites (152). Meanwhile, the genes that promote the initiation of autophagy, such as *Atg5*, *Atg7*, and *ULK1*, are significantly upregulated starting as early as 6 h and peaking at about 24 h after injury in a rat model of SCI (115).

Following the molecular and cellular changes, the morphological alteration of injured axons begins to emerge. In neurites of in vitro cultured mouse sensory neurons, the degradation of neurofilament (NF) proteins is detected about 8–12 h after injury, along with axonal fragmentation, which is indicative of the disintegration of the axoplasm membrane (161, 162). In in vivo models, breakdown of the injured nerve takes a little longer. Axonal beading and fragmentation are usually remarkable 1–2 days after injury (45, 83, 135, 145, 152). In human patients with cerebral infarction or SCI, NF loss close to the lesion is detected 12 days after injury, and NF proteins become almost undetectable in 1 year or longer (19).

Epilogue: Ever After

Glial and immune cells take an active role in axon injury and WD (**Table 1**). For simplicity, we have summarized their responses in the later events in the timetable in **Figure 4**; however, some glia and immune cells respond to injury almost instantly. For example, in zebrafish, macrophages arrive at the site of injury within minutes (118). In rodent models, Schwann cells release cytokines and chemokines within hours after injury to recruit macrophages (78, 101, 100, 136), which continue migrating and arriving at the lesion site for several days (13, 40, 111).

Macrophages, Schwann cells, and microglia, the resident phagocytic immune cells in the brain and spinal cord, all participate in the clearance of axonal and myelin debris once injured axons start the destruction. In mice, the clearance of fragmented axons in the injured sciatic nerve begins one day after injury, and the whole process can last for over a week (13, 17, 40, 61, 111). In the meantime, these cells form glial scars at the lesion site to enclose the damaged area and release regeneration-inhibitory chondroitin sulfate proteoglycans to the extracellular matrix (51). The

Table 1 Vertebrate glia and immune cells in axon injury

Cell type	Major response and function(s)	Timing	Injury model	Reference(s)
Macrophage	Migrate to the injury site; clear axon and myelin debris	From 1–3 min	Zebrafish, transection of SMN axons	118
		1–8 days	Mouse, SNI	40, 111
			Rat, SNI	13
		2–4 days	Rat, transection of SDR	4, 54
Microglia	Clear axon and myelin debris	24 h–3 days	Mouse, SCI	63
	Form glial scars	~1 week		12
Schwann cell	Release cytokines and chemokines to recruit macrophages	PAP-III, 12 h	Rat, HNI	100, 101
		LIF and IL-6, 3–10 h	Mouse, SNI	78, 136
	Glycolytic shift to support axon survival	24–48 h		9
	Axokinesis to promote axon degeneration	2–4 days		22
	Dedifferentiation to promote remyelination	1–5 days		62, 102, 105
	Clear myelin debris by autophagy	2–7 days		61, 67
	Clear myelin debris by phagocytosis			17
	Proliferation to promote clearance and remyelination	From 3 days	Rat, transection of LVR	99
Oligodendrocyte	Loss of oligodendrocytes	15 min–24 h	Rat, SCI	64
		24 h–2 days		114
	Activation of OPCs; oligodendrocytogenesis	Mostly in 1–7 days, continue to 42 days		114, 139
Astrocyte	Induction of reactive astrocytes to form glial scars	From 4 days	Human, SCI	19
	Produce inhibitory CSPGs	Several days to a few weeks	Rat, TBI	90
			Rat, SCI	70
	Repair the BBB; inhibit inflammation; protect neurons	14 days	Mouse, SCI	47
	Aid axon regeneration	8–10 weeks		3

Abbreviations: BBB, brain-blood barrier; CSPGs, chondroitin sulfate proteoglycans; HNI, hypoglossal nerve injury; IL-6, interleukin 6; LIF, leukaemia inhibitory factor; LVR, lumbar ventral root; OPC, oligodendrocyte precursor cell; PAP-III, pancreatitis-associated protein-III; SCI, spinal cord injury; SDR, spinal dorsal root; SMN, spinal motor neurons; SNI, sciatic nerve injury; TBI, traumatic brain injury.

complete development of glial scars can take a considerably longer time. For example, in patients with SCI, reactive astrocytes are found as early as four days after injury, whereas a late astrocytic reaction starts four months after injury and forms dense, long-lasting glial scars (19). There has been controversy as well as new developments related to the role of glia and glial scars in neural injury and axon regeneration (3, 9). This related topic has been reviewed elsewhere (39, 50, 138) and is not examined here, as this review focuses on the recent advances in our understanding of the molecular mechanisms of injury-induced axon degeneration.

CONCLUDING REMARKS

Our knowledge about the molecular and cellular mechanisms of WD has advanced substantially in the past three decades. Injury-induced changes along the NMNAT-NAD^+-SARM1 axis have become the major molecular paradigm for WD. A large body of evidence has demonstrated that the steady-state level of NAD^+ is crucial for maintaining axonal integrity. However, how exactly the NAD^+ depletion triggers the catastrophic collapse of injured axons is not completely understood.

This is in part because NAD^+ is widely involved in many cellular processes and functions critical for axonal survival, and it is challenging (and maybe impossible) to single out a main mechanism when it is in fact a combination of multiple downstream degenerative programs that are being activated.

The discovery that the TIR domain of SARM1 possesses the NADase activity represents a big step forward toward understanding the molecular mechanism of the prodegenerative function of SARM1 and once again confirms the central role of NAD^+ in WD. SARM1 has attracted interest from pharmaceutical companies and has become one of the hottest targets for treating axon degeneration in related neurological diseases. As mentioned above, NAD^+ is profoundly involved in cell survival and many cellular functions. Interrogating SARM1 with NAD^+ analogs may cause widespread side effects. Even for noncompetitive inhibitors, there are other potential problems to consider. For example, SARM1 is also a member of the Toll-like receptor adaptor family that participates in cellular stress and immune responses, and thus the systemic inhibition of SARM1 may bring unexpected adverse effects. Therefore, an inhibitor that specifically blocks SARM1 activation in injury and axon degeneration is much desired. However, so far, little is known about how SARM1 is activated in axon injury. This is clearly an important question that researchers in the field of axon injury and WD are pursuing.

Multiomics research is rapidly evolving today, which offers us the opportunity to understand the biology of WD with a global and comprehensive view. Single-cell sequencing and spatial transcriptomics can provide the cell identity and in situ spatial information about injury responses, while proteomics and metabolomics may discover novel players, including unknown proteins, protein modifications, and metabolites, and even draw a picture of the entire signaling pathways that orchestrate the drama of WD. Such studies may provide insights that were impossible to obtain from classical genetic screening in the past, and we look forward to what omics study will bring to the field.

Finally, axon degeneration is a common pathological feature of neurological disorders. The molecular players and mechanisms regulating axon self-destruction in WD, although not always exactly the same, may cast light on the understanding and development of new therapeutics to treat dying-back degeneration in human diseases. For example, clinical trials of SARM1 inhibitors for treating glaucoma, multiple sclerosis, amyotrophic lateral sclerosis, and other axon degeneration conditions are ongoing. With the molecular basis of axon injury and WD becoming more thoroughly understood in the future, we believe the presently incurable axon degeneration in human diseases will one day become curable.

FUTURE ISSUES

1. Is NAD^+ the answer to everything in Wallerian degeneration (WD)?
2. How is SARM1 activated by axonal injury?
3. Can omics research provide novel and useful insights into WD?
4. What can we learn from WD that might shed light on axon degeneration in disease?

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