Wallerian degeneration: an emerging axon death pathway linking injury and disease

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Preface

Axon degeneration is a prominent, early feature of most neurodegenerative disorders and can also be induced directly by nerve injury in a process known as Wallerian degeneration. The discovery of genetic mutations that delay Wallerian degeneration has provided insight into mechanisms underlying axon degeneration in disease. Rapid Wallerian degeneration requires the pro-degenerative molecules SARM1 (sterile α and HEAT/Armadillo motif containing protein 1) and PHR1 (PAM-Highwire-Rpm-1 ubiquitin ligase). Nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2) is essential for axon growth and survival. Its loss from injured axons may activate Wallerian degeneration, explaining why NMNAT overexpression also rescues them. Here, we discuss the roles of these and other proposed regulators of Wallerian degeneration, new opportunities for understanding disease mechanisms and intriguing links between Wallerian degeneration, innate immunity, synaptic growth and cell death.

Introduction

Axons are lost before neuronal cell bodies in many neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease (PD), Huntington's disease and glaucoma. It is the primary pathology in hereditary spastic paraplegia and many peripheral neuropathies and underlies progressive decline in multiple sclerosis (MS). The atrophy of many axonal arbors during normal ageing[1] exacerbates the effects of age-related disorders. Thus, it is essential to understand and devise means to target the mechanisms underlying axon degeneration.

The capacity of experimental models of these diseases to shed light on the mechanisms of axonal loss is often limited by a gradual and heterogeneous onset for individual axons within a population and by uncertainty over where and how pathogenesis begins. By contrast, Wallerian degeneration is a process in which axonal injury at a defined site and time affects all axons simultaneously (FIG. 1). These features have enabled significant progress to be made towards understanding the mechanisms underlying Wallerian degeneration.

During Wallerian degeneration, the distal portions of injured axons fragment after a predictable latent phase, which lasts 36-44h in mouse sciatic nerve[2]. A number of genetic mutations delay Wallerian axon degeneration for up to two weeks[3-5]. For example, NMNATs (BOX1), including the slow Wallerian degeneration protein (WLD^S) which contains NMNAT1 (FIG 2), delay Wallerian degeneration up to tenfold when overexpressed within axons[3,6-9]. Research focussed on such gain-

of-function effects for several years, but new loss-of-function studies have begun to ask 'how is Wallerian degeneration initiated?' and 'which proteins are required for its completion?'. For example, one endogenous isoform, NMNAT2, is present in axons (FIG 3) and its removal causes WLD^S-sensitive axon degeneration[10-12]. Conversely, deletion of SARM1 delays Wallerian degeneration as strongly as WLD^S, suggesting that it functions in axon death signalling[4]. Loss of PHR1 (also known as MYCBP2) has a protective effect *in vivo* that approaches this level[5]. Current evidence suggests that a range of other modifiers influence Wallerian degeneration more modestly (or their full efficacy *in vivo* remains to be established). The *in vivo* effects of NMNATs, SARM1 and PHR1 are closely reproduced both in primary cultures of mammalian neurons and by their *Drosophila melanogaster* orthologs dNmnat, dSarm and Highwire[4,13-15]. This has facilitated studies of exogenous agents and genetic manipulations that influence Wallerian degeneration.

Thus, experimental Wallerian degeneration is an excellent model system that can be used to understand one pathway of axon degeneration. Testing whether disease models are influenced by these mutations can then relate this mechanism back to those taking place in neurodegenerative diseases. Many different types of stress initiate axon loss. For example, inflammatory damage mediates MS, perturbations of mRNA metabolism are implicated in several types of motor neuron disease, poor mitochondrial quality control may be important in some PD cases and failure of glial support or disruptions of axonal transport are causative factors in some peripheral neuropathies[16]. Despite this diversity of causes, there is evidence of some convergence onto a common pathway. Overexpression of WLD^S delays axon degeneration in some peripheral neuropathy, PD and motor neuron disease models, suggesting that there is at least one shared step in the axon degeneration pathways in these disorders[17-21]. Even in models that are not influenced by WLD^S, such as some SOD1 transgenic ALS models[22], this approach helps to categorize degenerative mechanisms.

Here, we review recent studies that have increased our understanding of the relationship between Wallerian degeneration and axon loss in disease (TABLE 1) and how newly identified regulators of Wallerian degeneration should extend this. We begin by discussing progress towards a full understanding of the Wallerian degeneration pathway. Ordering events in this pathway, and defining the putative initiation and execution phases, is a key aim to help identify the best steps to target therapeutically. Finally, we ask whether the roles of SARM1 in innate immunity and cell death, and of PHR1 in synaptic growth, indicate mechanistic overlap between Wallerian degeneration and other important biological processes.

Wallerian-like and WLD^S-sensitive degeneration

Injury-induced axon degeneration, now known as Wallerian degeneration, was first described in 1850 by Augustus Waller, who predicted "important…applications…in relation to nervous diseases" [23]. The term 'Wallerian-like degeneration' traditionally describes morphologically similar axon degeneration seen in peripheral neuropathies, ALS and other disorders, exhibiting granular disintegration of the cytoskeleton, ovoids of degenerating myelin, fragmentation of distal axons and, in the CNS, large axonal swellings. Morphological similarity does not necessarily imply a related molecular mechanism but the serendipitous discovery of the *Wld*^{S} spontaneous mutant mouse[24], 139 years after Waller described injury-induced axon degeneration, finally made it possible to confirm that axon loss in some disease models is mechanistically related to Wallerian degeneration (TABLE 1)[17,20,25,26]. WLD^S protects axons after injury and in some (but not all) models that fit the classical definition of Wallerian-like degeneration. Thus, in this review we use the term 'WLD^S-sensitive degeneration' to refer to conditions in which WLD^S (or related NMNAT enzymes) delays the process.

Towards a Wallerian degeneration pathway

Injured axons in *Wld*^{S} mice show prolonged survival, challenging the earlier assumption that axon degeneration is a passive consequence of separation from cell bodies. Instead, this suggests that Wallerian degeneration and other types of WLD^S-sensitive axon degeneration are active processes involving an autonomous self-destruction pathway.

Although Wallerian degeneration appears to be largely distinct from apoptosis in molecular terms[27-29], there are some clear parallels. Both are conserved processes (BOX 2), each with a latent phase during which initiating events occur without morphological change, and an execution phase involving frank breakdown of the cell or axon. Apoptosis also features a particular stage after which a cell is committed to die but before which cell death can be prevented by removing the triggering insult[30]. Axon transection is irreversible, but there is evidence from a non-transecting, reversible model that WLD^S-sensitive degeneration can also be prevented before a commitment point is reached. The degeneration of uninjured superior cervical ganglion (SCG) neurites after blocking protein synthesis is only slightly slower than the degeneration seen after injury and is strongly delayed by WLD^S [10]. However, even wild-type neurites remain healthy if the reversible translation inhibitor cycloheximide is removed within 4-6 hours of addition. Later removal from overtly healthy cultures fails to prevent their subsequent degeneration.

The number of known modulators of Wallerian degeneration has grown significantly over the past few years, but attempts to integrate these into a cohesive pathway have been limited. Determining the sequence of events is crucial for understanding related disease mechanisms and for identifying

the most appropriate targets for therapeutic manipulation. Here, we propose that these events can be ordered into a conserved signalling pathway consisting of initiation and execution phases triggered by injury or other stresses (FIG. 4). We hypothesise that a series of core events receive a variety of modifying inputs and have putatively assigned molecules to these different groups based on the type and relative strength of reported phenotypes. Specifically, molecules involved in core events are defined here as those for which a gain- or loss-of-function either confers protection against Wallerian degeneration comparable to the archetypical tenfold delay provided by WLD^S expression, or induces WLD^S-sensitive degeneration of otherwise undamaged axons. Core events would thus include those influenced by WLD^S, SARM1/dSarm and PHR1/Highwire. Modifiers of the pathway are defined as those having more modest but significant effects. In some cases the full phenotype of animals with a gain- or loss-of-function of the proposed pathway modifier has not yet been tested *in vivo* so future data may suggest they are core components. Thus, our categorisations and relative positioning of different events should not be considered definitive; rather we hope they will act as a stimulus for future studies.

The initiation phase

The question of whether Wallerian degeneration is initiated by the absence of a survival signal from cell bodies or a pro-degenerative signal originating from the injury site has long been discussed[31]. The initiation of WLD^S-sensitive degeneration by a wide range of non-injury stresses now shows that an injury-derived signal such as calcium influx through the cut site is not necessary to initiate such a pathway. For example, genetic or toxic disruption of axonal transport, protein synthesis inhibition, NMNAT2 knockdown, and mutation of Schwann cell proteins all induce WLD^S-sensitive degeneration without physical injury[10,20,32,33].

<u>NMNATs and PHR1/Highwire:</u>

The robust delay of Wallerian and Wallerian-like axon degeneration conferred by ectopic expression of WLD^S, or ectopic overexpression or engineered mislocalisation of any of the three mammalian NMNATs, is highly reproducible both *in vivo* in varied organisms and in primary culture[3,7,8,13,34-36]. Together with the early depletion of NAD^+ in injured axons, which is prevented by WLD^S [(37)], this suggests that loss of a critical NMNAT function is a core event in the Wallerian degeneration pathway. As a number of different physical or toxic initiating stresses can trigger WLD^S-sensitive degeneration, we suggest that these pathways converge on or before this NMNAT step.

Data from our own work and that of others suggest that loss of endogenous NMNAT2 is a good candidate for the putative core NMNAT-reversible event. NMNAT2 is present in axons and

continuously trafficked by a population of Golgi-derived vesicles[10,38]. Like other endogenous NMNAT isoforms and WLD^S, exogenous overexpression of NMNAT2 delays Wallerian degeneration. However, because NMNAT2 is much more labile than the other NMNATs (its half-life is under an hour), the wild-type protein protects only when expressed at high levels[10,38]. *In vivo*, constitutive genetic depletion of NMNAT2 during development leads to stalled axon outgrowth resulting in axon truncation, failed peripheral innervation and perinatal lethality, consistent with a critical axonal function for this protein[11,12]. The levels of highly labile NMNAT2 decline rapidly after neurite transection in primary culture prior to degeneration, which led us to propose that early loss of this essential axon maintenance factor is a critical initiating event for subsequent degeneration[10]. Indeed, in primary culture, siRNA-mediated NMNAT2 knockdown is sufficient to initiate robust axon degeneration that is independent of cell death and not prevented by the presence of other endogenous NMNAT isoforms[10]. Consistent with our model, WLD^S rescues the NMNAT2 depletion phenotype both in primary culture and *in vivo*, probably by directly substituting its NMNAT function in axons[10,11]. Interestingly, WLD^S is far more stable than NMNAT2[10]. Our suggestion that this explains the prolonged survival of injured axons[10] is now supported by several studies in which the stabilities of WLD^S and NMNAT2 were altered and axon survival varied accordingly[7,38,39].

A key involvement of NMNAT2 loss in axon degeneration is consistent with recent data from multiple laboratories and experimental systems[5,11,12,15,40]. First, depletion of the single *Drosophila* NMNAT (dNmnat), like mammalian NMNAT2, causes spontaneous axon degeneration that is rescued by expression of WLD^S or mammalian or *Drosophila* NMNATs[40]. Second, depletion or loss-of-function of the orthologous E3 ubiquitin ligases *Drosophila* Highwire (Hiw) and murine PHR1, which influence turnover of dNmnat and NMNAT2, delays Wallerian degeneration[5,15]. In the case of the Hiw loss-of-function, the effect on axon protection is broadly equivalent to that of WLD^S [(15)]. In mammalian neurons, especially in primary culture, the effects of PHR1 depletion on axon survival and NMNAT2 stabilisation are significant but somewhat weaker[5]. This may reflect greater complexity in the regulation of NMNAT2 turnover in mammals, perhaps specifically in injured axons, although incomplete Cre-mediated deletion of PHR1 in the neuronal population may also have contributed. Finally, slowing the ubiquitination and turnover of NMNAT2 by disrupting its palmitoylation-dependent localization to Golgi-derived vesicles strongly enhances its protective capacity both in primary culture and *in vivo*[7,38]. Together these studies support the hypothesis that regulation of NMNAT2 turnover or activity could represent a critical, early node in the Wallerian degeneration pathway (FIG 4).

Although the 'NMNAT2 depletion model' has not been directly challenged by contradictory data, some important questions remain. Foremost is how this unstable protein reaches the ends of longer axons in sufficient quantities to ensure viability. Local protein translation is one possibility. Global suppression of protein synthesis does not discernibly alter Wallerian degeneration in injured primary culture neurites[10] but further investigation is needed *in vivo*. Alternatively, distinct pools of

NMNAT2 with different half-lives may exist. The ability of a cytoplasmic NMNAT1 variant to protect cut DRG neurites when virally transduced 4 hours after injury[41] (after substantial NMNAT2 loss has occurred[10,38]), may reflect slow progression of downstream events or suggest a second NMNAT-modifiable event later in the pathway. This may fit with some alternative models of axon degeneration (see below). Finally, WLD^S and a cytoplasmic variant of NMNAT1 confer strong protection against axon degeneration induced by NGF-withdrawal[27,42], but this *in vitro* model of axon pruning proceeds via activation of a seemingly different pathway that culminates in caspase activation[42-45]. It remains unclear whether NMNAT2 loss is also involved here or whether NMNAT activity impacts another event following NGF withdrawal.

There is still no absolute consensus regarding how NMNATs maintain axon health, although most reports (with exceptions[36]) suggest that enzymatic activity is critical for delaying Wallerian degeneration[8,46-51]. NAD^+, the product of the NMNAT activity, is thus a candidate effector, especially as it declines in the distal stump of injured wild-type neurites[37]. However, no other consistent correlation between neuronal NAD^+ levels, NAD^+-dependent events and axon survival has emerged. WLD^S and NMNAT overexpression does not increase basal NAD^+ levels[3,46]. NAD^+ applied exogenously has been reported to preserve neurites but very high and variable, non-physiological concentrations have been applied and some groups find no protection[37,46,52-54]. Exogenous NAD^+ may also enter cells not as NAD^+ but only after being converted to its precursor nicotinamide riboside (NR)[55]. Crucially, NR requires NMNAT to generate NAD^+, so as this enzyme is depleted in injured axons[10], it is unclear whether exogenous NAD^+ would actually increase NAD^+ inside them. Neither the NAD^+ dependent deacetylase SIRT1, nor the single *Drosophila* sirtuin, Sir2, is required for WLD^S to preserve axons[37,48] and increasing endogenous NAD^+ in mice by removing the NAD^+-consuming enzymes CD38 (ADP-ribosyl cyclase 1) and PARP1 (Poly [ADP ribose] polymerase 1) confers no protection[50].

Remarkably, pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD^+ salvage pathway (BOX1), lowers NAD^+ but confers moderate protection against neurite degeneration induced by injury or energy deprivation[50,56], rather than reversing the axon protection by NMNATs as might have been expected. WLD^S expression and NAMPT inhibition have both been shown to counter an early decline in ATP levels after energy deprivation, so it has been suggested that NMNATs might catalyze the reverse reaction to produce ATP and NMN from NAD^+ [(56)]. However, whether ATP loss is a cause or consequence of injury-induced axon degeneration remains unclear. Interestingly, stimulation of the NAD^+ biosynthetic pathway at multiple points, including NAMPT (NmPRT) overexpression prior to axotomy, also appears to have protective effects that are comparable to the effects of NAMPT inhibition by FK866 after axotomy[50,52], suggesting complex interactions of the different steps. Further studies of NAD^+ related metabolites, particularly NMN (FIG 3), should help to resolve these questions.

Intriguingly, enzymatically inactive NMNATs have been reported to delay non-injury and developmental axon degeneration in some models through an independent chaperone function, potentially underlying amelioration of pathology in some models of protein aggregation disorders[57-63]. It remains to be determined whether axon and synapse loss in these situations is mechanistically related to Wallerian degeneration or secondary to the effects of protein aggregates on cell bodies. Establishing which aspect of NMNAT function is important in any particular context will be critical when devising appropriate therapeutic strategies. Importantly, enzymatic activity-independent neuroprotection has so far only been demonstrated using overexpression of enzyme-dead NMNAT mutants or other chaperones, such as heat shock protein 26 (Hsp26) and Hsp70[62]. It will be important to test whether this alters enzymatic activity or stability of endogenous NMNATs.

SARM1/dSarm:

An absence of the Toll-like receptor (TLR) adapter SARM1 provides protection against Wallerian degeneration comparable in strength to WLD^S. Its involvement emerged from genetic screening for loss-of-function mutations delaying axon degeneration following antennal ablation in *Drosophila*[4] (BOX 2). Three nonsense alleles of the *dSarm* gene, the *Drosophila* ortholog of murine *Sarm1*, conferred robust axon protection and SARM1 was shown to be required for rapid Wallerian degeneration in mice[4]. Like Wld^S axons, $Sarm1^{-/-}$ axons survive for over two weeks after nerve injury and this striking protective effect is replicated in primary culture[4] demonstrating that both proteins influence axon degeneration cell-autonomously. $Sarm1^{-/-}$ DRG neurites also appear to be protected against axon degeneration induced by NGF-withdrawal[64], although this may depend on the culture time[4] and is more transient than protection conferred by WLD^S or cytoplasmic variant NMNAT1 in the same situation[27,42]. This might indicate that parallel SARM1-dependent and -independent degeneration pathways are activated after NGF withdrawal, each containing an NMNAT-regulated step.

The robust effect of SARM1 (or dSarm) loss-of-function after axotomy indicates a core involvement for this protein in the Wallerian degeneration pathway according to our suggested criteria. Its position in this pathway appears to be upstream of calpastatin degradation and calpain activation (see below)[65] but whether SARM1 has a role in initiation or execution remains unclear (FIG 4). Thus, determining its relationship to the proposed NMNAT-regulated step, as well as to mitochondrial and other events (below), should be revealing. In one recent report, overexpression of wild-type SARM1 did not cause axon degeneration in the absence of injury, suggesting that it requires an additional signal after injury to promote degeneration[64]. It is tempting to speculate that NMNAT2 loss or other events described below help generate this signal. Initial structure-function studies suggest that SARM1, which is a modular protein (FIG 2B), dimerizes through its SAM domains and this promotes interaction of the associated TIR signalling domains[64]. It will be important to refine these observations, to establish whether and how SARM1 functions within

axons themselves, and how its axonal role relates to its functions in promoting neuronal death after oxygen-glucose deprivation (OGD)[66] and cytokine regulation[67].

Other potential modulators of initiation:

Loss of superior cervical ganglion 10 (SCG10), a labile protein like NMNAT2, occurs early during the latent phase of Wallerian degeneration in DRG cultures[68]. Maintaining sufficient SCG10 in injured neurites confers moderate protection against degeneration and SCG10 depletion accelerates degeneration after injury, possibly by altering microtubule stability[68]. However, unlike NMNAT2, depletion of SCG10 alone is not sufficient to trigger degeneration. Thus, SCG10 loss appears to be a permissive signal for degeneration rather than causative[68]. Importantly, SCG10 turnover is c-Jun amino(N)-terminal kinase (JNK)-dependent, linking it to a JNK signalling cascade involving dual leucine zipper kinase (DLK; or its *Drosophila* ortholog Wallenda). Inhibiting this cascade, either genetically *in vivo* or pharmacologically in DRG cultures, also delays Wallerian degeneration, although protection is modest relative to that conferred by WLD^S or loss of SARM1[69]. Importantly, pharmacological inhibition is only effective if started early during the latent phase, supporting an initiation role[69]. Interestingly SCG10, like NMNAT2, at least partially localises to the Golgi apparatus and Golgi-derived vesicles via palmitoylation[70,71]. It will be interesting to test for any functional interaction between them.

Other signalling cascades may also have a modifying influence on Wallerian degeneration according to our criteria. IκB kinase (IKK) and a cascade involving glycogen synthase kinase 3 (GSK3) promote axon degeneration induced by injury or non-injury stresses, including NGF withdrawal, in DRG cultures[72,73]. However, protection following GSK3 or IKK inhibition is modest compared to $WLD^{S(72)}$ and pro-degenerative manipulation of GSK3 signalling has only limited effects on uninjured neurites[73]. As with JNK signalling (above), inhibitors of these kinases delay degeneration only when applied around the time of injury[72], suggesting that both impact early steps. Furthermore, the GSK3 cascade appears to act downstream of NMNAT as various manipulations can revert WLD^S-mediated protection of cut axons[73].

Finally, inhibiting serine proteases delays neurite degeneration in several situations[72,74]. TLCK, a trypsin-like serine protease inhibitor delays degeneration induced by injury, microtubule disruption or NGF withdrawal in SCG cultures[74]. Thus, like the NMNAT-regulated step, activation of these proteases appears to be a common event during degeneration triggered by multiple different stresses. A chymotrypsin-like serine protease inhibitor, TPCK, can also delay injury-induced neurite degeneration in DRG (although not SCG) cultures[72,74]. TPCK only delays degeneration when added around the time of injury, again suggesting a role in the initiation phase for serine proteases[72]. However, although there was no direct comparison, protection by both inhibitors

appears to be significantly weaker than that conferred by WLD^S suggesting a modifying influence based on these criteria.

The execution phase

It will be critically important to identify the event that defines the start of the execution phase of Wallerian degeneration, as this is likely to be the point at which axons become committed to degenerate. In apoptosis, mitochondria regulate an analogous step but in Wallerian degeneration their functions in initiation or execution have yet to be resolved (see below).

By contrast, increased intra-axonal Ca^{2+} and calpains (calcium-dependent cysteine proteases) activation are well-established events in the execution phase of Wallerian degeneration[28,65,72,75-78]. Recent support for calpain involvement comes from a study showing that endogenous inhibition of calpain by calpastatin influences axon degeneration in primary culture and *in vivo*[65]. Interestingly, calpastatin degradation occurs both after injury and following NGF withdrawal in DRG neurites, suggesting it could be a potential convergence point between the Wallerian degeneration and axon pruning pathways. However, its degradation seems to be primarily calpain- and caspase-mediated respectively in these pathways[65].

The same study also confirmed that increased intra-axonal calcium and calpain activation occur downstream of the proposed NMNAT-regulated step during Wallerian degeneration[65]. In injured DRG neurites, WLD^S expression significantly delays the increase in intra-axonal calcium during the latent phase of Wallerian degeneration, and exogenous expression of a cytoplasmic NMNAT1 mutant delays calpastatin degradation[65]. Regulation of neuronal excitability through changes in sodium and potassium channel conductance also appears to occur upstream of the changes in calcium levels[79]. The fact that the Ca^{2+} channel blocker nifedipine can delay Wallerian degeneration when added to DRG neurites 2 hours after injury further supports a relatively late role for calcium influx[72]. Given that a virally-produced cytoplasmic NMNAT1 mutant is protective when added to primary cultures 4 hours after injury[41], it appears likely that much later nifedipine addition, or indeed Ca^{2+} chelation, would also be effective.

Although pharmacological inhibition of calpains blocks cytoskeletal changes during the execution phase in injured SCG and DRG neurites, it does not prevent blebbing[28,72,76]. This probably reflects a failure to prevent microtubule destabilisation. Crucially, calpain inhibition is significantly less protective than calcium chelation[76]. This has led to the suggestion of other, as yet unidentified calcium-dependent events[76].

Interestingly, membrane occupation and recognition nexus (MORN) family member retinophilin, identified in a *Drosophila* RNAi screen for modifiers of Taxol-induced neuropathy, also appears to promote a later step during the execution phase of Wallerian degeneration, as depletion of the mouse ortholog MORN4 in DRG cultures also prevents fragmentation but not blebbing after injury[80]. Therefore, MORN4 may act downstream of intracellular Ca^{2+} but on the same pathway branch as calpain activation.

Mitochondrial events: early or late?

Mitochondria have been proposed to play one or more key roles in Wallerian degeneration but whether this relates to initiation, execution or both remains undetermined. Mutations in mitochondrial proteins cause neurodegenerative diseases with prominent axon degeneration, including PD, Charcot-Marie-Tooth disease (CMT) and Friedrich's ataxia[81]. Mitochondrial malfunction lowers ATP, generates reactive oxygen species (ROS) and impairs Ca^{2+} buffering, leading to cellular homeostasis imbalance, mitochondrial permeability transition pore (mPTP) activation, release of proapoptotic signals and other cell death mechanisms[81]. In a non-axotomy model, ATP depletion initiates axon degeneration and is attenuated by $WLD^{S(56)}$, consistent with an early and causative contribution to axon degeneration by mitochondria.

Early during Wallerian degeneration mitochondria swell, fragment, accumulate at paranodes and lose their membrane potential[82]. However, whether these changes are a cause or consequence of degeneration, or general markers of compromised axons remains unclear. WLD^S partially colocalizes with mitochondria[8,9,83] (BOX 1; FIG 3) and overexpression of the mitochondrial NMNAT isoform NMNAT3 (sufficient to increase total NMNAT activity at least fivefold) protects injured axons[8]. These observations led to suggestions that NMNATs act within mitochondria to confer axon protection. One proposal, based on observations in *Drosophila*, is that WLD^S increases mitochondrial motility and calcium buffering capacity, reducing calcium influx immediately after injury[84]. However, in zebrafish embryos WLD^S had only small effects on mitochondrial transport and its arrest after injury and a PTEN-induced putative kinase 1 (PINK1) mutation that increases mitochondrial transport does not delay Wallerian degeneration[85]. As injury is not necessary to induce WLD^S-sensitive axon degeneration (see above), calcium influx cannot be the only way to activate the pathway.

Importantly, the exact sites of action of WLD^S and overexpressed NMNAT3 in axons remain undetermined and both proteins clearly localize also outside mitochondria[8,83]. Endogenous NMNAT3 is present at low levels in brain despite the abundance of mitochondria[86,87] and cannot substitute for NMNAT2[10]. A recent study questions whether endogenous NMNAT3 is even present

in mitochondria[88], and targeting a cytosolic variant of NMNAT2 to mitochondria actually abolished its protective capability[38]. Depletion of mitochondria from axons has produced conflicting results. Dominant negative mutations of mitochondrial Rho GTPase (Miro), which decrease mitochondrial transport in *Drosophila* larvae, reduce the protective capacity of $WLD^{S(84)}$ but in loss-of-function mutants for Milton, a mitochondrial transport adapter, axons lacking mitochondria were slightly protected from Wallerian degeneration and mitochondria were dispensable for WLD^S protection[36]. Axons lacking functional Milton gradually degenerate spontaneously and neither WLD^S nor NMNAT3 overexpression can rescue them[40], suggesting they act either upstream of the mitochondrial defect or on a separate pathway consistent with the gradual atrophy after Milton downregulation compared to the rapid fragmentation after dNmnat depletion[40]. The recent finding that ATP from glycolysis can drive axonal transport[89] could explain how dNmnat continues to reach axons in the absence of mitochondria and indicates that sufficient ATP can be generated in the absence of mitochondria to support active axonal processes for some time. If the mitochondrial model is correct, these observations will need to be explained.

Whether SARM1 localizes to, and acts at, mitochondria is also unclear. Ectopically expressed SARM1-GFP localizes to mitochondria, where it recruits JNK3 from cytosol[66]. Based on this finding an N-terminal mitochondrial binding domain in SARM1 was identified[90]. However, there are conflicting reports regarding whether this domain is required for the protein's prodegenerative function[64,91]. A partial colocalization of endogenous SARM1 and mitochondria has been reported[64] although in comparison to overexpressed, tagged SARM1, the endogenous protein remains largely independent of mitochondria[4,64]. Intriguingly, SARM1 does relocalise to mitochondria in response to the encephalitis pathogen Bunyavirus, leading to mitochondrial damage and neuronal death. It will be important to determine whether similar relocalization occurs during Wallerian degeneration[92].

Mitochondria may, however, play a role in Wallerian degeneration downstream of the WLD^S-sensitive step. The mPTP may function during execution, as its pharmacological or genetic inhibition protects degenerating axons[93]. An increase in ROS before degeneration, potentially originating from mitochondria or cytoplasm, could lie upstream or downstream of mPTP activation in Wallerian degeneration[94-96]. ROS scavengers delay Wallerian degeneration and axon pathology associated with neurodegenerative disorders[94,97,98 99], and WLD^S reduces ROS accumulation[85]. Abnormalities in a variety of mitochondrial, peroxisomal or cytosolic enzymes or of intracellular antioxidant defenses can trigger ROS increases[94,100], and consequently the source of the ROS increase in Wallerian degeneration remains unidentified.

In summary, although a role for mitochondria appears likely in the execution phase, and mitochondrial changes are certainly a marker of degeneration, it remains unclear whether they also

help initiate Wallerian degeneration and whether mitochondrial roles of NMNATs or SARM1 are involved.

Relationship to disease mechanisms

It has been known for more than ten years that WLD^S prolongs axon survival in some motor neuron disease and peripheral neuropathy models, suggesting there is some pathway convergence[33,101]. However, as some disorders are unaffected by WLD^S, both the extent of the convergence and the molecular explanation for these differences are important to understand[21].

Many recent studies have investigated the relationship between Wallerian-like mechanisms and neurodegenerative disease. Here, we have grouped the neurodegenerative models studied into several categories: those in which WLD^S confers strong protection (defined as axon preservation at or near control levels that clearly alleviates symptoms), those where protection is moderate (defined as axon preservation with only a marginal or no improvement in symptoms), and those that are insensitive to WLD^S or NMNAT (TABLE 1). Most published studies report alleviation of axon degeneration in the presence of WLD^S or overexpressed NMNAT, although some negative results could remain unpublished. Prominent among the modifiable *in vivo* models are axon degeneration induced by neurotoxins[17-19], defective microtubule assembly or axonal transport impairment[17,20,25,26], and models of CMT[33,101], PD[18,19,102], and retinal disorders[25,26,103,104]. Neurites in WLD^S or NMNAT overexpressing primary cultures are resistant to vincristine, vinblastine, MPP^+ and rotenone[32,95,96], mirroring some of these themes.

Regarding disorders that are insensitive to WLD^S (TABLE 1), we previously noted that these were mostly disorders with a late onset and suggested that a 2-3 week delay in a slowly evolving disorder could be hard to detect[21]. However, recent examples of early onset disorders that are not rescued, such as the glycyl-tRNA synthase (*Gars*) model of CMT2D[105] and early onset models of spinal muscular atrophy[106,107], instead suggest there are distinct pathways to axon degeneration that can be distinguished by their sensitivity to WLD^S. However, it is important to note that WLD^S rescue of axons can be heavily dose dependent[11] and not all studies have tested its effects in homozygotes.

Axonal and nuclear effects of WLD^S in disease

These findings have led researchers to consider how WLD^S preserves axons in disease. Its effect is most easily understood in severe axonal transport disorders in which the delivery of cargoes from cell bodies is restricted (as it is after axotomy). In particular, we have proposed that the failure

to deliver NMNAT2 is a key determinant of Wallerian degeneration (see above). However, this appears less likely to explain why other models, such as CMT[33,101] and the wabbler-lethal mouse[108], are WLD^S-sensitive. Thus, we speculate that NMNAT2 or its enzyme activity could also be depleted by other mechanisms, such as changes in transcription, RNA processing, translation, Golgi processing, post-translational modification or turnover.

Some interesting developments suggest that Wallerian degeneration has a wider relevance to disease than previously expected. It now appears that WLD^S-sensitive axon degeneration can be caused not only by stresses to the axon itself but also by impairment of the soma. WLD^S blocks axon degeneration caused by excitotoxic damage to the retina[104] or by inhibition of protein synthesis in cell bodies in compartmentalized cultures[10]. In both cases an additional direct effect of the stressor on proximal axons cannot be ruled out; however, as knockdown of a single gene, *Nmnat2*, also causes WLD^S-sensitive axon degeneration[10] it seems likely that impairment of processes within the soma, and not only within the axon itself, can initiate a pathway related to Wallerian degeneration.

Surprisingly, WLD^S also preserves glucose tolerance in models of Type I and Type II diabetes[109,110]. Its preservation of pancreatic beta cells after streptozotocin treatment probably involves a mechanism distinct from axonal protection, where WLD^S clearly acts within the axon itself[9,39] (BOX 1; FIG 3). As this cell type has no axon, the abundance of WLD^S in nuclei appears likely to underlie its effect. Specifically, WLD^S may boost the capacity for nuclear NAD^+ synthesis, reducing NAD^+ depletion upon steptozotocin-induced DNA damage. Consistent with this, nuclear SIRT1 is required for the glucose homeostatic effect of WLD^S [(110)] but is dispensable for axon protection[37,48]. The effect of WLD^S on glucose tolerance makes it difficult to interpret whether WLD^S additionally preserves axons in diabetic peripheral neuropathy. Nevertheless, when diabetes was induced in Wld^S mice, neuropathy was less severe than in wild-type controls[109].

Similarly, nuclear WLD^S could sometimes preserve neuronal cell bodies in disease through a mechanism distinct from blocking Wallerian degeneration. In some disease models only axons are preserved[29,104], or neuronal cell bodies may be protected as a result of axon survival which ensures the continued delivery of neurotrophic factors[20,111]. However, some studies suggest a more direct effect of WLD^S in the attenuation of cell body death in ischemic injuries[103,112,113] or primary culture toxicity[114]. As in models of diabetes, WLD^S within neuronal nuclei could influence nuclear NAD^+ synthesis and thereby enzymes such as SIRT1 and PARP1 in addition to its axonal function. Moreover, NMNAT1 is known to influence SIRT1 and PARP1 through a direct interaction[115,116]. It will thus be important to investigate the nuclear and axonal roles of NMNAT and WLD^S, a process that may be aided by the use of NMNAT1 overexpressing[54], null and floxed mice[86].

It is important to extend these studies to the newly identified modifiers of Wallerian degeneration (see above). The outcomes may differ according to which step in the pathway is blocked, especially for any disease mechanisms converging downstream of the WLD^S-modifiable step. Moreover, whereas WLD^S may additionally prevent disruption of nuclear NAD^+ metabolism (see above) other modifiers of an axon-specific pathway may not. In this regard it is interesting to note that SARM1 has already been reported to modify ischemia-induced damage[66] and its genomic locus is associated with ALS[117].

In summary, the homogeneity and predictability of axon degeneration after injury (FIG 1C) has led to substantial advances in understanding this mechanism (below). This in turn sheds light on the more difficult challenge of understanding axon degeneration in disease, where onset is more heterogeneous, dispersed and multifactorial (FIG 1A, B). There is evidence for both WLD^S-sensitive and independent mechanisms, and perhaps for separate axonal and non-axonal actions of WLD^S. Novel modifiers of Wallerian degeneration are likely to help clarify these pathways.

Links to wider biological mechanisms

The recently identified regulators of Wallerian degeneration suggest links to other mechanisms not apparent from WLD^S studies. Apart from specific roles in cell and axon survival, WLD^S has little other notable effect. It influences neither synapse development[118] nor axonal pruning[14], although it may alter pruning of specific *Drosophila* dendrites[45]. Apoptosis is unaffected by the presence of WLD^S both in primary culture and *in vivo*[27,29], and retinal ganglion cells continue to die in some glaucoma and excitotoxic retinal injury models[26,119].

Against this background, the identification of the innate immunity signalling molecule SARM1[4,120] as a regulator of Wallerian degeneration was surprising. SARM1 is one of five mammalian TLR adapters. It appears not to induce NF-κB signalling like other TLR adapters[120], but does alter susceptibility to several specific viruses[92,121,122]. The most intensively studied role of innate immunity is the recognition of pathogens and induction of an inflammatory response to remove them, or of cell death to minimise their spread. However, in addition to pathogens, some host molecules also signal through TLRs to induce cell death: for example, the microRNA let-7 interacts with TLR7, providing a possible mechanism of cell damage signalling[123]. Thus, one could speculate that SARM1 is similarly activated by an intra-axonal injury signal. The nature of such a signal and its putative receptor remain unclear but as SARM1 requires its TIR signalling domain to induce axon degeneration, a TLR signalling mechanism appears likely[64].

SARM1 also influences virally-induced death in cortical neuron primary cultures[92]. An important question is whether Wallerian degeneration and some types of cell death are initiated differently but share a common, downstream mechanism, perhaps with SARM1 lying beyond the convergence point.

Finally, there are emerging links to neuronal development. First, NMNAT2 is required for axons to grow beyond a threshold length, as well as for survival of established axons[11,12]. Intriguingly, NMNAT2 levels also increase proximal to an axon lesion where NMNAT2 could function in regrowth[10]. Second, in primary hippocampal neurons, SARM1 knockdown strongly reduces dendritic branching, alters spine morphology and reduces axon growth[124] and SARM1 depletion *in vivo* reduces brain weight and dendritic branching[124]. Third, a loss-of-function mutation in Highwire results in synaptic overgrowth in *Drosophila*[125]. However, a different Highwire ubiquitination substrate, Wnd/DLK, mediates this developmental phenotype, as well as an increase in axon sprouting after injury and a modest effect on axon degeneration[15]. Thus, there may be some similarities between regulation of axon and dendrite growth and Wallerian degeneration, but some effects may reflect multiple functions for these proteins.

Evolution of Wallerian degeneration

Recent developments also demand that we rethink how Wallerian degeneration evolved. As rapid Wallerian degeneration is a necessary step towards axon regeneration in peripheral nerves, it is often assumed that this is its evolutionary advantage[126]. However, it is hard to see this as a major driver of evolution as free-living animals are unlikely to survive long enough after nerve injury for regeneration. Furthermore, it now appears that Wallerian degeneration evolved in a common ancestor of mammals and flies so its advantage must be considered in this context. As most invertebrates lack adaptive immunity, innate immunity confers a powerful survival advantage. Many viruses spread around the nervous system using axonal transport[127,128], so an intriguing possibility is that rapid axon degeneration originally arose as a mechanism to limit this spread and was only later adopted as a response to injury.

Although difficult to prove, there are interesting implications for neuropathology in humans and other mammals. West Nile Virus requires intact peripheral nerves to enter the CNS[129] and removal of SARM1 enhances its replication in the brainstem[122], consistent with a role for intact axons in virus replication. Thus, axon degeneration could limit the spread of this virus, a notion proposed previously for Theiler's murine encephalomyelitis virus[130]. SARM1 expression also responds to viral infections, rising during an infection with La Crosse Virus, where it may contribute to neuronal

death in paediatric viral encephalitis[92], and falling after infection with porcine reproductive and respiratory syndrome virus[121].

Other viral disorders are associated with axon damage, notably human immunodeficiency virus, which causes distal sensory neuropathy in the periphery and axonal transport disruption in the CNS[131,132]. Viral-induced axon degeneration could contribute to some cases of MS, potentially underlying the so-called 'inside-out' model, which proposes that demyelination and the autoimmune response are secondary events[133]. The potential roles of MHC expression in ALS and chemokine secretion in CMT could also indicate an innate immune role in axon survival[134,135]. It will be particularly important to determine whether SARM1, and degenerative pathways related to Wallerian degeneration, are required for many of these pathologies.

Conclusions and perspective

Until recently, expression of WLD^S or related NMNATs was the only known way to preserve severed axons for weeks *in vivo* and days in primary culture. In parallel with an improved understanding of their actions, several additional modulators with an equally robust effect have now emerged. We propose that they lie on the same putative core pathway, whereas proteins influencing Wallerian degeneration less strongly modify this pathway. Alternatively, there could be other equally important, but still unknown pathways in which some of these factors act. Morphologically, Wallerian degeneration involves a latent phase followed by abrupt fragmentation, likely with a commitment point prior to fragmentation. The updated list of disease models influenced by WLD^S and NMNATs (TABLE 1) shows clusters of some disease types but as yet no underlying principle. The additional functions of recently-identified modifiers dSarm/SARM1 and Highwire/PHR1 in innate immunity and synapse growth suggests possible links between the Wallerian degeneration mechanism and other important areas of neuroscience.

This is an exciting time for the field as this progress opens many new questions. Foremost among them is whether, as we propose, SARM1 and PHR1 lie on the same pathway as NMNAT and, if so, which steps are upstream and which downstream. Ongoing genetic screens in *Drosophila* and functional studies of existing modulators should reveal additional steps in the putative pathway. The involvement of mitochondria needs further clarification and the molecular identity of the commitment point is critically important. Any successful therapy will need to target a step preceding this point. It is important to determine what activates the prodegenerative action of SARM1 and whether this relates to its roles in innate immunity and cell death.

From a therapeutic viewpoint, the key questions are how, and in which disorders, to target the emerging pathway. Temporary periods of axon stress, such as chemotherapy-induced peripheral neuropathy, MS relapses or stroke, would appear to be better initial goals than chronic disease, as permanently compromised axons are protected only for limited periods. Inhibition of NAMPT, JNK, GSK-3β and other proteins can preserve injured axons in primary culture, albeit more modestly than genetic mutations, so it is essential to understand their modes of action pursue these studies *in vivo*. In parallel with attempts to mimic SARM1 and PHR1 loss-of-function pharmacologically, genetic studies to test which disease models they influence are needed. Unlike WLD^S, the SARM1 and PHR1 null phenotypes do not require the delivery of a protein into axons, suggesting that they may provide a more long-lasting protection in transport disorders. Actions at different points on the pathway could lead to different therapeutic profiles. For example, if disease pathways converge, blocking a step downstream of the convergence but before the commitment to degenerate should have a wider protective effect. Finally, it will be important to translate this knowledge into strategies for targeting human disease, especially as WLD^S preserves human axons[36] and NMNAT1 deficiency causes neurodegeneration in man[136-139]. The ability of SARM1 and WLD^S to influence cell body survival in some circumstances suggests the feasibility of combinatorial therapies to protect both cellular compartments. The genetic association of axonal disorders, or relative resistance to them, with haplotypes of NMNAT 1, 2 or 3, SARM1, PHR1 and other modulators is an important area for human studies, as is the identification of biomarkers that provide mechanistic insight and diagnostic capability.

Figure Legends

Figure 1. Systems for studying axon degeneration

Schematic representation of the causes and features of axon degeneration in different diseases and model systems. a,b| Early loss of axons is common in neurodegenerative disorders but the mechanisms can be difficult to study directly in disease models. In ALS (a) for example, a number of different causative factors have been proposed to lead to axonal degeneration in a population of vulnerable spinal motorneurons. This leads to disruption of the connectivity between the muscles and spinal cord and, eventually, to the death of the affected neurons. A population of 'resistant' motor axons, however, do not undergo degeneration in response to these stressors. Similarly, in Alzheimer's disease (b) the effects of amyloid beta peptide, tau pathology and a combination of other causative factors result in axonal dystrophy in vulnerable neurons. Other axons may encounter less of these pathogenic factors and do not degenerate. Progress towards understanding mechanisms of axon degeneration in these disorders is therefore limited by their multifactorial causes, by heterogeneity in the timing and sites of key pathogenic events, and by the

differential responses of vulnerable or resistant axons. c| By contrast, in Wallerian degeneration (injury-induced axon degeneration) the location and timing of the initial lesion is well understood and the process involves sudden degeneration of all axons in a nerve within a short time window following nerve injury[2]. Consequently, progress towards understanding this mechanism can be made relatively quickly.

Figure 2. Structure-activity relationships of WLD^S and SARM1.

a|. *Wld^S* is a chimeric gene that results from a tandem triplication[11] and encodes full length NMNAT1 (BOX 1) fused to 70 N-terminal amino acids (N70) from the ubiquitin ligase UBE4B (ubiquitin conjugation factor E4 B). N70 does not contain UBE4B catalytic activity, but retains a small motif within the N-terminal 16 amino acids that binds the ubiquitous cytoplasmic protein valosin-containing protein (VCP, also called p97). This sequence is important for the localisation of Wld^S in the cytoplasm and axoplasm[48,49] (BOX 1; FIG. 3). A variant WLD^S protein lacking this motif shows a predominant nuclear location and fails to confer axon protection[48,49]. An 18 amino acid-linker sequence (18AA) originates from the 5'UTR of *Nmnat1* and is unique to the WLD^S protein. Full length NMNAT1 sequence retains NAD^+-synthesis activity. This sequence and its catalytic activity are required for WLD^S axon protective activity[48,49].

b|. SARM1 is a modular protein comprising an N-terminal Heat/Armadillo (ARM) domain, two central sterile α motif (SAM) domains and a C-terminal Toll-interleukin-1 receptor (TIR) domain in common with other TLR signaling molecules. Several studies indicate a mitochondrial interacting sequence at the extreme N-terminus[64,66,90] but this remains to be confirmed for the endogenous protein (see text) and appears to be unnecessary for axon degeneration[64]. The SAM and TIR sequences are both essential for rapid axon degeneration, probably as sites for homodimerization and signaling respectively[64]. The N-terminal ARM domain appears to inhibit the prodegenerative function, as its removal appears to generate a constitutively active protein[64].

Figure 3. NAD^+ metabolism and compartmentalization in neurons

a|. The key steps of NAD^+ biosynthesis from nicotinamide (Nam) and nicotinic acid (Na). The NMNAT-catalyzed step is common to all the NAD biosynthetic pathways. FK866 and CHS-828 potently inhibit NAMPT, the rate-limiting enzyme. NaMN = nicotinic acid mononucleotide; NMN = nicotinamide mononucleotide; NaAD = nicotinic acid adenine dinucleotide.

b|. The compartmentalization of NAD^+ biosynthetic enzymes in neurons. NMNATs 1, 2, 3 (N1, N2, N3) are endogenous proteins that are localised to the nucleus, cytoplasm and mitochondria respectively, a distribution that is paralleled by that of NAD-metabolizing enzymes such as SIRTs and PARPs. NAMPT, another endogenous protein, is widely distributed throughout the cytoplasm and nucleus. WLD^S, which is naturally present only in *Wld^S* mice, is found in axons but with a much higher concentration in nuclei. Variant forms of WLD^S and NMNATs (not shown for reasons of clarity) have also been generated for structure-function studies. CytNMNAT1, Ax-NMNAT1 and

deltaNLS-WLD^S all increased the amount of WLD^S or NMNAT1 in axons[6,9,83], and NMNAT2ΔEx6 disrupted the tethering of NMNAT2 to Golgi-derived vesicles[7]. Each of these modifications increased the protective activity relative to NMNAT1, WLD^S and NMNAT2 respectively. ATX-WLD^S, replaced the VCP/p97 binding site with a motif derived from the protein ATX3, restoring the protective capacity of the N-terminally truncated WLD^S [(49)].

Figure 4. An emerging molecular pathway of Wallerian and WLD^S-sensitive axon degeneration.

We have assembled a speculative pathway of events that occur during Wallerian and WLD^S-sensitive axon degeneration in mammals based on our current assessment of the literature (see main text). Many events appear to be conserved in lower organisms. The variety of injury and non-injury stresses that are presumed to activate this putative pathway are listed to the left (NGF withdrawal additionally activates a presumed parallel pathway involving death receptor signaling and caspase activation that is required for axon pruning[43,44]). The pathway shown relates to events that occur in the main body of the axon and not to acute axon degeneration that occurs close to a site of physical injury. We propose that a core pathway, consisting of an initiation phase and an execution phase, receives additional input from other modifying signals. Many molecules and signals can be assigned to initiation or execution phases with some confidence (although their relative ordering remains open in many cases) but it is difficult to assign some participants, including SARM1 (thought to contribute to a core event), mitochondria and reactive oxygen species (ROS), to either phase based on current experimental data. As such they are shown as provisionally influencing steps in both phases although they likely act at only one point (question marks indicate alternative options). There are also likely to be several gaps in the pathway where important modulators have not yet been identified. We propose that regulation of NMNAT2 turnover and activity represents an early node in the pathway and the point at which WLD^S / NMNAT isoform overexpression or loss of PHR1 is likely to act to delay degeneration. Endogenous NMNAT2 is predicted to inhibit the pathway in healthy axons and rapid loss of this very labile protein could act as a trigger for pathway activation in the situations in which its delivery to axons fails. However, it remains possible that WLD^S / NMNAT overexpression could modulate other events in the pathway downstream of NMNAT2.

Table 1: **The effect of WLD^S and overexpressed NMNAT proteins on animal models of disease.** Strong protection is here defined as significant preservation of axons at or near control levels, accompanied by clear improvements in symptoms and/ or electrophysiological measures. Moderate axon protection is defined as preservation of axons with only marginal or no improvement of symptoms; in other disease models there was no effect. An additional small group showed some cellular preservation but axons were not studied.

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Table 1 The effects of WLD^S and overexpressed NMNAT isoforms on animal models of disease.

Disease modelled	Species	Insult or mutation	Onset age of degeneration or time after insult	NMNAT added	Level of axon protection	Effect on progression and symptoms	References
Studies showing strong axon protection							
Peripheral nerve injury	Mouse / Rat	Transection / Crush	Acute	WLD^S	2-3 week delay	N/A	24
Defective axon extension	Mouse	*Nmnat2* gene trap	<E12.5	WLD^S	> P1 / implied at 3 months	Improved survival to > 3 months	11
Optic nerve injury	Mouse	Crush	Acute	WLD^S	~ 50% protected after 2 weeks	N/A	26,111
Toxic neuropathy	Mouse	Taxol	Acute	WLD^S	> 2 week delay	Rescue of Rotarod performance	17
Glaucoma	Mouse	DBA/2J strain	6-16 months	WLD^S	at 12 months	Reduced	25
Glaucoma	Rat	Experimentally raised IOP	Acute	WLD^S	> 2 week delay (no protection at 4 weeks)	N/A	26
Glaucoma	Mouse	Experimentally raised IOP	Acute	cytNMNAT1	after 3 weeks	N/A	103
Excitotoxic injury to retina	Rat	NMDA injection	Acute	WLD^S	> 2 week delay	N/A	104
Retinal ischemia	Mouse	Experimentally raised IOP (transient)	Acute	cytNMNAT1	after 4 days	N/A	103
Hypoxic-ischemic injury	Mouse	Carotid artery ligation / hypoxia chamber	Acute	cytNMNAT1	implied from tissue damage / loss at 1-7 days after insult	N/A	112
Parkinson's disease	Mouse	6-hydroxydopamine	Acute	WLD^S	> 11 days delay for some	N/A	18
Parkinson's disease	Mouse	6-hydroxydopamine	Acute	WLD^S	6 day delay against anterograde degeneration (modest after 9 days)	N/A	102
Parkinson's disease	Mouse	MPTP	Acute	WLD^S	after 7 days	Enhanced mouse survival, but likely independent of axon protection	19
Wabbler-lethal (*wl*)	Mouse	*Atp8a2* mutation	12 days	WLD^S	at 2 months	No effect	108
Progressive motor neuronopathy	Mouse	*Tbce* mutation	< 2 weeks	WLD^S	2-3 week delay	Delayed by > 1-3 weeks	20
CMT (type 1B)	Mouse	*P0* (*MPZ*) null	< 6 weeks	WLD^S	at 3 months (but none at 5.5 months)	Modest improvements in conduction and muscle strength	33

Studies showing moderate axon protection							
CMT (type 1A)	Rat	*Pmp22* transgene	~ 2 months	WLD^S	at 13 weeks	Reduced electrophysiological abnormalities and increased strength gain	148
Gracile axonal dystrophy	Mouse	*Uchl1* mutation	6 weeks	WLD^S	reduction in axonal swelling at 4 months	No improvement	149
EAE	Mouse	Myelin oligodendrocyte glycoprotein	Acute	WLD^S	up to 8 weeks	Modest delay of behavioral defects	150
Studies showing neuronal protection; in which axons were not studied							
Global cerebral ischemia	Mouse	Transient carotid artery occlusion	Acute	WLD^S	No direct measure of axon health	Substantial reduction in neuronal damage	113
Diabetes	Mouse	Streptozotocin	Acute	WLD^S	No direct measure of axon health	Improved lifespan and reduced neuropathy and retinopathy; possibly secondary to rescue of pancreatic beta cells	109
Tauopathy	Mouse	P301L tau transgene (rTg4510)	2.5 months	NMNAT2	No direct measure of axon health	Reduced neurodegeneration at 5 months	61
No effect							
CMT (type 2D)	Mouse	*Gars* (glycyl-tRNA synthetase) mutation	~ 2 weeks	WLD^S	None at 4 weeks	Not reported	105
Spinal muscular atrophy	Mouse	*SMN* Δ *7*;*SMN2*;$Smn^{-/-}$ / *SMN2*;$Smn^{-/-}$	< P5 / before birth	WLD^S	no effect on distal axon & NMJ defects at P5	No	106
Spinal muscular atrophy	Mouse	*SMN* Δ *7*;*SMN2*;$Smn^{-/-}$	< P5	WLD^S	no axon loss in mutant at P12	No	107
ALS	Mouse	*SOD1* G93A transgene	> 11 weeks	WLD^S	None from 80 days	Modest extension of lifespan	151
ALS	Mouse	*SOD1* G37R transgene	4-5 months	WLD^S	None at 5-6 months	No	22
ALS	Mouse	*SOD1* G85R transgene	9-10 months	WLD^S	None at 1 year	No	22
Hereditary spastic paraplegia	Mouse	*Plp* null	8-18 months	WLD^S	None at 18 months	Not reported	152
Prion disease	Mouse	scrapie infection	> 140 days post infection	WLD^S	None at terminal stage >180 days post infection	No	153
Defective axon support	Mouse	*Cnp1* null	from P5	WLD^S	None at P20	No	154

Box 2 Conservation of the NMNAT survival role

Remarkably, overexpression of WLD^S confers similarly strong axon protective phenotypes in rats, flies and zebrafish, as does ectopic expression of any mammalian NMNAT isoform in flies[13,14,34,35]. WLD^S and dNmnat also protect lesioned human neurites in primary cultures[36]. Whether this evolutionary conservation of function extends to *C. elegans* is currently unclear. Overexpression of NMNAT does preserve *C. elegans* axons and cell bodies in the presence of constitutively open mechanosensory channels (degenerins), which cause axon degeneration and neuronal death by raising intracellular calcium[98]. However, neither WLD^S nor overexpression of the two endogenous NMNATs preserves *C. elegans* transected axons (A. Nichols & M. Hilliard, personal communication).

Conservation of the Wallerian degeneration pathway from flies to mammals (including man) validates *Drosophila* and zebrafish as systems to study its mechanisms. This brings a number of crucial experimental advantages. Importantly, these organisms are already widely used to model diseases and are highly amenable to genetic manipulation. For example, *Drosophila* genetic loss-of function screens, coupled in one case with MARCM (Mosaic analysis with a repressing cell marker) to avoid lethality[4], have identified novel modulators of Wallerian degeneration, such as SARM1/dSarm and MORN4/retinophilin[4,80]. *Drosophila* larvae, adult *Drosophila* wings and zebrafish embryos are all transparent, allowing time lapse fluorescent imaging to be performed *in vivo*[35,40]. Thus, live imaging in zebrafish of Wallerian degeneration and its consequences during development confirmed the sudden switch to axon fragmentation following a latent period[35] that was previously inferred from analysis of fixed mouse nerves[2]. Zebrafish also provide a platform for aqueous drug screening and manipulating axon degeneration in flies or zebrafish disease models is a promising avenue for mechanistic and therapeutic studies. Finally, the generation of Wld^S rats has widened the scope for studying neurodegenerative models[26,101,146] and provided a second mammalian system to test mechanistic hypotheses.

Nevertheless, there are important differences between species. *C. elegans* and *Drosophila* NMNATs are also required for the maintenance of neuronal cell bodies[57], possibly reflecting the fact that there are fewer isoforms in these species. The single *Drosophila* Nmnat probably fulfills the functions of all three mammalian homologs, preserving axons and cell bodies through its enzymatic activity and chaperone function respectively[58]. Mammalian evolution appears to have favored NMNAT expression from distinct genes and tight compartmentalization of each isoform (BOX 1). Thus, mammalian NMNAT2 is required for axon maintenance but NMNAT1 may be more instrumental in neuronal cell body survival: indeed, loss-of-function mutations in NMNAT1 cause retinal neurodegeneration[136-139]. Interestingly, other NAD^+ biosynthetic enzymes and their precursors also differ in some invertebrates[147]. These differences may be related to specialized NAD^+ metabolic reactions at specific locations[141] and smaller cellular dimensions.

BOX 1: NAD^+ metabolism and axon degeneration

NMNATs and NAD^+ metabolism. Our current understanding of the importance of NAD^+ metabolism for axonal survival emerged from the discovery of the slow Wallerian degeneration protein (WLD^S), a fusion protein that contains nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) (FIG 2). Like other NMNAT isoforms, NMNAT1 catalyses NAD^+ synthesis from ATP and nicotinamide mononucleotide (NMN, FIG 3A)[140]. In addition to its action as a co-enzyme involved in redox reactions, NAD^+ has key signalling roles: it mobilizes calcium through its metabolites ADP ribose (ADPR), cyclic ADPR and nicotinic acid adenine dinucleotide phosphate (NAADP), and contributes to post-translational protein modifications such as deacetylation, catalyzed by Sirtuins and mono- or poly-ADP ribosylation, catalyzed by mono- and poly (ADP-ribose) polymerases (FIG. 3).

Compartmentalization of NMNAT isoenzymes. NMNAT enzymes are found in all organisms. The three mammalian isoforms differ in enzymatic properties, tissue distribution and subcellular localization[141]: NMNAT1 is localized to the nucleus and widely expressed; NMNAT2 is found in the cytoplasm and axoplasm and is abundant in the brain; NMNAT3 is probably localized to mitochondria (although this has been questioned[88]) and is weakly expressed in the brain. Similarly, NAD^+ consuming enzymes, and hence probably NAD^+ metabolic pools, are also compartmentalized[141] (FIG 3B).

This compartmentalization is particularly evident in neurons, in which distal axons may be separated by long distances from cell bodies. NMNAT1 provides NAD^+ as substrate for nuclear enzymes and contributes to neuronal survival in the retina and probably elsewhere[136-139]. NMNAT2 undergoes fast bidirectional axonal transport and is essential for axon survival and growth[10,11,142]. Mitochondrial NAD^+, which may be synthesized by NMNAT3[88,143], is involved in regulating cellular energy balance through SIRTs and PARPs. The enzyme involved in the preceding step in the NAD^+ salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT) is homogeneously distributed within neurons[144], so localized distribution of NMNAT isoforms is the distinguishing feature of compartmentalized NAD^+ synthesis (FIG 3). These isoforms have at least partly non-redundant functions as deleting either NMNAT1 or NMNAT2 is lethal[12,86].

Where does WLD^S act to protect axons? WLD^S is predominantly nuclear, reflecting the nuclear localization of NMNAT1[3] (FIG. 2 and 3B). This led to the suggestion that it has a nuclear axon-protective action, which is mediated by sirtuin 1 (SIRT1)[46]. However, small amounts of WLD^S are also present in axons, and multiple lines of evidence indicate this is the location where WLD^S acts to delay injury-induced degeneration[39,41,83].

Although a mitochondrial site of action for WLD^S has also been discussed[8,84], it remains unclear which of the many cytoplasmic fractions containing WLD^S is its site of action[8,83]. WLD^S binding

partner VCP (FIGS. 2 and 3B) is widely distributed but is most abundant at the surface of cytoplasmic organelles[145]. A highly protective WLD^S variant in which nuclear localization is disrupted partially localizes to mitochondria and endoplasmic reticulum[83]. Other protective constructs, in which NMNAT1 is targeted to axons or cytoplasm are distributed among all subcellular and cytosolic compartments[9] or present with a diffuse extranuclear appearance[6], respectively. Lentivirally-transduced NMNAT1 does enter axons and strongly overexpressed NMNAT3 is observed in the cytosol and cytoplasm in addition to mitochondria[8]. Thus, overexpressed NMNAT1 and NMNAT3 may act in one of these ectopic locations to delay Wallerian degeneration rather than in nuclei or mitochondria. Interestingly, NMNAT2 is axonally transported on Golgi-derived vesicles, but its protective efficacy is greatly increased (to a level comparable with WLD^S) if it is detached from these vesicles[38]. This suggests a non-vesicular site of action for NMNAT enzyme activity.

Figure 1

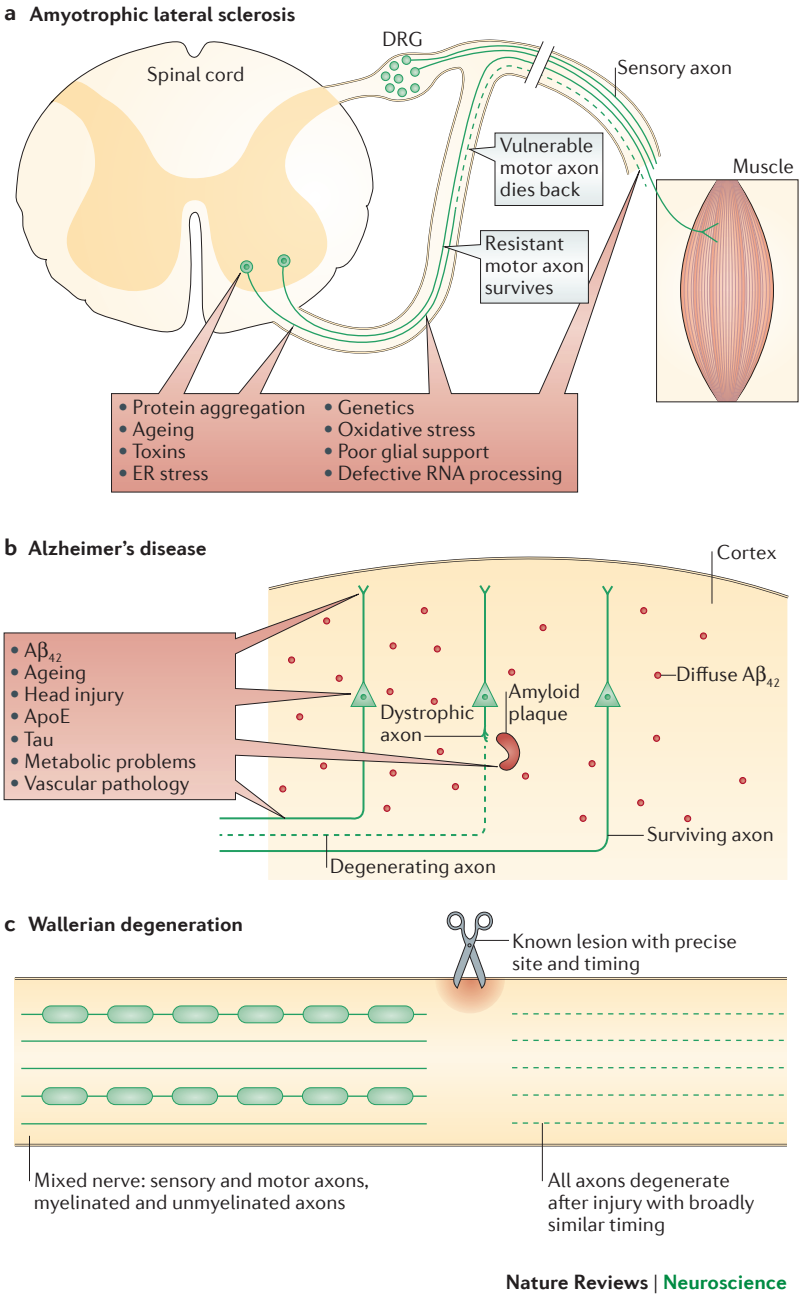

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Figure 2

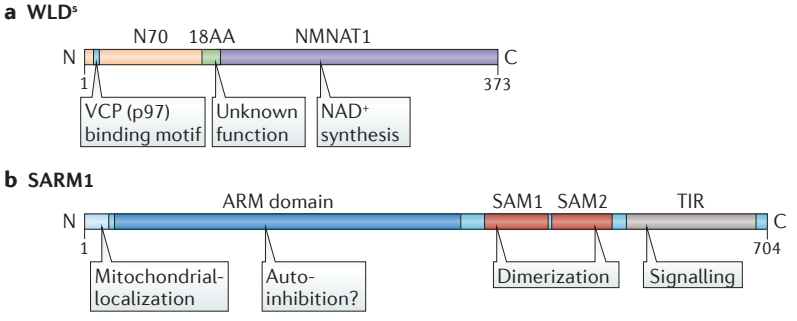

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Figure 3

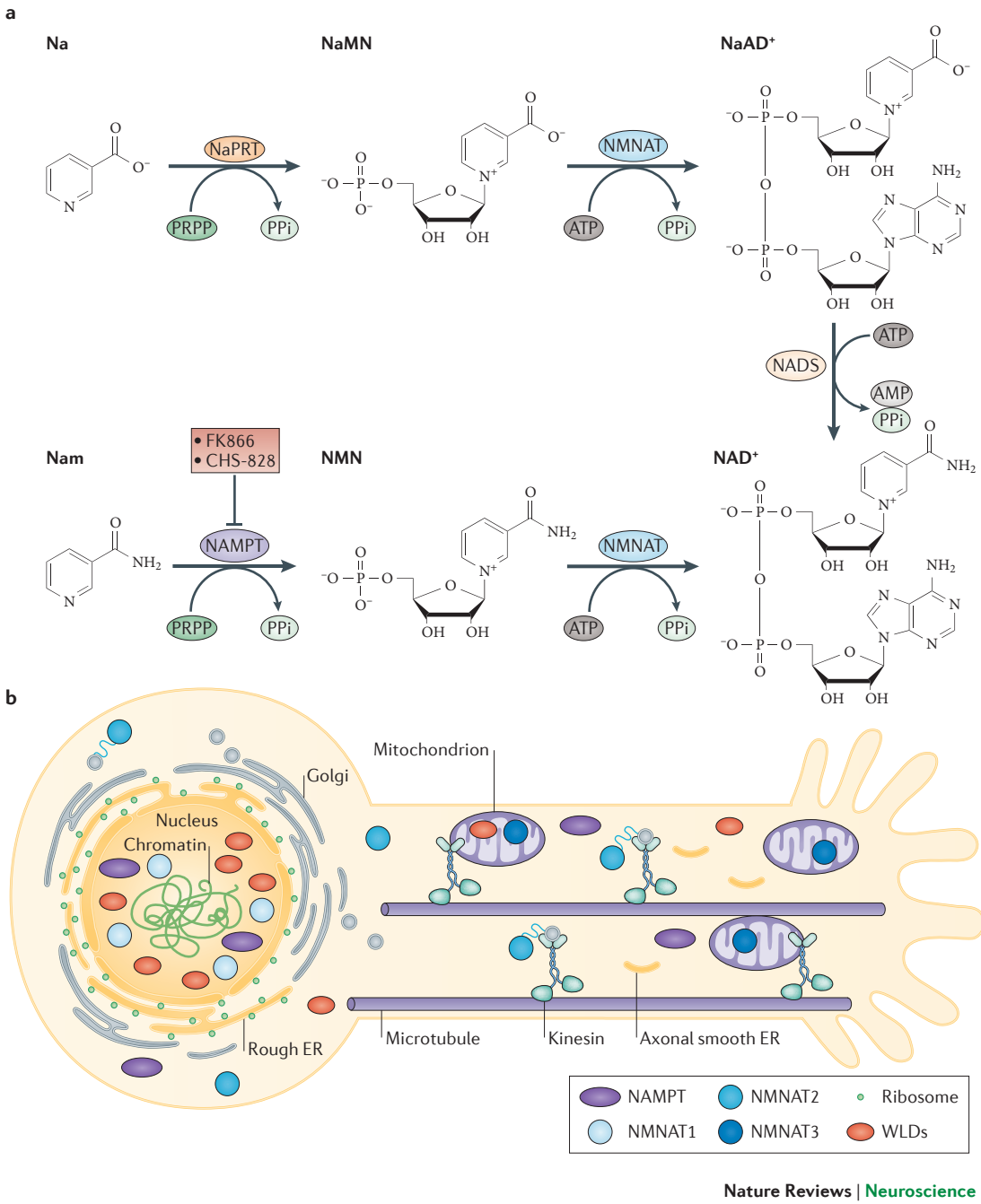

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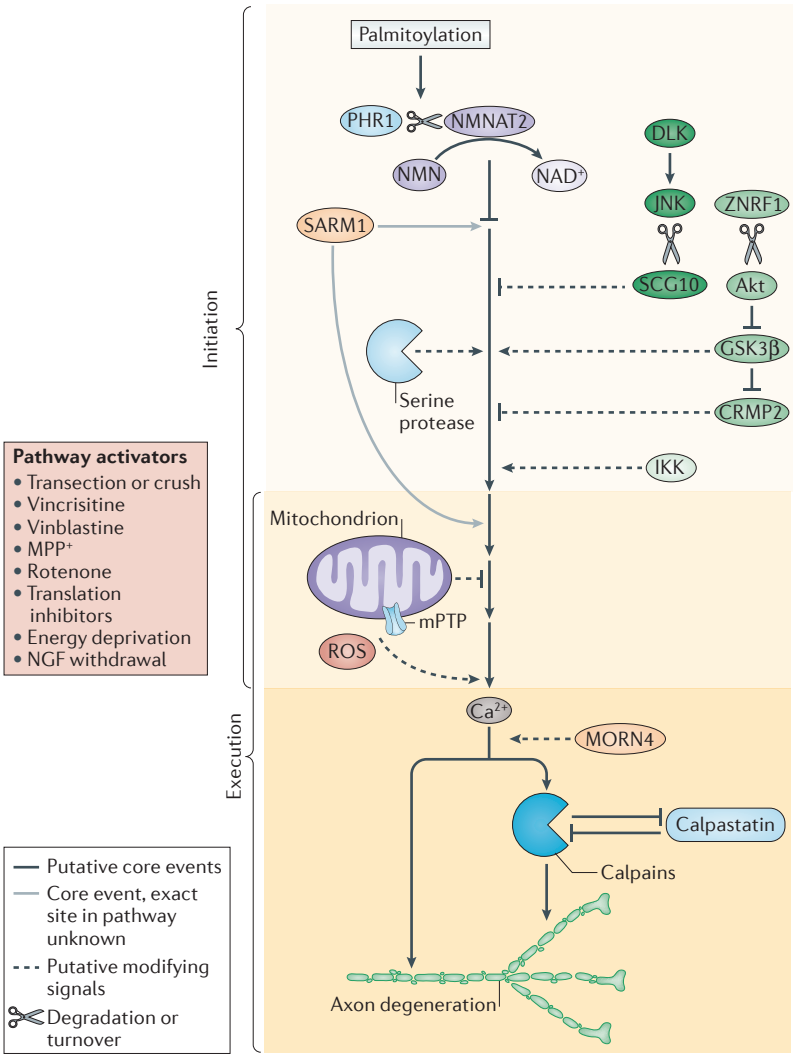
Figure 4



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