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MECHANISTIC INSIGHTS INTO AN AXON DEATH PATHWAY CONTROLLED BY NAD-RELATED METABOLISM

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**This dissertation is submitted for the degree of
Doctor of Philosophy**

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically stated in the text. The work presented here is not substantially the same as any I have submitted for a degree or diploma or other qualification at any other University.

Abstract

Studies of axon degeneration following an injury, known as Wallerian degeneration, and the axon-protective protein WLD^s revealed a strong link between NAD metabolism and the mechanisms resulting in axonal destruction. Current knowledge indicates that the labile axonal NAD-synthesising enzyme NMNAT2 is essential for axonal survival. Its depletion, following an injury or its genetic removal, causes axon degeneration or defective axon growth. We recently proposed a key role for NAD-precursor NMN, which rapidly accumulates in transected nerves as a consequence of NMNAT2 depletion, in initiating axon degeneration after injury. Pharmacological and genetic reduction of NMN levels, by inhibition of NMN-synthesising enzyme NAMPT or expression of the bacterial NMN-scavenging enzyme NMN deamidase, robustly delays Wallerian degeneration. Recent evidence suggests that NMNAT2 and the pro-degenerative protein SARM1 cooperate in a core pathway regulating axon degeneration. It is important to fully understand the mechanism of Wallerian degeneration because related mechanisms could contribute to axon loss in several disease models, including peripheral neuropathies, Parkinson's disease, multiple sclerosis and glaucoma.

This thesis focuses on elucidating the mechanism by which NMN promotes axonal degeneration. I show that downstream events of NMN accumulation after injury include an increase in intra-axonal Ca²⁺ that immediately precedes axonal fragmentation. NMN requires the recently identified pro-degenerative protein SARM1 to promote Ca²⁺ rise and axon degeneration. Whilst these data suggest that mitochondrial dysfunction has no influence on the timing of axonal degeneration after injury, they also indicate that NMNAT2 depletion and NMN accumulation promote degeneration of uninjured axons exposed to the mitochondrial toxins CCCP and rotenone.

These results identify critical steps of Wallerian degeneration where pharmacological intervention could be most successful. They also support the importance of NMNAT2 and NMN in controlling axonal health which goes beyond the simple regulation of Wallerian degeneration and potentially into

mitochondrial disorders, including Parkinson's disease, Friedreich's ataxia and multiple sclerosis.

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CHAPTER 1

Introduction

Introduction

Neurons are complex and highly compartmentalized cells which develop long processes called axons and dendrites from the cell body (also called the soma) to reach their targets. The efficient functioning of the whole neuronal cell depends on the correct functioning of every single compartment. Axons represent the extensions that connect neurons to each other and to other cell types and through which information is actively transmitted. They often show a very elongated shape (up to one meter in length in humans) and are quite vulnerable structures. In fact, axonal degeneration often occurs at early stages in several neurodegenerative diseases and is a recognized causative feature of these pathologies (Conforti *et al.*, 2007a; Hilliard, 2009; Adalbert & Coleman, 2013). It can precede cell body loss and frequently shows a retrograde pattern in which axons start to die from distal ends and proceed towards the cell body. This characteristic feature is referred to as “dying back” degeneration (Cavanagh, 1979) and underlies axonal pathology in a number of neurodegenerative disorders (Coleman & Freeman, 2010; Lingor *et al.*, 2012). Understanding mechanisms leading to axon degeneration is therefore essential to develop efficient therapeutic strategies and is the focus of the present thesis.

1.1 Wallerian degeneration

Neuronal complexity represents a huge challenge for experimentally modelling axon degeneration and for understanding degenerative mechanisms. Cell bodies and axons possess different and independent death mechanisms (Deckwerth & Johnson, 1994; Libby *et al.*, 2005; Adalbert *et al.*, 2006; Conforti *et al.*, 2007a) and more than one death pathway can be activated in the same cell (Simon *et al.*, 2016), depending on the type of insult (Geden & Deshmukh, 2016). Different neuronal subpopulations respond in diverse ways to toxic insults, resulting in asynchronous degeneration and difficulties when interpreting experimental results (Schaefer *et al.*, 2005; Pun *et al.*, 2006; Conforti *et al.*, 2007a).

Wallerian degeneration, first described by the neurophysiologist Augustus Waller in 1850, is defined as the degeneration of the axon distal to a site of physical injury (Fig. 1.1) (Waller, 1850). In this model, axon degeneration is initiated by an injury at a defined site/time and simultaneously affects all axons. This makes it a simple model which partially resolves some of these complications and is therefore widely used to study axon degeneration (Feng *et al.*, 2010a; Wang *et al.*, 2012; Conforti *et al.*, 2014). In the peripheral nervous system (PNS), Wallerian degeneration is characterized by three morphologically distinct phases. Minutes after transection, an acute degeneration process affects short axonal stumps proximal and distal to the site of injury. This process has been termed *acute axonal degeneration* (AAD) (Kerschensteiner *et al.*, 2005; Knoferle *et al.*, 2010; Koch *et al.*, 2010). This immediate response precedes a *latent phase* where no morphological changes occur and axons remain electrically excitable (Luttges *et al.*, 1976; Tsao *et al.*, 1994). The duration of the latent phase varies between different species, ranging from 24-48 hours *in vivo* in mice and rats (Miledi & Slater, 1970; Lubinska, 1977; Beirowski *et al.*, 2004; Beirowski *et al.*, 2005; Wang *et al.*, 2012) to several days in primates, including humans (Gilliat & Hjorth, 1972; Chaudhry & Cornblath, 1992; Gaudet *et al.*, 2011). This is followed by a rapid *disintegration phase* which is generally completed within 1-2 hours (Tsao *et al.*, 1999; Beirowski *et al.*, 2005; Wang *et al.*, 2012). Morphologically, the disintegration phase is characterized by the appearance of axonal swellings followed by fragmentation and appearance of myelin ovoids. The axonal cytoskeleton is degraded mainly due to Ca²⁺-dependent proteases called calpains (George *et al.*, 1995; Stirling & Stys, 2010; Ma, 2013; Ma *et al.*, 2013). In the course of the disintegration phase, bone-marrow-derived macrophages and Schwann cells cooperate to clear degenerated axons and myelin debris (Stoll *et al.*, 1989; Gaudet *et al.*, 2011; Rotshenker, 2011). Wallerian degeneration is similar in the central nervous system (CNS) but the removal of axonal fragments is slower and is performed by oligodendrocytes and microglia (Bignami & Ralston, 1969; Perry *et al.*, 1987; George & Griffin, 1994; Vargas & Barres, 2007). A similar sequence of events take place *in vitro*, however the whole process is faster and is completed within 6–16 hours in superior cervical ganglion (SCG) and dorsal root ganglion (DRG) explant rodent cultures, with variability depending on

culturing conditions (Glass *et al.*, 1993; Buckmaster *et al.*, 1995; Zhai *et al.*, 2003).

Axons undergoing Wallerian degeneration share some features with what is observed during an “apoptotic” type of degeneration. For instance, Annexin V positively stains the distal part of the cut axon. In addition, loss of mitochondrial membrane potential has been determined at an early stage of the degeneration process (Sievers *et al.*, 2003). Nevertheless, Wallerian degeneration is actually considered distinct from apoptosis as the activation of caspases, a hallmark of apoptosis (Saraste & Pulkki, 2000), is not involved in the degenerative process. In fact, knockout or inhibition of caspases (Finn *et al.*, 2000; Vohra *et al.*, 2010; Simon *et al.*, 2012), overexpression of members of the BCL-2 protein family (Burne *et al.*, 1996; Vohra *et al.*, 2010) or BAX and BAK deletion are unable to prevent it (Whitmore *et al.*, 2003).

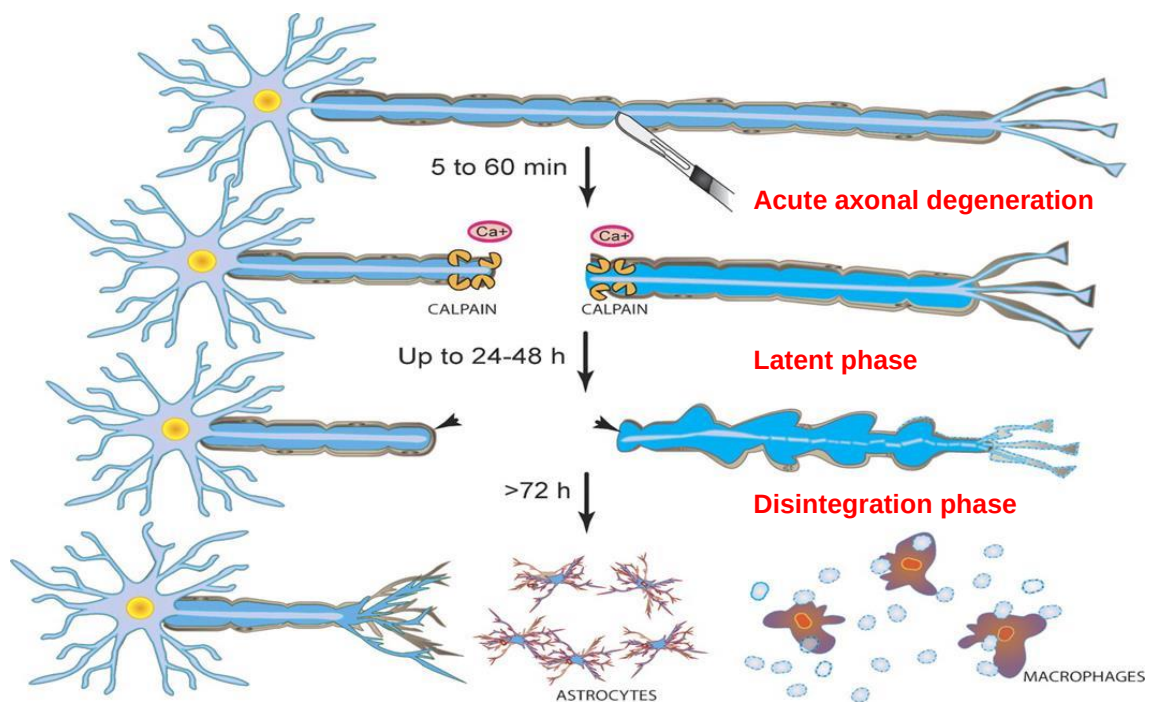


Figure 1.1 Wallerian degeneration, from (Wang *et al.*, 2012).

Graphical representation of the different phases of the degeneration of an axon distal to the site of injury. See text for more details.

1.2 The Wallerian Degeneration Slow (Wld^s) mutant mouse

Augustus Waller soon realized the likely relevance of Wallerian degeneration “particularly with reference to nervous diseases” (Waller, 1850; Coleman, 2005). Despite this early prediction, Wallerian degeneration was considered a passive degenerative response for decades, simply a consequence of the separation and loss of support of the axon from its cell body. The fortuitous discovery of a spontaneous mutant mouse whose transected axons remain intact ten times longer than normal (Lunn *et al.*, 1989) gave a significant impetus to the research in the Wallerian degeneration field. This mouse, later called Wallerian degeneration slow (Wld^s), fully overturned the beliefs regarding injury-induced axon degeneration, highlighting the existence of a regulated axon destruction process.

Wld^s transected axons, both in the PNS and CNS, remain intact and functional up to 2-3 weeks compared to the 2-3 days of wild-type axons (Lunn *et al.*, 1989; Perry *et al.*, 1991; Ludwin & Bisby, 1992; Mack *et al.*, 2001). Degeneration eventually occurs in a gradual and atrophic manner, at this point likely reflecting a passive depletion of structural proteins from long-term anucleated axons (Beirowski *et al.*, 2005; Coleman & Freeman, 2010). Synaptic terminals are also preserved in the Wld^s mouse. However, synapse withdrawal after axotomy occurs faster than the degeneration of the axon trunk, especially in older animals (Ribchester *et al.*, 1995; Gillingwater *et al.*, 2002; Adalbert *et al.*, 2005; Gillingwater *et al.*, 2006). Wld^s-derived SCG and DRG primary neuronal cultures also show remarkable protection for >72 hours (compare to survival for 6-16 hours of wild-type axons) (Buckmaster *et al.*, 1995; Wang *et al.*, 2005; Conforti *et al.*, 2009).

The Wld^s mouse shows no clear abnormality, except for a nerve regeneration defect and reduced plasticity after injury that is secondary to the delayed degeneration (Bisby & Chen, 1990; Brown *et al.*, 1994; Collyer *et al.*, 2014). Studies show that Wld^s phenotype is intrinsic to neurons rather than monocyte or glia cells (Perry *et al.*, 1990; Glass *et al.*, 1993). The above mentioned protection in primary neuronal culture lacking glial cells (Buckmaster *et al.*, 1995) further supports this notion.

To date, the role of glial cells in Wallerian degeneration is considered to be mainly restricted to removal of axonal fragments and myelin ovoids (Coleman & Freeman, 2010). However, whether the real contribution to Wallerian degeneration of glial cells is so limited is still under debate and seems unlikely. For instance, axonal death *in vivo* after injury is slower than *in vitro* (Wang *et al.*, 2012). It is possible that the lack of support from glial cells in *in vitro* cultures determines this difference, especially in view of key metabolic and neurotrophic support they provide to axons (Court *et al.*, 2008; Nave & Trapp, 2008; Nave, 2010; Funfschilling *et al.*, 2012; Lee *et al.*, 2012; Beirowski *et al.*, 2014; Campbell *et al.*, 2014). It would be surprising if this important axon-glia interaction is not involved in axonal survival, especially in transected *Wld^s* nerves, where some axons are still intact 3 weeks after their separation from the soma. Additionally, the role of glia in axonal maintenance under physiological conditions is well established (Nave, 2010; Beirowski, 2013). From a morphological point of view, whether myelination has an impact on the timing of degeneration is difficult to tell. After injury, all the different nerve fibers will eventually degenerate. However, many factors have been implicated in the timing of degeneration, including length, diameter, temperature and the criteria used to score degeneration. (Lubinska, 1975; 1982; Veronesi & Boyes, 1988; Blundon *et al.*, 1990; Beirowski *et al.*, 2004; Beirowski *et al.*, 2005). This causes variability and makes it difficult to interpret the small differences in degeneration timing that characterize the different fibers during Wallerian degeneration. In addition, non-myelinated axons are still in close contact and receive support from glial cells (Nave, 2010). To conclude, a better understanding of glial cells role in Wallerian degeneration remains a major goal in the field (Coleman & Freeman, 2010).

1.2.1 The *Wld^s* gene and protein

The unique feature of the *Wld^s* mouse is the presence of an uncommon genomic rearrangement, specifically a tandem triplication, which leads to the formation of a chimeric gene mapped to chromosome 4 (Fig. 1.2) (Lyon *et al.*, 1993; Coleman *et al.*, 1998; Conforti *et al.*, 2000). The resulting fusion protein (known as the *WLD^s* protein) comprises the full length nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) at the C-terminal end, a key

nuclear enzyme in the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway (Magni *et al.*, 2004b), linked to 70 N-terminal (N70) amino acids of the N-terminus portion of ubiquitin ligase E4B (UBE4B) (Conforti *et al.*, 2000; Mack *et al.*, 2001). An 18 amino acid sequence (the epitope of the specific Wld18 antibody), originally part of NMNAT1 5' untranslated region (UTR), links the two domains of the WLD^S protein (Fig. 1.2) (Samsam *et al.*, 2003; Coleman & Freeman, 2010). The expression of the fusion protein in transgenic mice reproduces the Wld^S phenotype and is therefore accountable for the protective effect (Mack *et al.*, 2001). Notably, the protective phenotype is strongly dose-dependent both in axons and synaptic terminals (Mack *et al.*, 2001; Gillingwater *et al.*, 2006; Wright *et al.*, 2010). Transgenic expression of WLD^S is protective in mice, rats, *Drosophila*, zebrafish and in primary human neuronal culture (Lunn *et al.*, 1989; Mack *et al.*, 2001; Adalbert *et al.*, 2005; MacDonald *et al.*, 2006; Martin *et al.*, 2010; Kitay *et al.*, 2013), indicating strong evolutionary conservation of the axon degeneration pathway with which it interferes. WLD^S shows intrinsic NMNAT enzymatic activity, as the presence of the NMNAT1 full sequence would suggest (Mack *et al.*, 2001). In contrast, only 6% of the UBE4B sequence is present in the fusion protein and the catalytic U-box sequence is excluded. Therefore WLD^S is unlikely to possess ubiquitin ligase activity (Coleman & Freeman, 2010; Conforti *et al.*, 2014).

1.2.2 Protective domains and subcellular site of action of WLD^S

After the identification and characterization of the WLD^S protein, understanding how and where, at subcellular level, WLD^S is acting to preserve axonal integrity is the next logical step to decode its mechanism of protection. The genomic mutation does not affect the endogenous NMNAT1 and UBE4B gene and protein expression levels (Gillingwater *et al.*, 2002; Conforti *et al.*, 2007b). In addition, the increased NMNAT enzymatic activity in Wld^S tissues argues against a dominant negative effect (Mack *et al.*, 2001; Coleman & Freeman, 2010). Therefore, gain rather than loss of function seems the most likely mechanism for WLD^S action (Coleman & Freeman, 2010). In 2004, Araki, Milbrandt and colleagues suggested a nuclear action of WLD^S, mediated exclusively by the NMNAT1 domain of the fusion protein (Araki *et al.*, 2004). At the time, this model was reinforced by evidence showing an abundant and

exclusively nuclear localisation of the WLD^S protein (Mack *et al.*, 2001; Samsam *et al.*, 2003; Fang *et al.*, 2005), a predicted site due to the nuclear localisation of endogenous NMNAT1. Subsequent studies confirmed the requirement of NMNAT1 enzymatic activity for WLD^S action, as suggested by the modest (Avery *et al.*, 2009) or absent (Conforti *et al.*, 2009; Yahata *et al.*, 2009) neuroprotective effect achieved *in vivo* in flies and mice expressing WLD^S variants in which NMNAT1 enzymatic activity is disrupted by mutation. Similar results are also observed *in vitro* (Araki *et al.*, 2004; Jia *et al.*, 2007). However, additional research failed to uphold the proposed nuclear action of WLD^S (Wang *et al.*, 2005) and revealed that the NMNAT1 domain alone is insufficient to confer the full Wld^S phenotype. In fact, NMNAT1 overexpression or downregulation in mice has no impact on the rate of Wallerian degeneration (Conforti *et al.*, 2007b; Yahata *et al.*, 2009; Conforti *et al.*, 2011). Additionally, NMNAT1 overexpression can delay injury-induced axon degeneration in flies, although its effect is significantly weaker than Wld^S (Avery *et al.*, 2009).

These data convincingly indicate a contribution of the N70 domain to the protective mechanism. Further supporting this notion, removing a short 16 amino acid N-terminal motif (N16) (Fig. 1.2) results in full suppression of WLD^S-mediated axon protection in mice (Conforti *et al.*, 2009) and significantly reduces it in flies (Avery *et al.*, 2009). Fusing N16 directly to NMNAT1 is sufficient to confer the same degree of axon protection achieved by WLD^S in flies (Avery *et al.*, 2009). This short sequence contains a binding site for valosin-containing protein (VCP) (Laser *et al.*, 2006; Morreale *et al.*, 2009), an AAA-ATPase, present both in the cytoplasm and nucleus, involved in several intracellular processes including protein degradation, autophagy and mitochondrial quality control (Wang *et al.*, 2004; Wojcik *et al.*, 2006; Stolz *et al.*, 2011; Bug & Meyer, 2012; Yamanaka *et al.*, 2012). Crucially, replacing the N16 motif with a different 16-amino acid VCP-binding sequence from protein ataxin-3 restores WLD^S protective effect (Conforti *et al.*, 2009). These studies led to the hypothesis that WLD^S could have an extra-nuclear action, with the N16 motif driving the re-localisation of WLD^S from the nucleus to the cytoplasm/axoplasm through its binding to cytoplasmic VCP (Avery *et al.*, 2009; Conforti *et al.*, 2009; Coleman & Freeman, 2010). Indeed, detailed analysis of WLD^S expression in

axons indicates low but measurable levels of the fusion protein (Beirowski *et al.*, 2009; Yahata *et al.*, 2009). Moreover, mutating the NMNAT1 nuclear localisation signal in WLD^S enhances its protective effect (Beirowski *et al.*, 2009) and axon degeneration is robustly delayed in mice expressing a cytoplasmic mutant of NMNAT1 (cyt-NMNAT1) (Sasaki *et al.*, 2009a). Targeting NMNAT1 into axons with an APP-derived peptide also confers axon protection even at low expression levels (Babetto *et al.*, 2010). Subsequent studies showed that a lentivirus-based transfection method unexpectedly leads to local transduction of NMNAT1 in the axon, which also confers protection (Sasaki & Milbrandt, 2010; Cohen *et al.*, 2012) and explains some of the previous contradictory results (Araki *et al.*, 2004). Crucially, destabilizing WLD^S specifically within axons abolishes its protective activity (Cohen *et al.*, 2012; Wang *et al.*, 2015).

Taken together, these studies provide compelling evidence for an extra-nuclear site of action of WLD^S, specifically at the axon level, as a result of the combined activity of the two protein domains. This model is now widely accepted in the field.

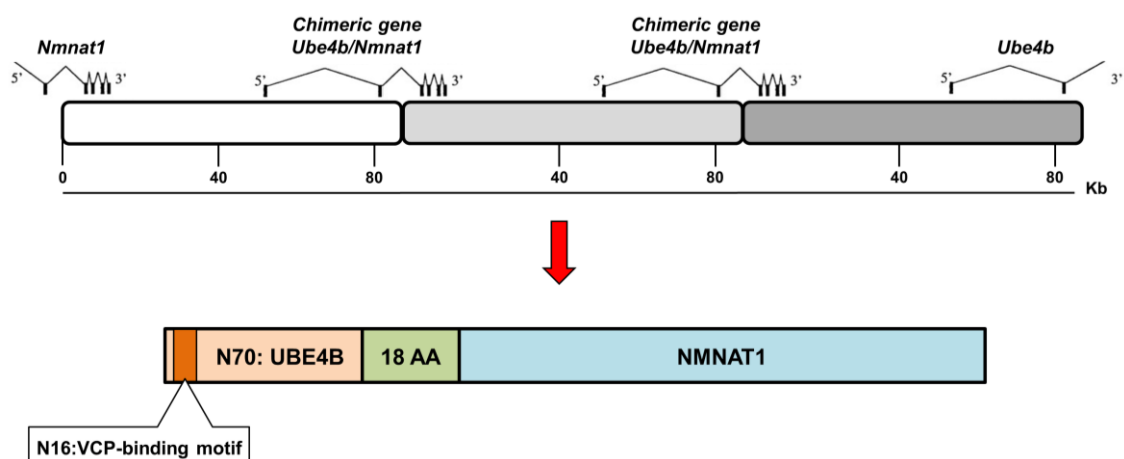


Figure 1.2 Schematic representation of *Wld^S* gene and protein.

An 85 Kb tandem triplication results in a chimeric gene that encodes for a chimeric protein in which the N-terminal 70 amino acids of the ubiquitination factor UBE4B is linked the full sequence of NMNAT1. An 18 amino acids linker-sequence, originally belonging to the 5' untranslated region of the *Nmnat1* gene, connects the two domains. The N16 VCP-binding motif is shown.

1.3 Wallerian-like degeneration

The discovery of Wld^S is important for two main reasons: **i)** It significantly facilitates the understanding of basic mechanisms of axon degeneration; **ii)** It gives us the opportunity to address a crucial question: “Is Wallerian degeneration relevant to neurodegenerative disorders?” With regards to this latter point, axon degeneration occurs in a number of neurodegenerative disorders when the axons are still connected to the cell body (Coleman & Freeman, 2010; Conforti *et al.*, 2014). Nevertheless, at least in some cases, axon pathology in disease shows a Wallerian-like degeneration. The term Wallerian-like degeneration classically describes axon degeneration which occurs in the absence of a cut (or crush injury) and presents morphological features similar to Wallerian degeneration (Griffin *et al.*, 1996). Wallerian-like degeneration characterises several *in vitro* models of axon degeneration as well as animal models of neurodegeneration (Conforti *et al.*, 2014). Wld^S enables us to test whether these other types of degeneration also share mechanistic rather than simply morphological features with Wallerian degeneration. The term Wallerian-like degeneration now has a broader meaning and is used to define axon degeneration in the absence of an injury which is sensitive to WLD^S (or other regulators of the Wallerian degeneration pathway that are discussed below).

1.3.1 *In vitro* models of axon degeneration sensitive to WLD^S

There are several toxic insults which cause WLD^S-sensitive axon degeneration *in vitro* (Conforti *et al.*, 2014). For instance, WLD^S delays axon degeneration caused by the chemotherapy agents vincristine and vinblastine (Mack *et al.*, 2001; Wang *et al.*, 2001; Watanabe *et al.*, 2007; Cohen *et al.*, 2012). This is particularly interesting as these drugs, used in cancer treatment, have been shown to cause peripheral neuropathy in humans (Armstrong *et al.*, 1967; Sandler *et al.*, 1969; Grisold *et al.*, 2012). Their main mechanism of action is thought to be impairment of axonal transport through microtubule destabilization (Green *et al.*, 1977; Stanton *et al.*, 2011; LaPointe *et al.*, 2013). Additionally, protein synthesis inhibition triggers WLD^S-sensitive axon degeneration (Gilley & Coleman, 2010). WLD^S also rescues axons from death caused by mitochondrial toxins (Antenor-Dorsey & O'Malley, 2012), which may be particularly relevant in

view of the involvement of mitochondria dysfunction in several neurodegenerative disorders (Schon & Manfredi, 2003; Court & Coleman, 2012; Johri & Beal, 2012). Whether the insults listed above re-activate the same axon death mechanism of Wallerian degeneration or share just a WLD^S-sensitive step is not fully understood. However, the degree of protection conferred by WLD^S suggests a mechanism that is closely similar to the Wallerian pathway. Finally, WLD^S (or cyt-NMNAT1) also delays axon degeneration caused by NGF deprivation, regarded as an *in vitro* model of axonal pruning (Nikolaev *et al.*, 2009). Importantly, WLD^S fails to rescue the cell body death in this model. (Deckwerth & Johnson, 1994; Vohra *et al.*, 2010). This is one of the clearest examples of how axons and soma possess independent mechanisms of death. However, at later stages, more than one pathway, involving caspase activation (absent in Wallerian degeneration) and a soma derived pro-degenerative signal, contributes to this model of axon death (Nikolaev *et al.*, 2009; Simon *et al.*, 2012; Gerdts *et al.*, 2013; Simon *et al.*, 2016). How these pathways cooperate is still unclear.

1.3.2 Wallerian-like degeneration in neurodegenerative diseases

To date, the Wld^S mouse has been used to test whether several animal models of neurodegeneration, characterised by “dying-back” axon pathology, follow a Wallerian-like mechanism. WLD^S expression is protective in genetic models of Charcot-Marie-Tooth (CMT) disease and progressive motor neuropathy (*pmn* mouse) (Ferri *et al.*, 2003; Samsam *et al.*, 2003; Meyer zu Horste *et al.*, 2011). WLD^S is also effective against axon degeneration caused by the chemotherapy drug Taxol (Wang *et al.*, 2002) and in a model of excitotoxic injury to the retina (Bull *et al.*, 2012). Axon degeneration is delayed in the Wld^S mouse in two acute models of Parkinson’s disease (caused by 6OHDA and MPTP administration) (Sajadi *et al.*, 2004; Hasbani & O’Malley, 2006; Cheng & Burke, 2010). This is interesting as it directly matches some of the results mentioned above in *in vitro* cultures.

Several additional studies extended the WLD^S (or cyt-NMNAT1) protective effect to other models of CNS pathologies, such as multiple sclerosis (experimental autoimmune encephalitis (EAE)), glaucoma and gracile axonal dystrophy (Mi *et al.*, 2005; Kaneko *et al.*, 2006; Howell *et al.*, 2007; Beirowski *et*

al., 2008; Zhu *et al.*, 2013; Williams *et al.*, 2017a; Williams *et al.*, 2017b). Of note, there are some models of neurodegeneration where WLD^S fails to protect axons, including mouse models of spinal muscular atrophy (SMA) (Rose *et al.*, 2008; Kariya *et al.*, 2009), prion disease (Gultner *et al.*, 2009), hereditary spastic paraplegia (Edgar *et al.*, 2004), type 2D CMT (Stum *et al.*, 2011) and different mouse models of amyotrophic lateral sclerosis (ALS) with mutations in superoxide dismutase 1 (SOD1) (Vande Velde *et al.*, 2004; Fischer *et al.*, 2005). This is important since it reveals the existence of WLD^S-independent mechanisms of axon degeneration in neurodegenerative disorders. A clear explanation for this is lacking; however, this might reflect the existence of a totally independent pathway of axon death or could be a consequence of defects in the neuronal cell body or in supporting cells.

WLD^S expression in the nucleus can sometimes have a beneficial effect in conditions involving excess activity of NAD-dependent nuclear enzymes (Conforti *et al.*, 2014). An example of this is WLD^S protection against diabetic neuropathy and retinopathy. In this circumstance, WLD^S protection has been attributed to the preservation of β -cells in the pancreas and maintenance of glucose homeostasis rather than a direct effect on neurons (Wu *et al.*, 2011; Zhu *et al.*, 2011). Potential extra-axonal beneficial functions of WLD^S will be discussed in more detail in the final chapter of this thesis. To conclude, these results prove the validity of Wallerian degeneration as a relevant model to understand mechanisms of axon degeneration which are likely relevant to a variety of neurodegenerative disorders.

1.4 The role of NMNAT2 in Wallerian degeneration

Studies on WLD^S exponentially increased our knowledge of axon degeneration, leading to the current vision that axons possess a local, genetically encoded self-destruction program. WLD^S is a mutant protein and is therefore not present in wild-type animals. However, a better understanding of its mechanism of action at a molecular level should help to identify important endogenous factors involved in maintaining axonal integrity under physiological conditions, or triggering the degeneration after injury. For instance, the requirement of the

enzymatically active NMNAT1 for WLD^S protective function revealed a previously unknown link between NMNATs/NAD biosynthetic pathway and axon degeneration.

One long standing hypothesis is that axons require constant delivery of a pro-survival signal from the cell body to maintain their integrity. Injury disrupts this supply and begins the degeneration process (Lubinska, 1977; Coleman & Freeman, 2010). In line with this hypothesis, Gilley and Coleman in 2010 proposed an attractive model which predicts that the survival factor that axons require is the endogenous, cytosolic and axonal isoform of NMNAT, NMNAT2 (Gilley & Coleman, 2010; Conforti *et al.*, 2014). Mammals have three NMNAT isoforms, all sharing the same enzymatic activity but with different subcellular distribution. NMNAT1 is the nuclear isoform (and the one present in WLD^S) (Magni *et al.*, 2004b), NMNAT2 is found in cytoplasm and axons associated with Golgi-derived vesicles, and NMNAT3 resides in mitochondria (Fig. 1.3) (Berger *et al.*, 2005; Di Stefano & Conforti, 2013; Conforti *et al.*, 2014). In lower organisms, such as *Drosophila*, only one NMNAT gene has been described and its product, dNMNAT, is widely distributed within the cell (Zhai *et al.*, 2006).

Among the three isoforms, only specific depletion of NMNAT2 through siRNA causes degeneration in uninjured SCG axons *in vitro*. WLD^S also confers protection in this experimental setting, whereas endogenous NMNAT1 and 3 fail to compensate for NMNAT2 loss. Inhibition of protein synthesis leads to rapid depletion of NMNAT2, indicating a fast turnover rate, and consequent WLD^S-sensitive axonal degeneration. A similar depletion is observed in the axon 4 hours after injury *in vitro*, which is compatible with the timing of initiation of axonal degeneration (Gilley & Coleman, 2010). Under physiological conditions, NMNAT2 is mainly synthesized in the soma and reaches the axons by fast axonal transport, in association with Golgi-derived vesicles (Gilley & Coleman, 2010; Milde *et al.*, 2013c). Importantly, injury disrupts the delivery of freshly soma-synthesized NMNAT2, explaining the observed depletion. Crucially, WLD^S is much more stable than NMNAT2 and is therefore not quickly depleted in transected axons (Gilley & Coleman, 2010). NMNAT2 mRNA has been found in axons (Dr. Jose Sotelo-Silveira, personal communication), indicating a possible involvement of local translation for more subtle regulation of local NMNAT2

levels. However, specific inhibition of protein synthesis in the soma using compartmentalised cultures results in distal axon degeneration. In contrast, no degeneration is detected when the inhibitor of protein synthesis is added exclusively to the axonal compartment (Eng *et al.*, 1999; Gilley & Coleman, 2010). These observations suggest no or minor contribution of local translation to total axonal NMNAT2 levels, at least *in vitro*.

These results led to the hypothesis that WLD^S can substitute NMNAT2 locally in the axon after injury due to its higher stability (Gilley & Coleman, 2010). This model is consistent with the above mentioned studies that indicate an axonal action of WLD^S. Additionally, it would easily explain the protection conferred by WLD^S against at least some of the toxic insults mentioned above which cause Wallerian-like degeneration. For instance, toxins interfering with axonal transport (Goshima *et al.*, 2010) are likely going to affect the delivery of soma-synthesized proteins, mimicking what happens after injury. Whether NMNAT2 transport is impaired, however, has not been directly tested yet. NMNAT2 depletion after injury is also observed in WLD^S expressing axons, arguing against the possibility that WLD^S acts by stabilizing NMNAT2 (Gilley & Coleman, 2010). This model can be extended to NMNAT variants which, as a consequence of mutation or overexpression, acquire a cytoplasmic localisation. In fact, in addition to cyt-NMNAT1 (Sasaki *et al.*, 2009a), overexpressed mitochondrial NMNAT3 is also found in the cytoplasm and confers robust protection from Wallerian degeneration (Avery *et al.*, 2009; Yahata *et al.*, 2009). Importantly, both endogenous NMNAT1 and 3 are more stable than NMNAT2 (Gilley & Coleman, 2010).

The “NMNAT2 model” is now supported by several studies. NMNAT2 half-life has been confirmed to be extremely short (<1 hour) and its turnover is mediated by the ubiquitin-proteasome system (Milde *et al.*, 2013c). NMNAT2 overexpression is protective *in vitro* (Gilley & Coleman, 2010; Yan *et al.*, 2010) and in zebrafish (Feng *et al.*, 2010b). However, high overexpression is needed to overcome its fast turnover (Gilley & Coleman, 2010). This might explain why no protection is found in *Drosophila* 5 days after axotomy in olfactory receptor neurons (Avery *et al.*, 2009). NMNAT2 variants which are characterized by longer stability are indeed able to confer robust protection both in mice and

Drosophila (Milde *et al.*, 2013a; Milde *et al.*, 2013c). Furthermore, wild-type NMNAT2 confers moderate protection in the *Drosophila* wing model of axon injury (Fang *et al.*, 2012). Knocking down dNMNAT in *Drosophila* triggers WLD^S/NMNATs-sensitive (when used in this context, NMNATs refer to cytosolic variants of endogenous NMNATs) axon degeneration (Fang *et al.*, 2012). Finally, knocking out two pro-degenerative ubiquitin ligases, *Drosophila* Highwire and murine PHR1, implicated in dNMNAT and NMNAT2 turnover respectively, confer strong axon protection after injury (Xiong *et al.*, 2012; Babetto *et al.*, 2013). The role of PHR1 and Highwire will be discussed in more detail below.

1.4.1 NMNAT2 is a critical survival factor for axons

The finding that specific knock down of NMNAT2 *in vitro* is sufficient to initiate WLD^S-sensitive axon degeneration in the absence of an injury is somewhat surprising (Gilley & Coleman, 2010). Axotomy likely leads to the depletion of several other axonal proteins, but NMNAT2 seems to be critical and sufficient to initiate degeneration. These findings indicate a possible role of NMNAT2 in regulating axonal health which goes beyond regulation of Wallerian degeneration. Indeed, recent studies revealed a fundamental role of NMNAT2 in axon growth and maintenance (Hicks *et al.*, 2012; Gilley *et al.*, 2013). Knocking out NMNAT2 leads to severe defects in peripheral innervation and perinatal lethality in mice. Truncated axons are observed both in the PNS and CNS. Primary neuronal cultures derived from NMNAT2 null mice fail to develop full length axons. This reflects a failure of axon extension rather than “dying-back” degeneration. WLD^S expression corrects all the above mentioned defects and, at a homozygous dose, extends life span to at least 10 months (Gilley *et al.*, 2013; Gilley *et al.*, 2017). Of note, it remains to be determined whether NMNAT2 deficiency affects the transport of other molecules along the axon.

Recent studies looked more carefully at NMNAT2 localisation and turnover. As mentioned above, NMNAT2 is transported attached to Golgi-derived vesicles. Surprisingly, disrupting its palmitoylation-dependent attachment to vesicles results in broad NMNAT2 cytoplasmic distribution and increased protection, both *in vitro* and *in vivo*. These variants of NMNAT2 are still able to reach the axon and manifest an increased stability, which explains their protective effect

(Milde *et al.*, 2013a; Milde *et al.*, 2013c; Milde & Coleman, 2014). However, these findings raise several questions regarding the actual role of membrane binding for NMNAT2 axonal transport.

In summary, these studies strongly support a crucial role for NMNAT2 to preserve axonal health and offer a model to explain WLD^S/NMNATs-mediated protection. Of note, NMNAT2 is predominantly expressed in the nervous system (Raffaelli *et al.*, 2002; Yalowitz *et al.*, 2004; Mayer *et al.*, 2010; Di Stefano & Conforti, 2013).

1.5 The role of NAD biosynthesis in Wallerian degeneration

1.5.1 NMNATs and NAD biosynthesis

NMNAT enzymes catalyse the final step of NAD biosynthesis common to both the *de novo* and the salvage pathways (Fig. 1.3). There are three salvage pathways which use different substrates: nicotinic acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR). They are all part of the vitamin B family (more specifically and collectively known as vitamin B₃) and found in several foods such as meat, eggs, fish, dairy, some vegetables, whole wheat, corn and cow's milk (Bieganowski & Brenner, 2004; Bogan & Brenner, 2008; Chi & Sauve, 2013). The pathway utilising NA is also known as the Preiss-Handler pathway (Preiss & Handler, 1958b; a). NA is converted to NaMN which is then used by NMNATs to produce nicotinic acid adenine dinucleotide (NaAD). NaAD can finally be converted to NAD by NAD synthetase. In the other salvage pathway, NAM is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT, sometimes called NamPRT). NMN is then directly converted to NAD by NMNATs. The *de novo* pathway, also known as the kynurenine pathway, starts from the essential aromatic amino acid tryptophan, which is converted to NaMN and then further to NAD, reconnecting to the Preiss-Handler pathway (Magni *et al.*, 2004a; Hassa *et al.*, 2006; Di Stefano & Conforti, 2013). However, tryptophan on its own is insufficient to completely fulfil NAD requirements (Bogan & Brenner, 2008; de Figueiredo *et al.*, 2011), highlighting the importance of the salvage pathways.

In mammals, NAM is the product of NAD hydrolysis by several enzymes such as mono-ADP-ribosyl transferases (MARTs), poly-ADP-ribose polymerases (PARPs), sirtuins (SIRTs), and ADP-ribosyl cyclases (Rongvaux *et al.*, 2003; Magni *et al.*, 2004a; Hassa *et al.*, 2006; Bogan & Brenner, 2008; Di Stefano & Conforti, 2013) and is the predominant source for NAD synthesis in most mammalian cells (Collins & Chaykin, 1971; 1972; Gross & Henderson, 1983; Revollo *et al.*, 2007; Imai, 2009; Di Stefano & Conforti, 2013; Imai & Yoshino, 2013; Imai & Guarente, 2014; Mori *et al.*, 2014).

NAD is involved in a variety of intracellular processes; it is well known as a co-factor for redox reactions with a crucial role in both mitochondrial and glycolytic ATP synthesis. More recently, its function in the regulation of post-translational protein modifications and Ca^{2+} signalling has been highlighted (Belenky *et al.*, 2007; Chiarugi *et al.*, 2012; Di Stefano & Conforti, 2013). In addition, a decline in NAD levels with ageing seems to play a role in the development of age-related pathologies (Imai & Guarente, 2014).

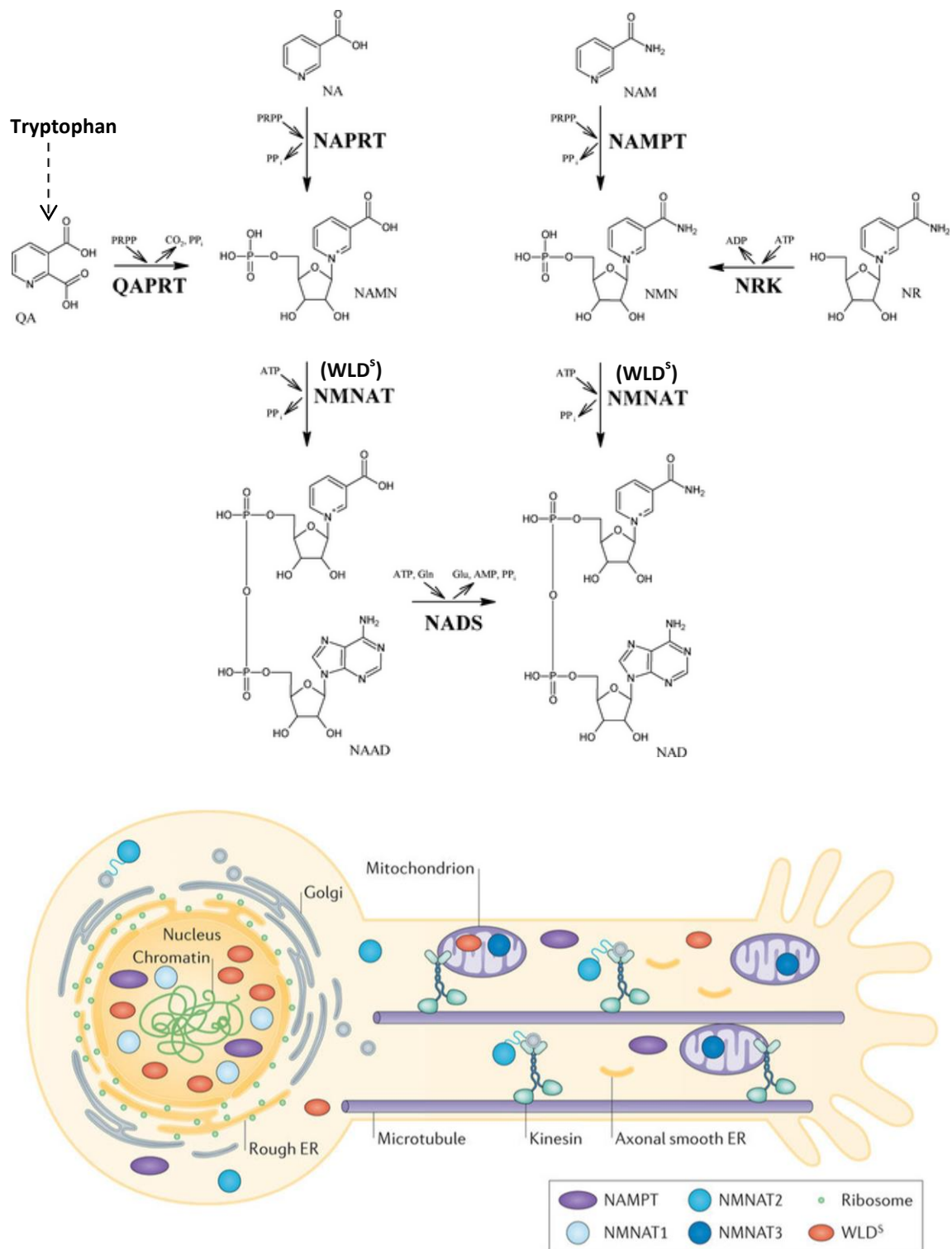


Figure 1.3 NAD biosynthesis in mammals; from (Di Stefano & Conforti, 2013; Conforti *et al.*, 2014) with modifications.

NAD is synthesised either *de novo* from tryptophan or through the three NAD salvage pathways from nicotinic acid (NA), nicotinamide (NAM), or nicotinamide riboside (NR). WLD^S position in the pathway is shown. See text for more details. Below, the compartmentalization of NAD biosynthetic enzymes in neurons.

1.5.2 NAD role in Wallerian degeneration

WLD^S, or at least the fraction of it that preserves injured axons, is essentially a cytoplasmic NMNAT isoform which relies on its NAD-synthesizing activity to confer protection. High levels of NAD are regarded as beneficial and needed for the intracellular processes mentioned in the previous section. Therefore, the first and most straightforward hypothesis is that increased NAD production is the means by which WLD^S protects against Wallerian degeneration. However, after years of contradictory results, a general consensus on the importance of NAD for the degenerative mechanism and the protection conferred by WLD^S/NMNATs is still lacking (Gerdtz *et al.*, 2016). In support of this model, a marked depletion of axonal NAD following axotomy, rescued by WLD^S and NMNAT1, occurs before any morphological change (Wang *et al.*, 2005). Moreover, exogenous addition of NAD itself and its precursors, as well as the overexpression of biosynthetic enzymes upstream of NMNATs (e.g. NAMPT), is reported by some groups to delay Wallerian degeneration (Araki *et al.*, 2004; Wang *et al.*, 2005; Sasaki *et al.*, 2006; Wang *et al.*, 2015). However, the protection conferred by these strategies is markedly weaker than that achieved with WLD^S/NMNATs, and the concentrations used for NAD and its precursors are far from the physiological range and require 24 hours pre-incubation (Wang *et al.*, 2005; Sasaki *et al.*, 2006). In another study, neither transected axons, nor DRG neurons treated with vincristine, which causes WLD^S-sensitive axon degeneration, are protected by exogenous NAD addition (Conforti *et al.*, 2007b). Of note, one important experimental limitation, which could lead to misinterpretation of some of these data, is that exogenous NAD (and some of its precursors) likely does not enter the cells directly but requires a conversion to its cell-permeable precursor NR (Billington *et al.*, 2008b; Nikiforov *et al.*, 2011). Intriguingly, no increase in NAD levels is measurable in Wld^S tissues (Mack *et al.*, 2001; Araki *et al.*, 2004; Wang *et al.*, 2005) or when NMNAT activity is increased by 15 fold (Sasaki *et al.*, 2009a). Furthermore, increasing cellular NAD levels by knocking out the NAD catabolizing enzymes CD38 and PARP1 does not alter the rate of Wallerian degeneration (Sasaki *et al.*, 2009b). A strong argument against the “NAD model” is the absence of an expected decline in cytNMNAT1-mediated protection following pharmacological inhibition of NAMPT with the potent inhibitor FK866 (Fig. 1.4) (Sasaki *et al.*, 2009b),

notwithstanding that it lowers intracellular NAD, and the failure of NAD-depleted wild-type axons to degenerate after exposure to FK866 on a timescale resembling Wallerian degeneration (Di Stefano *et al.*, 2015). As a matter of fact, FK866 surprisingly confers moderate protection against Wallerian degeneration and axon degeneration caused by energy deprivation (Sasaki *et al.*, 2009b; Shen *et al.*, 2013; Di Stefano *et al.*, 2015).

In summary, despite reinforcing the importance of NAD biosynthesis in Wallerian degeneration, these studies fail to prove NAD as the major player behind the WLD^s phenotype.

1.5.3 NAD precursor NMN and axon degeneration

The studies mentioned above support a potential NAD-independent mechanism for the axon protection conferred by WLD^s/NMNATs, or a mechanism involving both NAD and other metabolites. The finding that the NAMPT inhibitor FK866 protects axons after injury is unexpected and does not fit with the “NAD model”. FK866 is a highly specific and potent inhibitor of NAMPT (Hasmann & Schemainda, 2003; Khan *et al.*, 2006), the rate limiting enzyme in NAD salvage pathway from NAM (Fig. 1.4A) (Micheli *et al.*, 1990; Rongvaux *et al.*, 2002; Revollo *et al.*, 2004). Its administration leads to strong depletion of NAD in neurons as well as in other cell types (Billington *et al.*, 2008a; Billington *et al.*, 2008b; Sasaki *et al.*, 2009b; Di Stefano *et al.*, 2015), a predictable result in view of the predominant role of the salvage pathway using NAM in mammalian cells (Di Stefano & Conforti, 2013). Both WLD^s (increases NAD) and FK866 (depletes NAD) protect from axon degeneration, while having opposite effects on NAD levels. The protective effect upon NAMPT inhibition after injury is also surprising since FK866 long-term administration is toxic to neurons, likely due to complete depletion of intra-cellular NAD. However, this takes much longer to occur than Wallerian degeneration.

In a recent study in our laboratory, we focused on understanding the mechanism of FK866-mediated protection. We confirmed that FK866 confers protection from Wallerian degeneration up to 36-48 hours after injury in SCG and DRG neuronal cultures (Fig. 1.4B) (Di Stefano *et al.*, 2015). The protection, despite being significantly weaker when compared to WLD^s/NMNATs

phenotype, is very consistent and among the strongest obtained with a pharmacological treatment (Di Stefano *et al.*, 2015). Of note, whilst the protective effect of FK866 has now been reported in several studies, the degree of protection achieved does vary (Sasaki *et al.*, 2009b; Shen *et al.*, 2013; Di Stefano *et al.*, 2015). Different sources of FK866 could account for this variability. While searching for a common action between WLD^s and FK866, we reasoned that both can maintain low levels of NMN in the cell, FK866 by directly inhibiting NMN synthesis and WLD^s by using it as a substrate to produce NAD (Fig. 1.4A). Intriguingly, NMNAT2 depletion in axons following injury can lead to an accumulation of NMN. Crucially, we found that NAMPT, the enzyme that synthesises NMN, is still present in axons long after NMNAT2 depletion following injury (Di Stefano *et al.*, 2015). This indicates that axonal NMN synthesis likely continues even when it can no longer be converted into NAD. We therefore tested the hypothesis that injury leads to a toxic accumulation of NMN in axons, likely as a consequence of NMNAT2 depletion. We did indeed detect an accumulation of NMN in mouse sciatic nerves long before axons start to degenerate. Both WLD^s and FK866 block this rise. Exogenous addition of NMN in primary culture overcomes FK866 inhibition and, as expected, restores degeneration. Of note, NAMPT mutants that are resistant to FK866 restore degeneration, confirming the specificity of FK866 effect (Di Stefano *et al.*, 2015). These data support a model in which FK866 and WLD^s/NMNATs maintain axonal integrity by preventing NMN toxic accumulation.

Probably the most convincing evidence in support of the “NMN model” is the robust protection achieved after injury by expressing the bacterial enzyme NMN deamidase. This enzyme, absent in eukaryotic cells, converts NMN into its deamidated form NaMN (Galeazzi *et al.*, 2011). NaMN could eventually be converted into NAD; however its conversion is dependent on the presence of an NMNAT (Fig. 1.4A). In contrast to WLD^s, NMN deamidase cannot synthesise NAD directly and is unlikely to maintain NAD levels after injury, since NaMN cannot be converted into NAD due to the degradation of NMNAT2. Again, the two neuroprotective proteins share only the NMN scavenging activity. Despite this, NMN deamidase expression *in vitro* protects transected SCG axons to an extent comparable to WLD^s (Fig. 1.4C) (Di Stefano *et al.*, 2015). During the

course of this thesis project, our group generated a transgenic mouse expressing NMN deamidase. Despite very low levels of expression, Wallerian degeneration *in vivo* in sciatic nerves of NMN deamidase mice is delayed by up to 3 weeks (Fig.1.4D and E). NMN deamidase expression also fully rescues axonal defects and perinatal lethality caused by NMNAT2 deficiency, with several mice still healthy at 10 months of age (Dr. Jon Gilley, personal communication) (Di Stefano *et al.*, 2017). Both effects on Wallerian degeneration and on NMNAT2 deficiency are similar to those already described with Wld^s (Mack *et al.*, 2001; Gilley *et al.*, 2015). A profile of nucleotide levels in sciatic nerves after injury shows that NMN deamidase expression prevents NMN accumulation. As expected, no differences were found in NAD levels with respect to wild-type nerves. Crucially, we found an accumulation of NaMN and a depletion of NaAD in transected sciatic nerves of NMN deamidase mice, which is the expected consequence of NMNAT2 depletion and the prolonged activity of NMN deamidase (Di Stefano *et al.*, 2017).

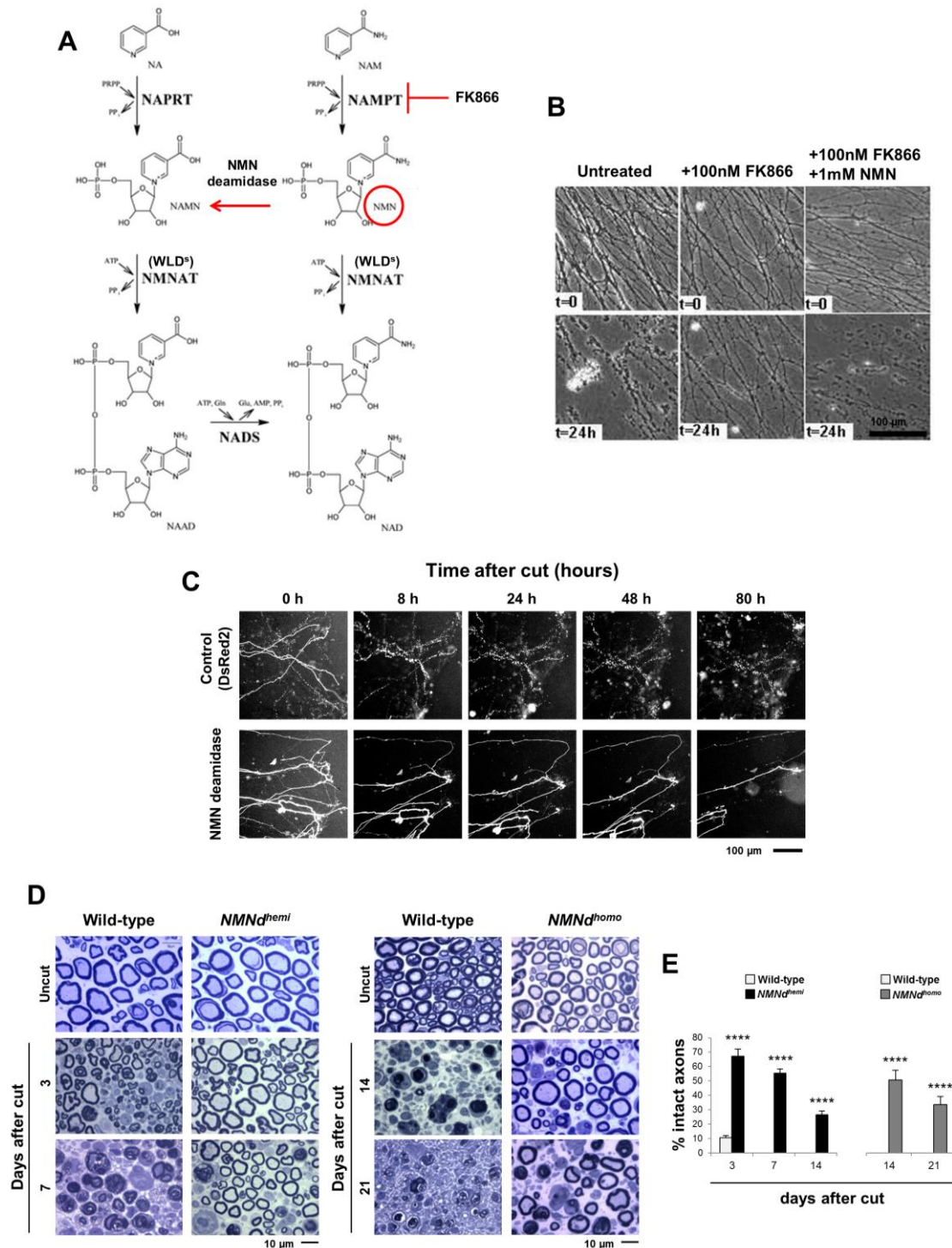


Figure 1.4 The protective effect of FK866 and NMN deamidase expression against Wallerian degeneration, from (Di Stefano *et al.*, 2015; Di Stefano *et al.*, 2017).

(A) The salvage pathway of NAD biosynthesis from NAM and NA. NMN-synthesising enzyme NAMPT is the target of FK866 potent and specific inhibition as shown. The reaction catalysed by bacterial NMN deamidase is also shown. (B) FK866 delays Wallerian degeneration in SCG neuronal cultures. Exogenous NMN addition overcomes FK866 and restores degeneration. (C) Expression of the bacterial NMN deamidase robustly protects SCG axons after injury. (D)

Sciatic nerves of NMN deamidase transgenic mice are protect up to 3 weeks against Wallerian degeneration. Similar to WLD^S, the protection conferred by NMN deamidase is dose-dependent.

These results provide compelling evidence for an important role of NMN in axon degeneration and predict that WLD^S/NMNATs maintain axonal integrity mainly through regulation of NMN levels. These findings are particularly unexpected since NMN can have cytoprotective properties in other contexts, mainly through stimulating NAD production ([Wang et al., 2011](#); [Yoshino et al., 2011](#); [Bi et al., 2012](#); [Gomes et al., 2013](#)), but in an axonal context this requires it to be added before axons have been injured so NMNAT2 is still present.

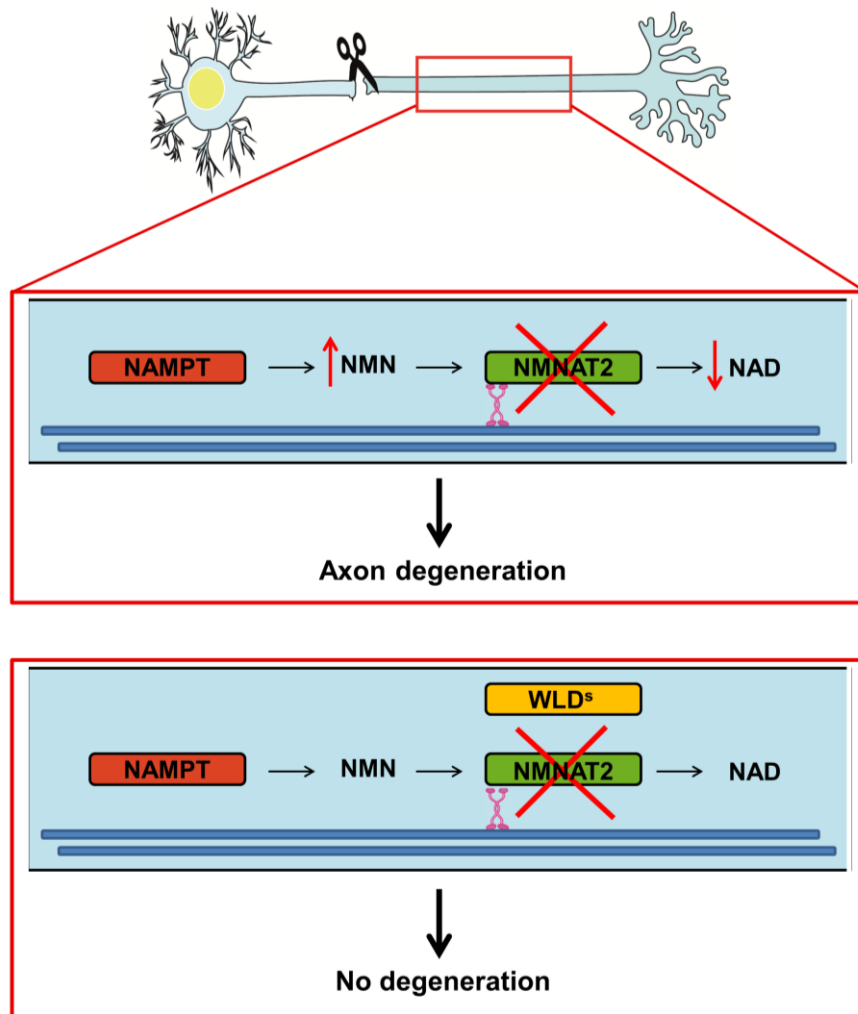


Figure 1.5 A molecular model of WLD^s-mediated axon protection.

NMNAT2 levels rapidly decline in injured axons due to its fast turnover and the disruption of freshly soma-synthesized NMNAT2 delivery through axonal transport. This leads to accumulation of NMN and depletion of NAD, followed by axonal fragmentation. When present, the more stable WLD^s substitutes NMNAT2 and maintains axonal integrity.

1.6 Other modulators of Wallerian degeneration and their relation to WLD^s/NMNATs

1.6.1 Initiation and execution phase

The discussion above establishes an essential role of NMNAT2 and NMN in Wallerian degeneration and, more generally, in preserving axonal health. Recently, several other endogenous regulators of Wallerian degeneration have been discovered. Some of them confer levels of protection which are broadly

similar to WLD^S/NMNATs, while others show a weaker phenotype. Key aims in the field are: defining **i)** if/how these endogenous regulators cooperate; **ii)** if they act together in single or in independent pathways; **iii)** the precise chronological steps of this pathway/s.

A shared vision is that the Wallerian degeneration pathway can be divided into two phases: initiation and execution. The first one represents an early step where axons still appear intact but undergo intracellular processes that commit them to degeneration; the latter follows and is a rapid destruction phase accountable for the full fragmentation of the axon (Saxena & Caroni, 2007; Wang *et al.*, 2012; Conforti *et al.*, 2014). At present, the molecular explanation for the two phases is merely speculative (Conforti *et al.*, 2014; Gerdtz *et al.*, 2016). Although a precise mechanism is missing, regulators that confer robust protection against Wallerian degeneration are thought to be critical regulators of the pathway and are therefore assigned to the initiation phase (although some of them could come into play at a late stage in the degeneration process). In some cases there is experimental support for this. For instance, in SCG neuronal cultures, FK866 confers maximum protection when added up to 3 hours after axotomy. Since FK866 is thought to block the toxic consequence of NMNAT2 depletion (NMN increase), it is likely that NMNAT2 acts at early stages after injury (Di Stefano *et al.*, 2015). Similarly, replacing NMNAT2 by directly transducing NMNAT1 in the axon up to 4 hours after injury confers strong protection (Sasaki & Milbrandt, 2010). In fact, strong axonal NMNAT2 depletion is observed as early as 2.5 hours after cut (Di Stefano *et al.*, 2015). Therefore, NMNAT2 depletion is likely to be a crucial event of the initiation phase, possibly the first signal which activates the pathway (Conforti *et al.*, 2014).

It is also important to define when the execution phase starts, as it is likely that axons commit to degeneration at this step. The execution phase itself can be delayed; however this is likely to be a short-lived protection. More than one mechanism is expected to act at later stages and it would be surprising if the axon still retains its functionality at this point (Conforti *et al.*, 2014). Below, I will introduce additional regulators of Wallerian degeneration, where in the pathway they are thought to act and their potential link to NMNAT2.

1.6.2 Ubiquitin proteasome system (UPS), Highwire and PHR1

Multiple studies show that pharmacological or genetic UPS impairment markedly delay Wallerian degeneration (Zhai *et al.*, 2003; MacInnis & Campenot, 2005; Hoopfer *et al.*, 2006; Barrientos *et al.*, 2011; Xiong *et al.*, 2012; Babetto *et al.*, 2013). As mentioned above, WLD^S interacts directly with VCP via its N-terminal domain. Given the role of VCP in the degradation of several substrates through the UPS (Dai & Li, 2001), an impairment of proteasome function is a possible direct mechanism of action of WLD^S. However, the protective effect displayed by cytosolic NMNATs variants, which do not require interaction with VCP, does not fit with this model (Sasaki *et al.*, 2006; Avery *et al.*, 2009; Sasaki *et al.*, 2009a).

Subsequent studies offer a clear explanation for the protection achieved upon UPS inhibition, which is likely due to maintaining high levels of NMNAT2 for longer by preventing its degradation rather than a specific action of WLD^S (Gilley & Coleman, 2010; Milde *et al.*, 2013c). UPS efficiency is actually required for axonal health (Coleman & Ribchester, 2004), as proteasome inhibition causes spontaneous neurite degeneration in culture and peripheral neuropathy in humans (Kane *et al.*, 2003; Laser *et al.*, 2003), and several mouse axonopathies are linked to UPS impairment (Saigoh *et al.*, 1999; Kaneko-Oshikawa *et al.*, 2005). It is likely that this is caused by accumulation of many damaged proteins that would normally be cleared by the UPS.

Further support to the “NMNAT2 model” comes from the discovery of the function in Wallerian degeneration of the *Drosophila* E3 ubiquitin ligase Highwire, previously described in connection with neural development (DiAntonio *et al.*, 2001; Bloom *et al.*, 2007), and its mammalian homologue PHR1. Highwire loss of function strongly delays Wallerian degeneration in *Drosophila* (Xiong *et al.*, 2012). Highwire mediates the degradation of both dNMNAT and ectopically expressed mammalian NMNAT2 (Xiong *et al.*, 2012). This activity is shared by its mammalian counterpart PHR1, whose conditional deletion in mice substantially delays Wallerian degeneration both in the PNS and CNS by slowing down NMNAT2 turnover (Babetto *et al.*, 2013). Intriguingly, NMNAT2 eventually drops also in the absence of PHR1 long before fragmentation (Babetto *et al.*, 2013). A possible explanation for this is that

NMNAT2 loss is required early after injury, with a later decrease being unable to induce degeneration. However, an additional, perhaps more likely, explanation is that there are still high enough levels of the protein that cannot be detected easily due to the poor quality of the detection systems (Milde *et al.*, 2013b). Highwire and PHR1 deletion are among the few modifications that result in axonal protection approaching the efficacy of WLD^S. The connection with NMNAT2 is straightforward, with the two ubiquitin ligases directly involved in the degradation of dNMNAT and mammalian NMNAT2. Therefore, these regulators are likely central components of the initiation phase (Conforti *et al.*, 2014).

1.6.3 SARM1

Recently, large-scale genetic screening in *Drosophila* identified an essential component of the Wallerian degeneration pathway, a protein known as Sterile α and TIR motif-containing protein 1 (SARM1 in mammals, dSARM in *Drosophila*). SARM1 is a Toll-like receptor adaptor family member involved in the innate immunity response (Mink *et al.*, 2001; Couillault *et al.*, 2004; Carty *et al.*, 2006; Dalod, 2007). Its deletion robustly delays Wallerian degeneration in PNS and CNS neurons in both mice and *Drosophila*. Of note, the extent of protection achieved with SARM1/dSARM deletion after injury resembles that of WLD^S/NMNATs (Osterloh *et al.*, 2012; Gerdts *et al.*, 2013). *Sarm1*^{-/-} axons are also protected from vincristine and NGF withdrawal toxicity (Gerdts *et al.*, 2013). Importantly, mice lacking SARM1 are healthy and do not show any clear abnormality. This makes SARM1 an ideal therapeutic target and its mechanism of action is consequently the focus of intensive studies.

In retinal ganglion cells (RGC), excitotoxicity, which leads to Wallerian-like degeneration, up-regulates SARM1 expression (Massoll *et al.*, 2013). However, over-expression of SARM1 does not seem to be the mechanism underlying Wallerian degeneration (Gerdts *et al.*, 2013). Recently, Gerdts, Milbrandt and colleagues proposed an attractive working model to explain SARM1 action. This model predicts that SARM1 exists in a multimer conformation (mediated by the two sterile α (SAM) domains (Gerdts *et al.*, 2013)) which is kept in an inactive state in uninjured axons through an N-terminal Armadillo (ARM) auto-inhibitory domain. After injury, an unknown signal releases the auto-inhibition enabling the association of Toll-interleukin receptor homology (TIR) domains that then

transmit the pro-degeneration signal (Fig. 1.6). Consistent with this model, deletion of the N-terminal domain (leaving just 2 x SAM-TIR domains) produces a constitutively active form of SARM1 that promotes death of axons and cell bodies. On the contrary, SARM1 mutants in which SAM and/or TIR domains have been deleted are unable to initiate degeneration. Finally, chemical induced dimerization of TIR domains alone is sufficient to trigger degeneration (Gerdts *et al.*, 2013; Gerdts *et al.*, 2015; Gerdts *et al.*, 2016). To date, this elegant model is based on artificial and overexpressed SARM1 constructs and evidence of such behaviour of the endogenous, intact protein is still lacking. SARM1 activation seems to occur within the first 4 hours after injury *in vitro*. This and the strength of the protective phenotype place it as a critical regulator of the initiation phase of Wallerian degeneration.

An interesting question is whether SARM1, WLD^S/NMNATs and NAD metabolism are linked in a single pathway regulating Wallerian degeneration, or if they act independently. A recent study shows that SARM1 acts directly downstream of NMNAT2 loss or in a parallel branch of a convergent pathway (Fig. 1.7). Crucially, similar to expression of WLD^S and NMN deamidase, SARM1 deletion fully rescues axonal defects and perinatal lethality caused by NMNAT2 deficiency. Double NMNAT2 and SARM1 knock out mice are healthy into adulthood (Gilley *et al.*, 2015). In addition to this, SARM1 activation has been shown to induce a rapid depletion of NAD (Gerdts *et al.*, 2015), which could, together with NMNAT2 loss, explain the depletion of axonal NAD after injury (Wang *et al.*, 2005). Based on these results, Gerdts, Milbrandt and colleagues proposed that WLD^S/NMNATs preserve axons by counteracting this SARM1-dependent toxic NAD depletion. This model seems to directly contradict the “NMN model”. In reality, these results support the existence of strong crosstalk between SARM1 and NAD metabolism to regulate axon degeneration. A discussion on how the “NMN model” and these latest findings on SARM1-induced-NAD depletion could be unified will be discussed in more detail in the final chapter of this thesis.

Of note, SARM1 deletion results in attenuated axonal damage in a model of traumatic brain injury (Henninger *et al.*, 2016; Hill *et al.*, 2016) and prevention of vincristine induced neuropathy in mice (Geisler *et al.*, 2016). In addition, in a

genome-wide association study, variants of SARM1 have been associated to ALS (Fogh *et al.*, 2014). To conclude, studies on SARM1 confirmed once again how regulators of Wallerian degeneration could be useful targets to treat other neurodegenerative conditions.

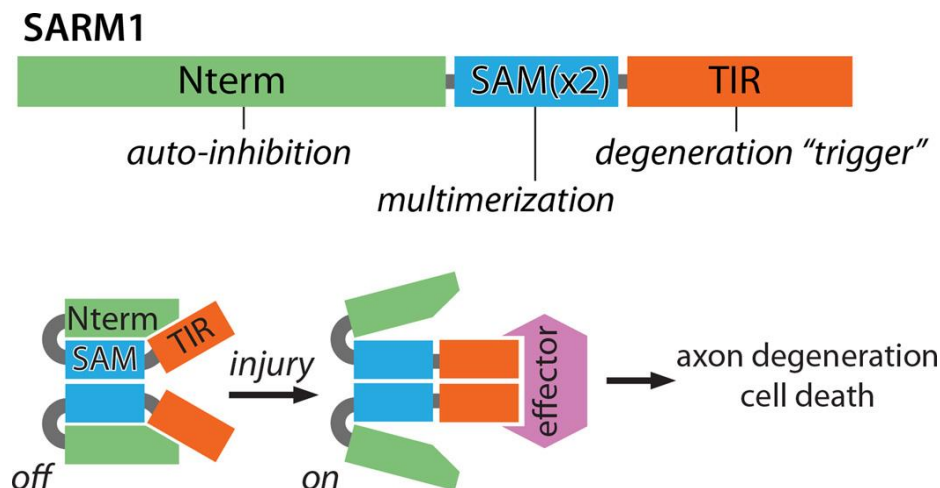


Figure 1.6 A hypothetical model of SARM1 activation upon injury, from (Gerdtz *et al.*, 2016).

The SARM1 protein consists of three different regions with different functions. The proposed working model predicts that the N-terminal domain inhibits the pro-degenerative activity of the TIR domain in uninjured axons. A signal after injury releases this inhibition, exposing the TIR domains which in turn activate a pro-destructive process. SAM domains are involved in the multimerization of the protein, which is required to initiate degeneration.

1.6.4 Mitogen-activated protein kinase (MAPK) cascade

MAPKs are a family of proteins involved in signal transduction pathways regulating a wide range of physiological processes. They are generally activated (or inhibited) by phosphorylation at specific residues and cooperate following a precise sequential scheme: **i)** a MAPK kinase kinase (MAPKKK) is often the first to be activated; **ii)** MAPKKK activation regulates the phosphorylation of MAPK kinase (MAPKK); **iii)** MAPKKs finally activate the MAPKs, the final members of the cascade (Cargnello & Roux, 2011).

The first member of this family of proteins described to have a role in Wallerian degeneration is dual leucine zipper-bearing kinase (DLK), a MAPKKK with a function in synaptic development (Nakata *et al.*, 2005; Collins *et al.*, 2006). Mice

lacking DLK show significant delay of axon degeneration after injury. A similar result is observed in *Drosophila* mutants lacking the DLK ortholog, *Wallenda* (*Wnd*). DLK likely acts through the downstream MAPK c-jun n-terminal kinase (JNK), as pharmacological inhibition of JNK confer a similar degree of protection against Wallerian degeneration (Miller *et al.*, 2009). However, loss of DLK confers a degree of protection that is significantly weaker than WLD^S/NMNATs.

In a recent study, Yang, Tessier-Lavigne and colleagues showed that the weaker axon protection phenotype upon loss of DLK is due to a functional redundancy with related MAPKs. They identified two other MAPKKs involved in axon degeneration after injury, MEKK4 and MLK2. Genetic disruption of all three MAPKKs leads to greatly improved protection. DLK, MEKK4 and MLK2 are then responsible of the activation of the MAPKKs MKK4 and MKK7 that in turn activate the JNK family of MAPKs (JNK 1-3), completing the MAPK cascade activation (Yang *et al.*, 2015).

MAPKs are likely acting at early stages in the initiation phase of Wallerian degeneration, possibly triggering local energy depletion. In support of this, activation of MAPKs is surprisingly observed just minutes after injury and inhibition of JNK is effective only within the first 3 hours after injury (Miller *et al.*, 2009; Yang *et al.*, 2015). However, the link between this MAPK cascade, WLD^S/NMNATs and SARM1 is still controversial. A study shows that SARM1 is required to activate this signalling cascade and expression of cyt-NMNAT1 blocks MAPK activation (Yang *et al.*, 2015). However, it is still unclear how SARM1 and NMNATs can regulate this cascade since MAPKs are shown to be activated minutes after injury. Both SARM1 and cyt-NMNAT1 can act hours after injury, despite controlling early steps in the Wallerian degeneration pathway. Deletion of SARM1 within 4 hours of injury still confers strong protection (full protection when it is deleted within 2 hours of transection) (Gerdtz *et al.*, 2015). Similarly, axonal transduction of NMNAT1 and FK866 treatment in the first 4 hours after injury still confers strong protection (Sasaki & Milbrandt, 2010; Di Stefano *et al.*, 2015). A more recent study instead suggests that MAPKs act upstream of NMNAT2 and SARM1, regulating and accelerating NMNAT2 turnover (Walker *et al.*, 2017).

The MAPK cascade also has interesting connections with other regulators of the Wallerian degeneration pathway. First, DLK/Wnd is a target of PHR1/Highwire. In fact, in PHR1 knock out mice and Highwire mutant flies DLK/Wnd levels are increased (Xiong *et al.*, 2012; Babetto *et al.*, 2013). However, in these mutants axonal degeneration is still delayed, indicating the requirement of an upstream signal to activate the MAPKs pathway. Moreover, JNK influences the turnover of superior cervical ganglion 10 (SCG10), another short-lived protein which has also been implicated at early time points in Wallerian degeneration (Shin *et al.*, 2012). The role of SCG10 in Wallerian degeneration will be discussed in more detail in the introductory section of chapter 4 of this thesis. Finally, AKT acts as a negative regulator of MKK4. Interestingly, AKT is targeted to proteasomal degradation by the E3 ubiquitin ligase ZNRF1. This leads to a depletion of AKT in injured axons, which likely contributes to the activation of the MAPKs cascade. Consistently, ZNRF1 deletion confers modest protection against Wallerian degeneration (Wakatsuki *et al.*, 2011; Gerdts *et al.*, 2016).

1.6.5 Ca²⁺

An intra-axonal Ca²⁺ increase has long been implicated in Wallerian degeneration (Schlaepfer & Bunge, 1973; Schlaepfer & Hasler, 1979; LoPachin *et al.*, 1990; George *et al.*, 1995). Ca²⁺ elevation after injury is known to activate a family of proteases known as calpains (Ca²⁺-dependent cysteine proteases), ultimately responsible for axonal cytoskeleton degradation (Ma, 2013). Consistently, blocking Ca²⁺ increase and calpain inhibition delay Wallerian degeneration (George *et al.*, 1995; Finn *et al.*, 2000; Zhai *et al.*, 2003; Ma, 2013; Ma *et al.*, 2013; Yang *et al.*, 2013). Earlier studies placed Ca²⁺ accumulation downstream of WLD^s as a late event in Wallerian degeneration, likely a key modulator of the execution phase (George *et al.*, 1995; Gerdts *et al.*, 2011; Adalbert *et al.*, 2012; Yang *et al.*, 2013; Conforti *et al.*, 2014). However, recent studies suggest a possible role of Ca²⁺ early in the degeneration process (Avery *et al.*, 2012; Summers *et al.*, 2014). In addition, the exact triggers of Ca²⁺ increase after injury are still unclear and it remains elusive how WLD^s can delay these processes (Fig. 1.7). In chapter 3 of this thesis, I study the role of Ca²⁺ in axon degeneration after injury and its relation to NMN accumulation and

SARM1. The introduction to chapter 3 provides a more detailed view of the role of Ca^{2+} in Wallerian degeneration.

1.6.6 Mitochondria

Mitochondria regulate several intracellular processes ranging from ATP production, intracellular Ca^{2+} levels, reactive oxygen species (ROS) production, release of pro-apoptotic signals and other cell death mechanisms, which ultimately affect cell fate (Ernster & Schatz, 1981; Lehninger *et al.*, 2013). Mitochondria dysfunction is involved in the development of several neurodegenerative disorders (Lin & Beal, 2006; Court & Coleman, 2012; Johri & Beal, 2012). Mitochondria have been reported as central executors in Wallerian degeneration through mitochondrial permeability transition pore (mPTP) opening (Barrientos *et al.*, 2011; Villegas *et al.*, 2014). However, Wallerian degeneration is only modestly delayed in axons devoid of mitochondria in some models and mitochondria are dispensable for WLD^s protection (Kitay *et al.*, 2013). Therefore, the role of mitochondria in Wallerian degeneration and the WLD^s mechanism, as well as the relationships between NMN increase, SARM1, Ca^{2+} and mitochondrial dynamics remain unresolved (Fig. 1.7). In chapter 4 and 5 I delve more deeply into the actual contribution of mitochondrial dysfunction in degeneration of injured and uninjured axons. More details on the role of mitochondria in axon degeneration can be found in the introductory sections of these 2 chapters.

1.7 NMNATs chaperone activity

In recent years, NMNATs are emerging as central regulators of neuronal maintenance. In addition to the above described involvement in axon degeneration, recent studies have revealed a chaperone activity of these proteins which seems to play a neuroprotective role (Zhai *et al.*, 2008; Ali *et al.*, 2013; Ali *et al.*, 2016; Lavado-Roldan & Fernandez-Chacon, 2016). In 2008, Zhai and colleagues showed that dNMNAT is acting as a molecular chaperone to protect against spinocerebellar ataxia 1 (SCA1)-induced neurodegeneration, independently from its enzymatic activity (Zhai *et al.*, 2008). More recently, NMNAT2 has been shown to act as a chaperone to protect against proteotoxic

stress (Ljungberg *et al.*, 2012; Ali *et al.*, 2016) (typical of several neurodegenerative disorders (Douglas & Dillin, 2010)). These results reveal a double neuroprotective function of NMNATs depending on the type of insult. Surprisingly, studies in *Drosophila* have shown that enzyme-dead versions of dNMNAT still confer protection from axon degeneration induced by crush (Kitay *et al.*, 2013) and from degeneration caused by dNMNAT loss (Zhai *et al.*, 2006). Nevertheless, at least in mammals, it seems unlikely that NMNAT chaperone activity has a crucial role in WLD^s-mediated protection in Wallerian degeneration, as suggested by the well-established importance of NMNAT1 enzymatic activity in WLD^s and the absence of protection of the enzyme-dead WLD^s mutant where the chaperone activity is still present (Conforti *et al.*, 2009; Coleman & Freeman, 2010). In addition, it still needs to be established whether residual enzyme activity is responsible for the protective phenotypes, especially in cases where the protein is heavily overexpressed. Past experience with WLD^s and NMN deamidase suggest that minimal levels of protein expression (and activity) are sufficient to strongly protect axons.

1.8 Aims of this thesis

Two major goals in the Wallerian degeneration field are:

- Fully characterizing the pathway regulating axon death after injury.
- Identifying circumstances where the same pathway regulates axon loss in the absence of an injury, a critical feature of several neurodegenerative disorders.

Achieving these goals should lead to an accurate identification of axonal disorders characterized by Wallerian-like degeneration and the most suitable targets in the pathway for therapeutic intervention.

Along this line, the present thesis aims to expand our current knowledge of the mechanisms controlling Wallerian degeneration. I focus on understanding how the accumulation of NMN leads to the death of the axonal stump distal to the site of injury. Specifically, I investigate the relationship between NMN, SARM1 and Ca^{2+} , and the role of mitochondria in axon degeneration after injury (chapters 3 and 4) (Fig. 1.7). I then test whether the same mechanisms control the degeneration of uninjured axons exposed to mitochondrial toxins (chapter 5).

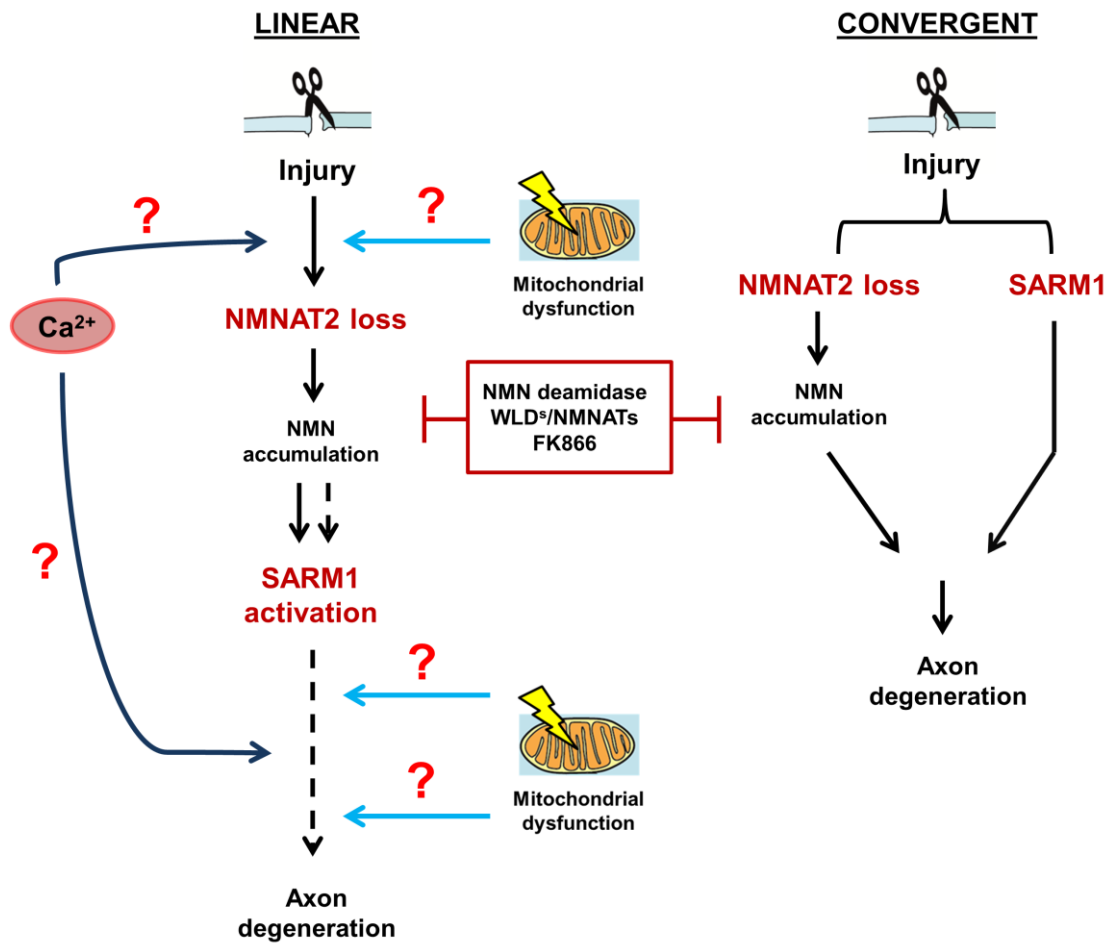


Figure 1.7 Schematic representation of the Wallerian degeneration pathway.

NMNAT2 and SARM1 are emerging as major players cooperating in a core pathway leading to axon degeneration after injury. NMNAT2 loss leads to the toxic accumulation of NMN and the activation of the pathway. SARM1 action is required downstream of NMNAT2 for axons to degenerate. It is still unclear whether SARM1 acts directly downstream of NMNAT2 loss in a linear pathway, or whether they converge on a common downstream step. This will be discussed in more detail in chapter 6.

Several other questions remain unanswered. In this thesis, I focus on the contribution of Ca^{2+} and mitochondria to the degeneration process. Whether changes in intracellular Ca^{2+} and mitochondrial homeostasis are early or late events in the pathway is still debated. In addition, their relationship with NMNAT2, NMN and SARM1 has not been fully studied and will be the focus of chapters 3 and 4.

CHAPTER 2

Materials and Methods

Materials and Methods

The materials and methods used in this thesis are described below. The numbers in brackets in the headings denote the chapters of this thesis in which the method was used. All studies conformed to the institution's ethical requirements in accordance with the 1986 Animals (Scientific Procedures) Act.

2.1 Primary neuronal cultures (chapters 3, 4, 5)

2.1.1 Superior cervical ganglia (SCG) explant cultures

C57BL/6 or CD1 (referred to as wild-type, Charles River, UK), *Nmnat2*^{+/+}, *Nmnat2*^{+/gtE}, *Nmnat2*^{gtBay/gtE} and *Sarm1*^{-/-} (kindly provided by Prof. Michael Coleman, University of Cambridge) mouse SCG explants were dissected from P0-2 pups. Explants were cultured in 35 mm tissue culture dishes or ibidi μ -dishes (Thistle Scientific) pre-coated with poly-L-lysine (20 μ g/ml for 1 h; Sigma) and laminin (20 μ g/ml for 1 h; Sigma) in Dulbecco's Modified Eagle's Medium (DMEM D6546, Sigma) with 2 mM glutamine, 1% penicillin/streptomycin, 100 ng/ml 7S NGF (all Invitrogen) and 2% B27 (Gibco). 4 μ M aphidicolin (Sigma) was used to reduce proliferation and viability of small numbers of non-neuronal cells. Despite this, glial cells are still present in explant cultures and their potential impact on the experimental outcomes needs to be taken into account. Culture media was replenished every 3 days. Axons were allowed to extend for 6-7 days before performing the experiments.

2.1.2 Superior cervical ganglia (SCG) dissociated cultures

For cultures of dissociated SCG neurons, wild-type and *Sarm1*^{-/-} SCG explants were incubated in 0.025% trypsin (Sigma) in PBS (without CaCl₂ and MgCl₂) (Sigma) for 30 minutes followed by incubation with 0.2% collagenase type II (Gibco) in PBS for 20 minutes. Ganglia were then gently dissociated using a pipette. Dissociated neurons were plated in a poly-L-lysine and laminin-coated area of ibidi μ -dishes for microinjection experiments. Dissociated cultures were maintained as explant cultures except that B27 was replaced with 10% fetal

bovine serum (Sigma). Axons were allowed to extend for 3 days in culture (for Ca^{2+} experiments) before microinjection and cut the following day.

In our experimental system (Di Stefano *et al.*, 2015), axons originating from wild-type SCG explants degenerate within 8 hours after cut. For the purpose of comparison, no morphological changes occur in wild-type axons within the first 4 hours after cut; clear morphological damages and fragmentation occur at 6-8 hours after cut. After 4 days in culture, axons originating from dissociated cells degenerate within ~3 hours after cut; this difference could be due to the different time in culture (Buckmaster *et al.*, 1995). I refer to time 0 as the first time point acquired immediately after cut and/or drug treatment.

2.1.3 Dorsal root ganglia (DRG) explant cultures

C57BL/6 mouse DRG explants were dissected from E16.5 embryos. Explants were cultured in 35 mm tissue culture dishes pre-coated with poly-L-lysine (20 $\mu\text{g/ml}$ for 1 h; Sigma) and laminin (20 $\mu\text{g/ml}$ for 1 h; Sigma) in Dulbecco's Modified Eagle's Medium (DMEM D6546, Sigma) with 2 mM glutamine, 1% penicillin/streptomycin, 100 ng/ml 7S NGF (all Invitrogen) and 2% B27 (Gibco). 4 μM aphidicolin (Sigma) was used to reduce proliferation and viability of small numbers of non-neuronal cells. Culture media was replenished every 3 days. Axons were allowed to extend for 7 days before performing the experiments.

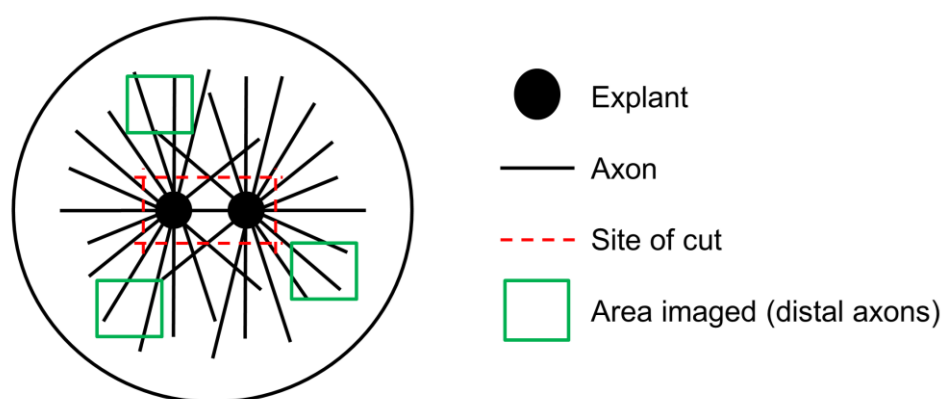
2.1.4 Cortical neurons cultures

C57BL/6 mouse cortices were dissected from E16.5 embryos and carefully triturated. Neurons were cultured into microfluidic devices (Xona microfluidics) with 150- μm -long microgrooves placed on glass coverslips pre-coated with poly-L-ornithine (Sigma). Neurons were maintained in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Gibco) and 1% GlutaMAX (Gibco). Culture media was replenished every 2 days. Axons were allowed to extend for 7 days before performing the experiments.

2.2 SCG and DRG explant axotomy experiments (chapters 3, 4)

Wild-type SCG axons were cut around the cell bodies using a disposable scalpel under a dissection microscope. FK866 was administered immediately

after axotomy. NMN was added at time 0 hours (for experiments with CCCP) or 12 hours after cut and FK866 administration (Di Stefano *et al.*, 2015); all the drugs tested were pre-incubated for 30-45 minutes before NMN administration. The same protocol was followed for DRG, with the only difference that NMN was added 24 hours after cut. The drugs tested were used at the following concentration: 100nM FK866 (kind gift of Prof. Armando Genazzani, University of Novara); 100 μ M-1mM NMN (Sigma); 5mM EGTA (Sigma), 10 μ M verapamil (MP Biomedicals); 10 μ M KB-R7943 (Sigma); 30 μ M nifedipine (MP Biomedicals); 10 μ M ryanodine (Tocris bioscience); 20 μ M cyclosporin A (Sigma); 50 μ M ruthenium red (Calbiochem), 50 μ M CCCP. The time of pre-incubation and the concentrations used for every experiment are indicated in the figures and/or figure legends. The concentration of each pharmacological agent was determined based on previous studies., where similar doses were reported to confer protection or induce axon degeneration in similar experimental paradigms (FK866 and NMN (Di Stefano *et al.*, 2015); EGTA, nifedipine, verapamil, KB-R7943, ryanodine, cyclosporin A, ruthenium red (Zhai *et al.*, 2003; Barrientos *et al.*, 2011; Gerdtts *et al.*, 2011; Villegas *et al.*, 2014); CCCP (Summers *et al.*, 2014)).



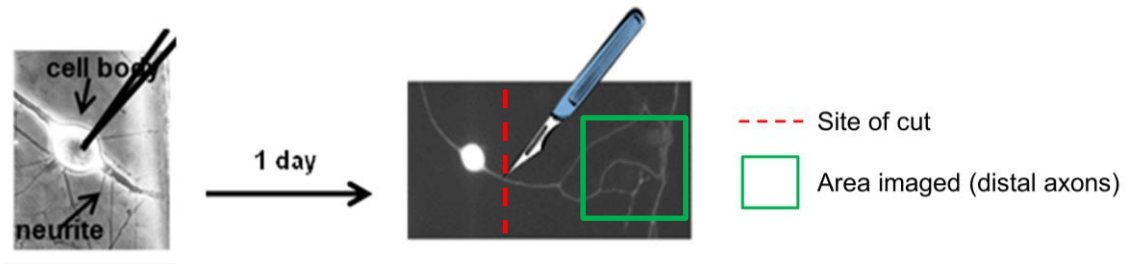
Schematic diagram showing an axotomy experiment.

2.3 Acquisition of phase contrast/bright field images and quantification of axon degeneration (chapters 3, 4, 5)

Phase contrast/bright field images were acquired on a DMRB upright fluorescence microscope (Leica microsystems) coupled to a monochrome digital camera (Hamamatsu C4742-95) using Volocity software (PerkinElmer) or on a Zeiss TIRF microscope coupled to an EMCCD (Photometrics PVCam) camera using Axiovision software (Carl Zeiss Inc.). The objectives used were HCXPL 20X/0.40 Corr and Zeiss EC Plan Neofluar 20X/0.5NA. To measure the axon degeneration index (Sasaki *et al.*, 2009b) an ImageJ plugin was used (Schneider *et al.*, 2012) (<http://rsb.info.nih.gov/ij/download.html>). Images were binarized and the total number of black pixels was defined as the total axon area. Intact axons show a continuous tracts (>200 pixels); axonal fragments <200 pixels were scored as degenerated. The degeneration index was calculated as the ratio of fragmented axon area over total axon area, therefore accounting for differences in axonal density in the ROI. Axons that detached from the dish were considered completely degenerated and scored as 1.

2.4 Microinjection and cloning (chapters 3, 4)

Dissociated cells were microinjected using a Zeiss Axiovert S100 microscope with an Eppendorf FemtoJet transjector and 5171 micromanipulator system. Plasmids were diluted in 0.5×PBS (without CaCl₂ and MgCl₂) and filtered using a Spin-X filter (Costar). The mix was injected directly into the nuclei of SCG neurons using Eppendorf Femtotips. Approximately 100 neurons were injected per dish approximately along a line so that proximal and distal axons can be easily defined. Injected plasmids were allowed to express for 1 day before axotomy. Plasmids were injected at the following concentrations: 100ng/μl GCaMP5, 100ng/μl TagRFP-mito, 30-50ng/μl pDsRed2-N1, 100ng/μl NMN deamidase, 100ng/μl pCDNA 3.1 (+) (Empty vector). *E. Coli* NMN deamidase ORF was subcloned from a bacterial expression plasmid (kind gift of Prof. Nadia Raffaelli) into pCDNA3.1 (+) vector using EcoRI/NotI restriction enzymes (NEB).



Schematic diagram showing a microinjection experiment.

2.5 Ca^{2+} imaging and quantification (chapters 3, 4)

Wild-type and *Sarm1*^{-/-} dissociated SCG neurons were cut and then imaged in a controlled environment (37°C and 5% CO_2). Treatments were applied 30 minutes before cut or immediately after cut at the following concentrations: 100nM FK866, 1mM NMN (Sigma), 5mM EGTA (Sigma). The time of pre-incubation and the concentrations used for every experiment are indicated in the figures and/or figure legends. Time-lapse fluorescent images were acquired every 5 minutes up to 260 minutes on a Zeiss LSM710 confocal microscope coupled with an EMCCD (Photometrics PVCam) camera using ZEN 2010 software (Carl Zeiss Inc.). The objectives used were Neofluar 40X/0.6 oil and Zeiss EC Plan Neofluar 63X/1.25 oil. At the end of each sample recording, 10 μM Ionomycin (Sigma) was added to each sample to control for the correct functioning of the Ca^{2+} indicator GCaMP5.

Axons showing membrane swellings more than twice the width of the associated axon were scored as abnormal. The number of healthy DsRed2-labeled axons remaining at each time point is shown as a percentage of the total number (both healthy and abnormal). For GCaMP5 quantification, the intensity of uncut axons was subtracted from the intensity of cut axons at each time point (ΔF). All measurements were corrected for background by subtracting the background intensity (F_0) at each time point. A region of interest (ROI) was manually drawn in an area populated by axons and identical regions were analysed in all experiments. The background was calculated in an axon-free region of the same field of view. For quantification of Ca^{2+} peak intensity, the

time point showing the highest signal intensity prior to any visible presence of axonal swellings was used.

Ca²⁺ intensity inside axonal swellings was measured from 10 different, still continuous axons in 3 independent experiments. 3 individual swellings per each axon were analysed. For comparison, Ca²⁺ intensity in axonal segments of the same area of the swellings and adjacent to them was calculated. The mean intensity inside axonal swellings was normalised to the mean intensity in axonal fragments immediately adjacent to the correspondent swelling. Signals intensities were determined using ZEN 2010 software.

2.6 Mitochondrial motility imaging and quantification (chapter 4)

Wild-type and *Sarm1*^{-/-} SCG explants were pre-incubated with 100nM mitotracker red CMXRos (Invitrogen) for 15 minutes at 37 °C before axotomy. When used, 100nM FK866 and 1mM NMN (Sigma) were administered immediately after axotomy. Time-lapse fluorescent images were acquired at 37 °C on a Zeiss TIRF microscope coupled with an EMCCD (Photometrics PVCam) camera using ZEN 2010 software (Carl Zeiss Inc.). Images were acquired at the rate of 1 frame/sec for 5 minutes at the specified time points. The objective used was 100X/1,46 AlphaPlanAPO.

Quantification of mitochondrial motility was performed using the ImageJ plugin *Difference tracker* ([Andrews et al., 2010](#)). Differences in contrast between each movie were minimized by applying automated contrast normalization to all images using ImageJ.

2.7 Mitochondrial membrane potential quantification (chapter 4)

Wild-type and *Sarm1*^{-/-} SCG neurons were incubated with the cell-permeant dye TMRM (VWR International) for 30 minutes at 37 °C before axotomy and imaging. When used, 100nM FK866 and 1mM NMN (Sigma) were administered immediately after axotomy. TMRM, added to a final concentration of 50nM, is readily sequestered by active mitochondria and released during depolarization

(Villegas *et al.*, 2014). Fluorescent and bright field images were acquired at 37 °C on a Zeiss TIRF microscope coupled with an EMCCD (Photometrics PVCam) camera using ZEN 2010 software (Carl Zeiss Inc.). The objective used was 63X/1.4 PInAPO. At the end of the experiment 50µM CCCP (Sigma) was added to control for the correct functioning of the mitochondrial membrane potential indicator TMRM. Signal intensities were determined using ZEN (blue edition) software (Carl Zeiss Inc.) and normalised to uncut controls.

2.8 Mitochondrial length quantification (chapter 4)

Wild-type and *Sarm1*^{-/-} SCG explants were incubated with 100nM mitotracker red CMXRos (Invitrogen) for 15 minutes at 37 °C before cutting. When used, 100nM FK866 and 1mM NMN (Sigma) were administered immediately after axotomy. Fluorescent and bright field images were acquired at 37 °C on a Zeiss TIRF microscope coupled with an EMCCD (Photometrics PVCam) camera using ZEN 2010 software (Carl Zeiss Inc.). The objective used was 100X/1.46 AlphaPInAPO. Mitochondrial length was determined manually by measuring the longest dimension using ZEN (blue edition) software (Carl Zeiss Inc.). Different treatment groups were analysed singularly comparing time 0 to time 4 hours after cut.

2.9 CCCP and rotenone treatment (chapter 5)

Uncut wild-type, Wld^S, *Nmnat2*^{+/+}, *Nmnat2*^{+/gtE}, *Nmnat2*^{gtBay/gtE} and *Sarm1*^{-/-} SCG neurons were treated with CCCP, rotenone or vehicle (DMSO) just prior to imaging (time 0). The drugs were used at the following concentrations: 25µM, 50µM, 100µM CCCP (Sigma); 1µM, 5µM, 10µM, 25µM rotenone (Sigma). In most experiments, 100nM FK866 and 1mM NMN were added together with CCCP/rotenone. In some experiments, 100nM FK866 was added 4, 8 and 12 hours after CCCP addition, and 12, 16 and 20 hours after rotenone addition. The time of incubation and the concentration used for every experiment are indicated in the figures and/or figure legends. To check that CCCP treatment was triggering full depolarization of axonal mitochondrial, neurons were incubated for 30 minutes at 37 °C with TMRM.

2.10 Cortical neurons axotomy and immunostaining (chapter 5)

Axotomy of cortical neurons in microfluidic chambers was performed via vacuum aspiration of the cell bodies in the soma compartment (Taylor *et al.*, 2005). Following axotomy, neurons were fixed with 4% paraformaldehyde (Sigma) and incubated overnight with mouse anti- β -tubulin III (T8578 Sigma, 1:200), then washed and incubated for 1 hour at room temperature with Alexa fluor 488 secondary antibody (Thermo Fisher Scientific). Images were acquired on a Zeiss TIRF microscope using Zeiss EC Plan Neofluar 20X/0.5NA objective. % of intact axons was scored manually.

2.11 Ca²⁺ imaging and quantification in zebrafish larvae (chapter 3)

Zebrafish larvae were grown in E3 buffer and 0.003% 1-phenyl 2-thiourea (PTU) (Sigma) was added before 24 hours post fertilization (hpf) to block pigmentation. To obtain mosaic labelling of somatosensory neurons, approximately 10 pg each of Isl1:Gal4:UAS:DsRed and UAS:GCaMP-HS plasmids (kind gifts of Prof. Alvaro Sagasti and Dr. Mauricio Vargas) were injected into one-cell stage embryos. At 48-54 hpf, larvae were pre-treated in 0.003% PTU Ringer's solution with 400 μ M FK866 (Sigma) in DMSO/pluronic (1% and 0.05%, Sigma and ThermoFisher scientific respectively) or with vehicle alone 6 hours prior to axotomy and subsequently anaesthetized in 0.01% tricaine and mounted in 1% low melt agarose. Two-photon laser axotomy was performed with a 710nm laser at an intensity of ~20% transmission using Zeiss C Apochromat 40x/1.2 water objective (O'Brien *et al.*, 2009; Di Stefano *et al.*, 2015). Time-lapse images were obtained at 2 minutes intervals starting 20 minutes after axotomy using a Zeiss LSM 880 confocal microscope with a Zeiss C Apochromat 40x/1.2 water objective. Images are shown as Maximum Intensity Projections. Larvae were kept at 28.5 °C in the same buffer used for the pre-treatment during the imaging period. Single axons separate into several branches, which were scored as abnormal when they showed membrane swellings that were more than twice the width of the associated axon. The number of healthy DsRed-labeled axonal branches remaining at each time point

is shown as a percentage of the total number. For GCaMP-HS quantification, a ROI was manually drawn around the transected axons and the intensity at each time point (F) was divided by the intensity of the first time point imaged (F0). For quantification of Ca^{2+} peak intensity, the time point showing the highest signal intensity prior to appearance of axonal swellings was used. Signal intensities were determined using ZEN 2012 software (Carl Zeiss Inc.).

2.12 NMN deamidase expression in zebrafish larvae (chapter 5)

To obtain mosaic labelling of somatosensory neurons expressing NMN deamidase, approximately 10 pg each of *Isl1:Gal4:UAS:DsRed* and *UAS:NMN deamidase* plasmids were injected into one-cell stage embryos. Laser axotomy, imaging and quantification of axon degeneration were performed as described above. Time-lapse images were obtained with intervals of 10 minutes starting 20 minutes after axotomy. Imaging of axons injected with the empty vector was blocked soon after degeneration occurred. Axons expressing NMN deamidase were imaged up to 12 hours. Some axons were followed up to 24 hours (n=3).

2.13 Western blot (chapter 5)

Following treatment with CCCP, rotenone and DMSO, SCG explants were separated from their axons with a scalpel. Axons originating from 15 ganglia were collected per condition, washed in ice-cold PBS, and lysed directly in 15 μl 2x Laemmli buffer (Bio-Rad) containing 5% 2-Mercaptoethanol (Sigma). For NMNAT2 immunoblots, 14 μl of protein samples were loaded on a 12% SDS polyacrylamide gel. For SCG10 and NAMPT immunoblots, 1:10 dilutions of the original samples were loaded on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane using a semi-dry blotting apparatus (Bio-Rad). Membranes were blocked for 3 hours in 5% milk in TBS (50mM Trizma base and 150mM NaCl, PH 8.3, both Sigma) plus 0.05% Tween-20 (Sigma) (TBST), incubated overnight with primary antibody in 5% milk in TBST at 4°C and subsequently washed in TBST and incubated for 1 hour at room temperature with HRP-linked secondary antibody (Bio-Rad) in 5% milk in TBST. Membranes were washed, treated with ECL prime (Enhanced

Chemiluminescence detection kit; GE Healthcare) and exposed to film for an appropriate time. The following primary antibodies were used: mouse anti-NMNAT2 (WH0023057M1 Sigma, 1:250), mouse anti-NAMPT (clone OMNI 379, Cayman Chemical Company, 1:2000) and rabbit anti-SCG10 (10586-1-AP Proteintech, 1:3000). As loading control, mouse anti- β -actin was used (A5316 Sigma, 1:5000).

2.14 Statistical analysis (chapters 3, 4, 5)

Data are expressed as Mean $\pm$ SEM or SD. Statistical analysis was performed using ANOVA, Kolmogorov-smirnov test or Student's t test with P-values less than 0.05 being considered significant for any set of data. Stars represent statistical significance with $p = *$ <0.05, $**$ <0.01, $***$ <0.001, $****$ <0.0001. When less than 3 experiments were performed, data are indicated as preliminary in the main text and no statistical analysis test was carried out.

CHAPTER 3

NMN-induced Wallerian degeneration is executed by a SARM1-dependent late influx of Ca^{2+}

3.1 Introduction

As previously mentioned, one of the key aims in the Wallerian degeneration field is to define the exact pathways leading to degeneration after injury, linking together the endogenous regulators that have been identified so far. Our research group recently proposed a key role for NAD precursor NMN in promoting axon degeneration after injury (Di Stefano *et al.*, 2015). To our knowledge, NMN had no previously known function in mammals other than as an NAD precursor. In our experimental system, we can block endogenous NMN production by administering FK866, an inhibitor of NMN-synthesizing enzyme NAMPT. Cut axons treated with FK866 are strongly protected against injury-induced axon degeneration. Of note, exogenous co-administration of NMN overcomes FK866 inhibition, restores NMN accumulation in cut axons and triggers degeneration (see Fig. 1.4A and B in chapter 1). This experimental model has advantages for further mechanistic studies as we can initiate degeneration by adding a molecule (NMN) to transected axons held in a state where they do not degenerate (FK866 treated), rather than simply by injury. It is a useful experimental setting to investigate the downstream mechanism of NMN-induced Wallerian degeneration. In addition, it is an indirect way to confirm the toxic role of NMN; if accumulation of endogenous NMN promotes degeneration in cut axons, its exogenous administration should replicate the same series of events that occur after injury.

For clarification purposes, unlike the induced degeneration of transected axons protected by FK866, NMN addition to uncut axons does not cause degeneration. Healthy neurons express all the three NMNAT isoforms. Therefore, NMN is unlikely to accumulate in intact axons where these NMN consuming enzymes will rapidly convert NMN to NAD. This is not the case in cut axons protected by FK866, where NMNAT2 is lost around 4 hours after injury and the conversion to NAD can no longer occur.

In this chapter, I will address the relationship between NMN, SARM1 and Ca²⁺ after injury. In the next sections, I will discuss in more detail what is currently

known about the role of Ca^{2+} in Wallerian degeneration and I will introduce the use of zebrafish as a model to study Wallerian degeneration *in vivo*.

3.1.1 Ca^{2+} homeostasis in neurons

Ca^{2+} is one of the most important second messengers and regulates many intracellular processes in neurons (as well as in other cell types), including synaptic transmission, control of energy production, gene transcription, neuronal development and programmed cell death (Orrenius *et al.*, 2003; Gleichmann & Mattson, 2011; Brini *et al.*, 2014). Ca^{2+} regulation in neurons is extremely complex and maintaining its homeostasis requires energy expenditure (Gleichmann & Mattson, 2011). Many proteins are involved in the regulation of intracellular Ca^{2+} levels, ranging from Ca^{2+} channels, ATPases (generally called pumps) and exchangers (Brini *et al.*, 2014). Some of them are localised to the plasma membrane; others are present in intracellular organelles which function as important intracellular stores of Ca^{2+} (e.g. the endoplasmic reticulum (ER) and mitochondria). Below is a brief description of those most relevant to the experimental work of this chapter.

3.1.2 $\text{Na}^+/\text{Ca}^{2+}$ exchangers and voltage-dependent Ca^{2+} channels

An important class of Ca^{2+} channels expressed on the plasma membrane are the voltage-dependent Ca^{2+} channels (VDCC). VDCC are crucial transducers of membrane potential changes into intracellular Ca^{2+} levels variations which then promote many physiological events. Their name reflects the characteristic activation in response to membrane depolarization and action potentials (Catterall, 2011). In neurons, VDCC are involved in several processes, including synaptic transmission and regulation of gene expression (Tsien *et al.*, 1988; Dunlap *et al.*, 1995; Catterall & Few, 2008; Flavell & Greenberg, 2008). However, under excessive stimulation or toxic conditions and in the presence of lower levels of ATP, VDCC could mediate significant increases in cytosolic Ca^{2+} , which could eventually lead to cell death (Tanaka *et al.*, 2004; Zundorf & Reiser, 2011).

Another important role is covered by the $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX). NCX are a class of membrane protein that play an important role in regulating $\text{Na}^+/\text{Ca}^{2+}$ homeostasis in the cell. Under physiological conditions, NCX work in the

forward mode and excludes one molecule of Ca^{2+} from the cell in exchange of three molecules of Na^+ . However, in the presence of high intracellular Na^+ , NCX switches to the reverse mode, starting to pump Ca^{2+} inside the cell (Blaustein & Lederer, 1999). The activity of NCX is considered one of the most important mechanisms to remove Ca^{2+} from the cell (DiPolo & Beauge, 2006).

3.1.3 ER and mitochondria crosstalk in Ca^{2+} regulation

The ER and mitochondria play a critical role in regulating intracellular Ca^{2+} levels. The role of the ER as an intracellular store of Ca^{2+} has long been known. (Gleichmann & Mattson, 2011). Levels, influx and efflux of Ca^{2+} in the ER are determined by the coordinated activity of different Ca^{2+} channels and pumps. Among these, the most well-known are the ryanodine receptors (RyRs) and inositol 1,4,5-triphosphate receptors (IP3Rs) channels and a family of ER Ca^{2+} -ATPase called SERCA (Verkhratsky, 2005). $[\text{Ca}^{2+}]_{\text{ER}}$ is much higher than cytosolic free $[\text{Ca}^{2+}]$. This difference is maintained by the activity of SERCA. Ca^{2+} leaves the ER mainly through RyRs and IP3Rs. These two classes of receptors regulate an important process called Ca^{2+} -induced Ca^{2+} release (CICR). This phenomenon consists in the release of ER Ca^{2+} stimulated by the increase of Ca^{2+} , or other signalling processes, in the cytosol, which is in turn regulated by the activity of VDCC and NCX (DiPolo & Beauge, 2006). CICR is mediated mainly by RyRs and IP3Rs which open in response to Ca^{2+} and allow further Ca^{2+} release in the cytosol (Endo, 2009; Gleichmann & Mattson, 2011). This process has been described to contribute to Ca^{2+} elevation associated with the action potential (Usachev & Thayer, 1997; Sandler & Barbara, 1999). In addition to this, ER and mitochondria are in close proximity in the axon (Tsukita & Ishikawa, 1976; Villegas *et al.*, 2014) and they crosstalk to regulate many intracellular processes by transferring Ca^{2+} from one organelle to the other (Rizzuto *et al.*, 1998; Csordas *et al.*, 2010; Giacomello *et al.*, 2010; Marchi *et al.*, 2014). However, an overload of Ca^{2+} in the mitochondria can lead to the opening of the so called mitochondrial permeability transition pore (mPTP), resulting in further release of Ca^{2+} into the cytosol. The role of mitochondria in intracellular Ca^{2+} buffering will be discussed in more detail in the introductory section of chapter 4.

3.1.4 The role of Ca^{2+} in Wallerian degeneration

A role for intra-axonal Ca^{2+} increase in Wallerian degeneration has long been known (Schlaepfer, 1977; Schlaepfer & Hasler, 1979; LoPachin *et al.*, 1990; George *et al.*, 1995). Injury triggers two distinct Ca^{2+} increase events: **i)** an immediate Ca^{2+} elevation is observed close to the cut site without further progression, and leading to the so called acute axon degeneration (AAD) of a short axonal stump (Kerschensteiner *et al.*, 2005; Wang *et al.*, 2012); **ii)** a marked increase of Ca^{2+} occurs hours after axotomy which spreads all along the distal axonal stump (Adalbert *et al.*, 2012). It is still debated whether AAD shares common mechanistic features with Wallerian degeneration of the distal axon. Both Ca^{2+} channels and extracellular Ca^{2+} influx from the cut membrane have been implied in AAD (Knoferle *et al.*, 2010; Adalbert *et al.*, 2012; Wang *et al.*, 2012). A study in 2005 showed that WLD^S is also protective against AAD (Kerschensteiner *et al.*, 2005). Another study in *Drosophila* suggests that WLD^S could protect against Wallerian degeneration by attenuating this early Ca^{2+} rise (Avery *et al.*, 2012). However, this is not reproducible in mammalian primary cultures as WLD^S is unable to prevent the first Ca^{2+} wave in mouse SCG (Adalbert *et al.*, 2012). Of note, WLD^S-sensitive axon degeneration can be triggered without injury (e.g. selective NMNAT2 depletion or genetic or toxic disruption of axonal transport) (Conforti *et al.*, 2009; Gilley & Coleman, 2010) (Hung *et al.*; unpublished); hence an influx of Ca^{2+} from the cut end is not required to induce a Wallerian-like degeneration process.

Nevertheless, the subsequent intra-axonal Ca^{2+} rise in the distal stump is still central for Wallerian degeneration. Extracellular Ca^{2+} influx through ion-specific channels and its release from intra-axonal organelles both contribute to this delayed Ca^{2+} rise (George *et al.*, 1995; Zhai *et al.*, 2003; Barrientos *et al.*, 2011; Gerdtz *et al.*, 2011; Yang *et al.*, 2013; Villegas *et al.*, 2014). Ca^{2+} elevation activates a family of proteases known as calpains, ultimately responsible for axonal cytoskeleton degradation (Ma, 2013). All three neurofilament isoforms (NEFL, NEFM, NEFH) and microtubules-associated components are calpain substrates (Kamakura *et al.*, 1983; Kamakura *et al.*, 1985; Schlaepfer *et al.*, 1985; Billger *et al.*, 1988; Johnson *et al.*, 1991). Many studies show that calpain inhibition delays Wallerian degeneration (George *et al.*, 1995; Finn *et al.*, 2000;

Wang *et al.*, 2000; Zhai *et al.*, 2003; Ma, 2013; Ma *et al.*, 2013; Yang *et al.*, 2013). Under physiological Ca^{2+} concentration, calpains are kept in an inactive state by the endogenous inhibitor calpastatin. However, a marked and prolonged increase in Ca^{2+} levels causes the degradation of calpastatin mediated by calpains themselves, which are then “free” to initiate fragmentation. The calpastatin/calpain system also plays a role in models of axon degeneration caused by trophic factor deprivation (Yang *et al.*, 2013), placing Ca^{2+} and calpains as a convergent step for different toxic insults (Ma, 2013). Intriguingly, Ca^{2+} chelation is more effective than calpain inhibition (Zhai *et al.*, 2003), suggesting that additional Ca^{2+} -dependent events could be taking place.

The delayed Ca^{2+} rise is considered a late event in Wallerian degeneration, likely responsible for the fragmentation of the axon during the “execution phase” (Wang *et al.*, 2012). In support of this, WLD^S suppresses the secondary intracellular Ca^{2+} rise after axotomy and exogenous addition of Ca^{2+} is sufficient to abolish WLD^S-mediated protection, placing it downstream of the WLD^S regulated step (Glass *et al.*, 1994; Adalbert *et al.*, 2012). In addition, the Ca^{2+} -channel blocker nifedipine protects when added hours after axotomy (Gerdtz *et al.*, 2011) and inhibition of calpains delays cytoskeletal fragmentation but not the appearance of neuritic swellings (Zhai *et al.*, 2003; Gerdtz *et al.*, 2011; Yang *et al.*, 2013), indicating them as late factors. However, recent studies suggest instead that Ca^{2+} rise may occur early after injury (Avery *et al.*, 2012), possibly acting upstream of SARM1 (Summers *et al.*, 2014). Therefore, there are still some open questions on the role of Ca^{2+} in Wallerian degeneration. Specifically, a precise temporal correlation between Ca^{2+} rise and axonal fragmentation is still lacking and the molecular player that promotes intra-axonal Ca^{2+} increase is yet unidentified (Fig. 1.7).

3.1.5 Zebrafish as a model to study Wallerian degeneration *in vivo*

The zebrafish (*Danio rerio*) is a model organism widely used to test specific questions *in vivo* and offers many advantages over other models. First, zebrafish are vertebrate and share a high degree of genetic similarity to mammals, including humans. It is easy to introduce genetic modifications (either transiently or permanently) and express them in specific tissues using the appropriate promoters. The embryos are transparent meaning that they can be

imaged under a microscope. As they live in an aqueous environment, they can absorb pharmacological treatments directly added to the water. Finally, they produce a high number of offspring that develop at a fast rate (Dooley & Zon, 2000; Xi *et al.*, 2011). Specific to the field of Wallerian degeneration, in 2009 O'Brien, Sagasti and colleagues developed a method to lesion zebrafish trigeminal and Rohon-beard sensory axons by using two-photon laser axotomy (O'Brien *et al.*, 2009). Using this approach, they later showed that WLD^S also robustly protects against Wallerian degeneration in this model (Martin *et al.*, 2010).

Zebrafish are now also used to model neurodegenerative disorders. Despite clear differences between the zebrafish and human nervous system, the overall organization is maintained (Panula *et al.*, 2010). Zebrafish has been used to model neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and Huntington's disease (Xi *et al.*, 2011). For instance, knockdown of parkin in zebrafish, a protein known to be involved in the development of Parkinson's disease, causes dopaminergic neuronal cell loss and reduction in mitochondrial complex 1 activity (Flinn *et al.*, 2009). Such a striking effect has not been reproduced in another study, which instead shows a role for parkin in reducing apoptosis induced by proteotoxic stress (Fett *et al.*, 2010). Impairment of dopaminergic neuron development and motor function is also reported following PTEN-induced putative kinase 1 (PINK1) knockdown (Xi *et al.*, 2010). In addition, PINK1 deficient zebrafish are more vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) toxicity (Sallinen *et al.*, 2010). Finally, MPTP and rotenone treatment in zebrafish cause loss of dopaminergic neurons, locomotor defects and a decline in dopamine levels, which is consistent with findings in humans (Bretaud *et al.*, 2004; Lam *et al.*, 2005; McKinley *et al.*, 2005; Wen *et al.*, 2008; Sallinen *et al.*, 2009; Wang *et al.*, 2017).

Thus, zebrafish constitute a potentially good alternative model to study molecular mechanisms leading to neurodegeneration which could be increasingly adopted for high-throughput drug screening in the near future. Related to my work, it is a valuable model to study *in vivo* the relative timing of Ca²⁺ changes and axonal fragmentation after injury.

3.2 Hypothesis and aims of this chapter

Based on the evidence discussed above, the aim of this chapter is to elucidate some of the contradictions present in the field regarding the role of Ca^{2+} in Wallerian degeneration and to determine a chronological sequence of events leading to axon death (Fig. 1.7). Taking advantage of our model of NMN-induced axon degeneration, I will test the hypothesis that NMN and SARM1 cooperate in a unique axon degeneration pathway terminating with a rise in intracellular Ca^{2+} . The aims of this chapter are as follows:

- To determine whether Ca^{2+} increases late after injury both *in vitro* and *in vivo*.
- To test that NMN accumulation is the signal required to promote this intra-axonal Ca^{2+} rise.
- To determine whether NMN and SARM1 cooperate upstream of Ca^{2+} increase.
- To assess whether extracellular influx and intracellular release of Ca^{2+} both contribute to NMN-induced Wallerian degeneration.

3.3 Results

These data are reported in a manuscript produced as a result of this work; (Loreto et al., 2015) Wallerian degeneration is executed by an NMN-SARM1-dependent late Ca^{2+} Influx but only modestly influenced by mitochondria. Cell reports, 13, 2539-2552.

3.3.1 Pharmacological and genetic data indicate that NMN induces a rise in intra-axonal Ca^{2+} rapidly followed by axon fragmentation

After the identification by our research group of a key role for NAD-precursor NMN in promoting axon degeneration after injury (Di Stefano et al., 2015), an important next step is to determine the mechanisms by which NMN could lead to axon fragmentation and how this relates to other regulators of Wallerian degeneration. To address events downstream of the NAD-precursor NMN, I used SCG primary neuronal cultures, a widely used *in vitro* model in the field and in our laboratory. SCG neurons can either be cultured as explants or they can be dissociated to single neurons. In both conditions, neurons extend long axons (see Materials and Methods for additional details on this *in vitro* model). I first addressed two questions: **i)** Is Ca^{2+} increase an early or a late event in injury-induced axon degeneration? **ii)** Is NMN accumulation necessary and sufficient to evoke changes in intra-axonal Ca^{2+} concentration? To test this, SCG neurons were microinjected with plasmids encoding the Ca^{2+} indicator GCaMP5 and the fluorescent marker DsRed2. GCaMPs are a class of genetically encoded Ca^{2+} sensors which consist of a circularly permuted enhanced green fluorescent protein (EGFP) linked to the Ca^{2+} -binding protein calmodulin and the calmodulin-binding peptide M13. When Ca^{2+} is present at sufficiently high concentrations, calmodulin-M13 interactions induce changes in the fluorophore environment that result in increased emitted fluorescence (Nakai et al., 2001; Tian et al., 2009). DsRed2 is a red fluorescent protein which spreads homogeneously in the cytoplasm and allows the detection of morphological changes. Axons were separated from the cell bodies the day after microinjection, to allow sufficient time for transcription and translation to occur. Changes in GCaMP5 fluorescence signal in distal axonal stumps were measured and normalised to their corresponding uncut controls, which remained relatively stable for the entire duration of the experiment in all the conditions tested in this experimental chapter (Fig. 3.1B-G).

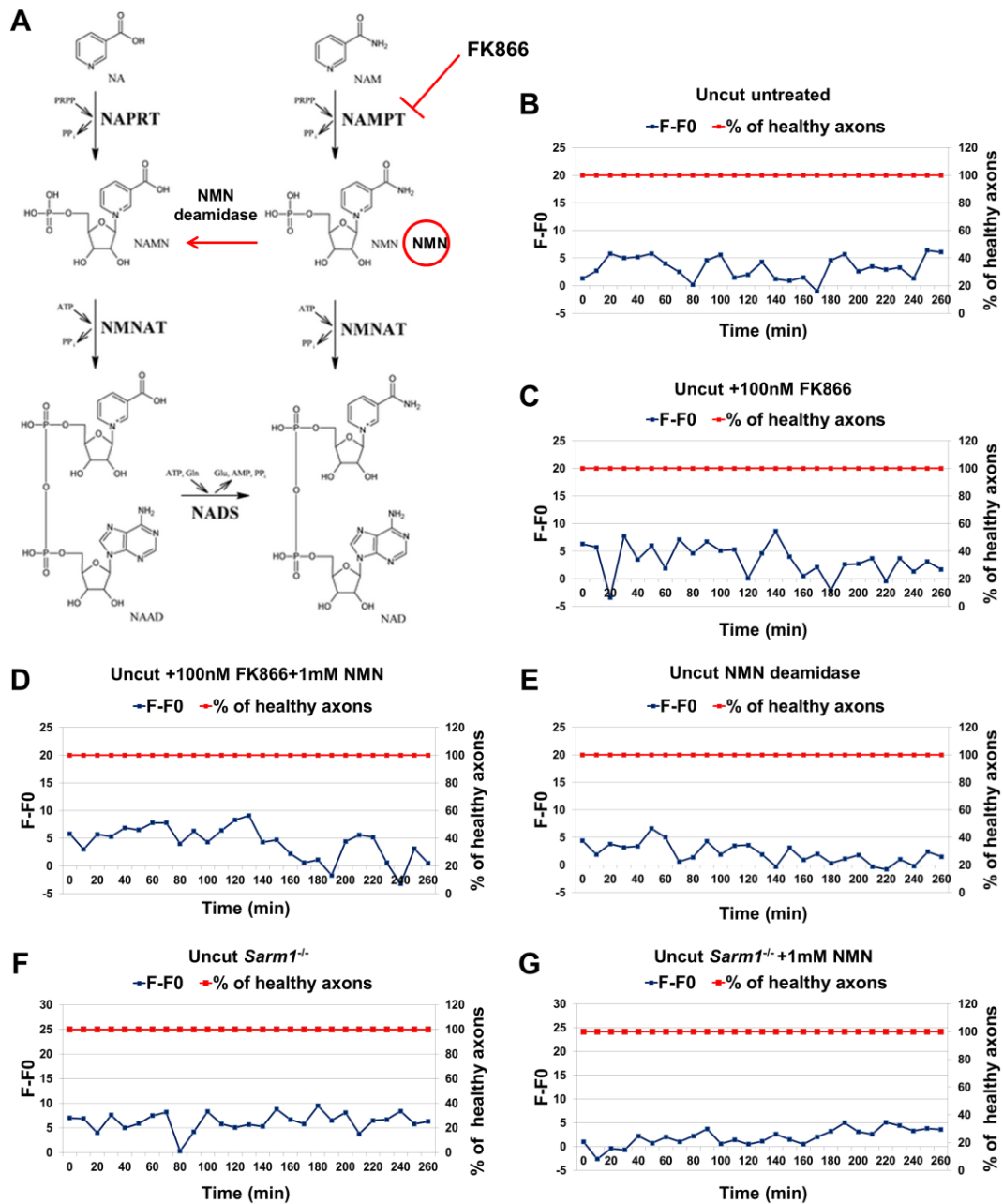


Figure 3.1 NAD-biosynthetic pathway and Ca^{2+} signals in wild-type, NMN deamidase expressing and *Sarm1*^{-/-} uncut axons.

(A) The salvage pathway of NAD biosynthesis from nicotinamide. NMN-synthesising enzyme NAMPT is the target of FK866 potent and specific inhibition as shown. The reaction catalysed by bacterial NMN deamidase is also shown. **(B-D)** Representative traces showing Ca^{2+} baseline signal in uncut wild-type SCG axons treated as indicated. **(E)** Representative traces showing Ca^{2+} baseline signal in uncut wild-type SCG axons expressing NMN deamidase. **(F-G)** Representative traces showing Ca^{2+} baseline signal in uncut *Sarm1*^{-/-} SCG axons expressing treated as indicated.

Consistent with previous studies ([Adalbert *et al.*, 2012](#)), I found a marked increase in Ca^{2+} levels in distal injured axons starting 2-3 hours after axotomy (Fig. 3.2A and B; Movie S1). This Ca^{2+} rise shortly preceded the appearance of morphological damage and axon integrity was lost 10 to 25 minutes after Ca^{2+} started to increase (Fig. 3.2A and C). To test whether this Ca^{2+} rise was induced by NMN accumulation, I treated axons with FK866 to inhibit the NMN-synthesizing enzyme NAMPT (Fig. 3.1A). We have previously shown that FK866 blocks NMN accumulation in injured sciatic nerves ([Di Stefano *et al.*, 2015](#)). FK866 completely abolished the Ca^{2+} rise and, consistent with our previous report ([Di Stefano *et al.*, 2015](#)), preserved axonal integrity for the entire duration of the experiment (Fig. 3.2A-C; Movie S1). Co-administration of exogenous NMN fully reverted FK866 protection, restoring Ca^{2+} rise and axonal fragmentation (Fig. 3.2A-C; Movie S1).

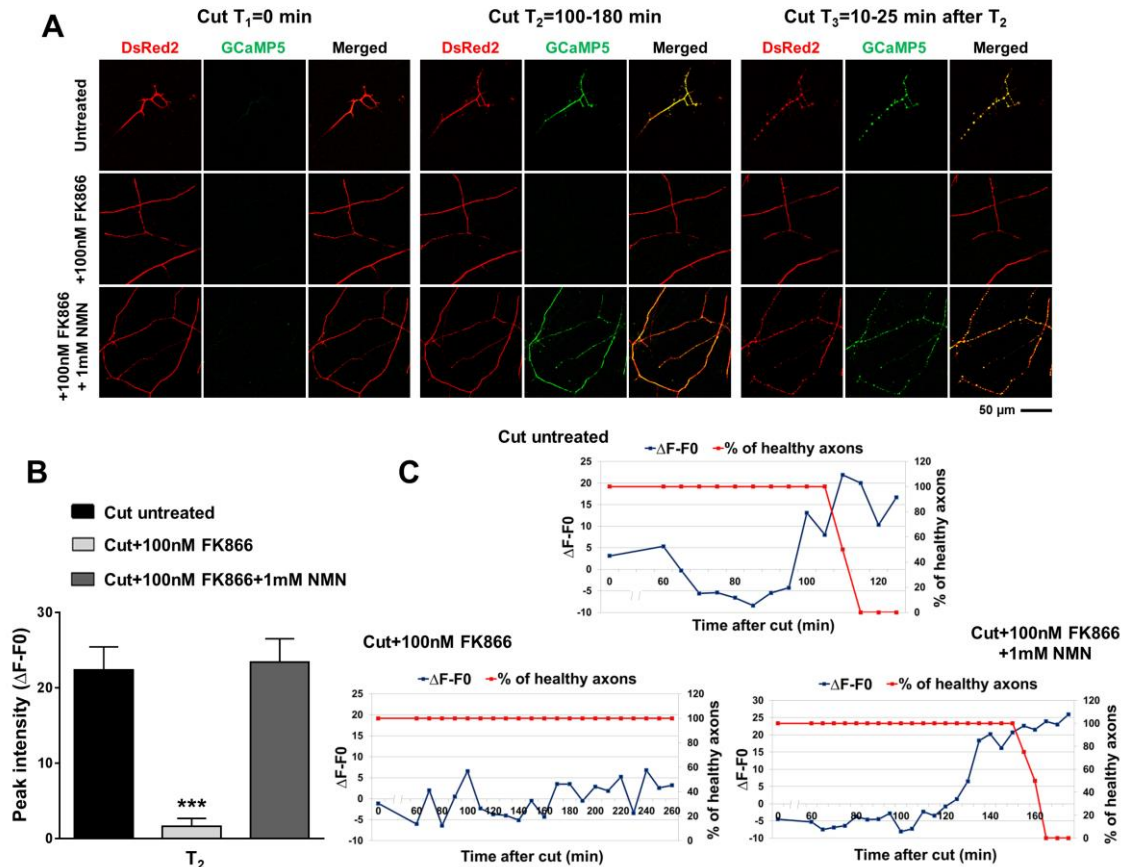


Figure 3.2 NMN induces a rise in intra-axonal Ca^{2+} rapidly followed by axon fragmentation.

(A) Plasmids encoding for the Ca^{2+} indicator GCaMP5 and for the axonal marker DsRed2 were microinjected into wild-type SCG nuclei; axons were cut 1 day after microinjection and left untreated or treated as shown with FK866 and NMN, added immediately after cut. Representative time-lapse fluorescent images acquired every 5 minutes up to 260 minutes. **(B)** Quantification of Ca^{2+} peak intensities (see Materials and Methods). All measurements were normalised to uncut controls (Mean $\pm$ SEM; $n=6$, 2 fields in 3 independent experiments; one-way ANOVA followed by Bonferroni post-hoc test; ***, $p < 0.001$). **(C)** Representative traces showing Ca^{2+} responses in axon fragments distal to the site of injury over time, in correlation with the percentage of healthy axons.

In our previous reports, we showed that scavenging NMN by expressing the bacterial enzyme NMN deamidase (Galeazzi *et al.*, 2011) significantly delays axon degeneration after injury (Di Stefano *et al.*, 2015). I therefore tested whether this genetic intervention to deplete NMN could also block intra-axonal Ca^{2+} rise and found that expressing NMN deamidase completely abolished this

increase and preserved axonal integrity for the entire duration of the experiment (Fig. 3.3A-C; Movie S1).

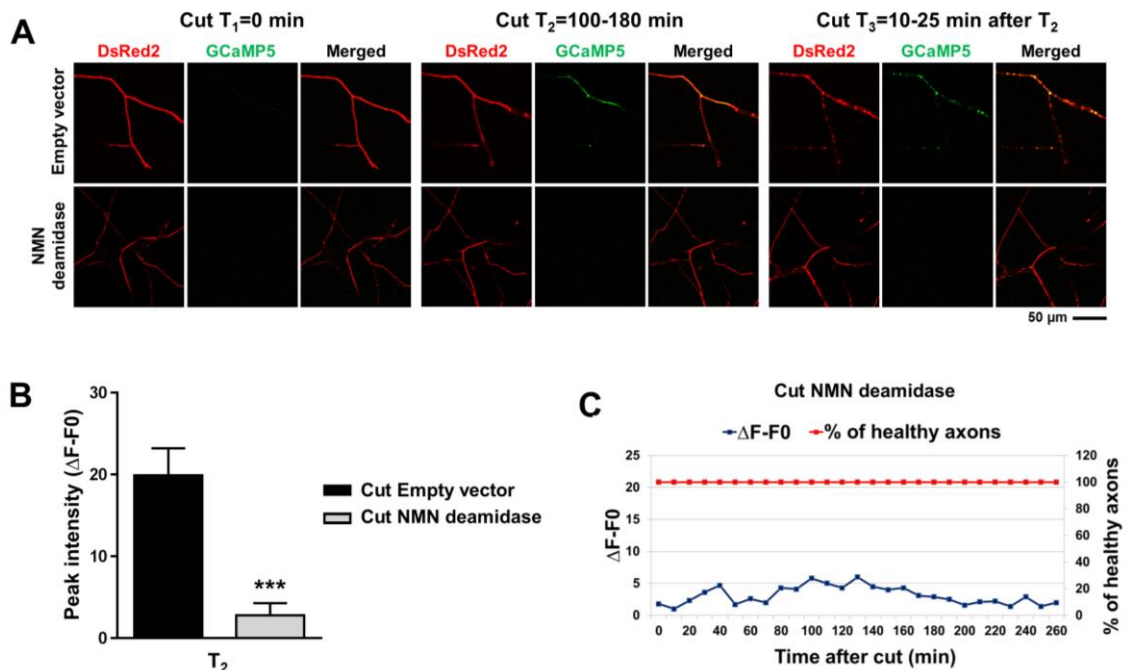


Figure 3.3 Scavenging NMN with the bacterial enzyme NMN deamidase blocks Ca^{2+} increase and delays axon degeneration.

(A) Representative fluorescent images of wild-type SCG axons expressing GCaMP5 and DsRed2 and microinjected with plasmids encoding either NMN deamidase or Empty vector, at the indicated time points after transection. **(B)** Quantification of Ca^{2+} peak intensities. All measurements were normalised to uncut controls (Mean $\pm$ SEM; $n=6$, 2 fields in 3 independent experiments; one-way ANOVA followed by Bonferroni post-hoc test; ***, $p < 0.001$). **(C)** Representative traces showing Ca^{2+} responses in axon fragments expressing NMN deamidase distal to the site of injury over time, in correlation with the percentage of healthy axons.

Taken together, these data demonstrate that injury-induced Ca^{2+} increase is a late step in the degeneration program, shortly preceding axonal fragmentation. NMN accumulation evokes this late intra-axonal Ca^{2+} rise, which can be blocked by NAMPT inhibitor FK866 or by genetically scavenging NMN.

3.3.2 NMN requires SARM1 to induce degeneration and intra-axonal Ca^{2+} increase

Of note, NMN addition to uncut axons is insufficient to directly promote an intra-axonal Ca^{2+} elevation (Fig. 3.1D). We therefore hypothesized that NMN required a downstream effector to stimulate Ca^{2+} increase. A recent report shows that injured *Sarm1*^{-/-} nerves have high NMN levels but do not degenerate (Gilley *et al.*, 2015). It is therefore likely that SARM1 is required for NMN to induce axon death. I tested this possibility by adding NMN to transected *Sarm1*^{-/-} axons and found that they remained intact for at least 4 days, even in the presence of high concentrations of exogenous NMN (Fig. 3.4A and B). In contrast, FK866-protected wild-type axons degenerate as soon as 3 hours when exposed to greatly lower concentrations of NMN (Di Stefano *et al.*, 2015). Thus, NMN requires SARM1 to induce axon degeneration after an injury.

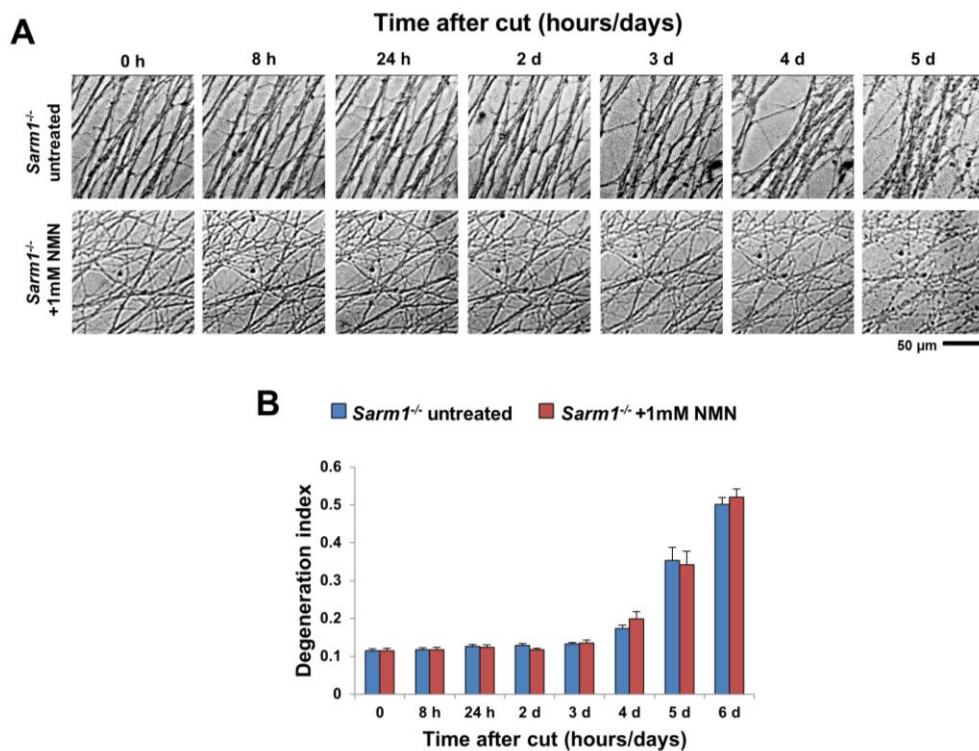


Figure 3.4 NMN requires SARM1 to induce axon degeneration after injury.

(A) Representative phase contrast images of *Sarm1*^{-/-} SCG axons left untreated or treated with NMN as shown, at the indicated time points after cut. **(B)** Degeneration index was calculated from 3 fields per sample in 3 independent experiments (Mean $\pm$ SEM; n=3; two-way ANOVA followed by Bonferroni post-hoc test).

I then asked whether SARM1 is also required for the increase in intra-axonal Ca^{2+} after cut. This is important in view of a recent report that SARM1 acts downstream of Ca^{2+} (Summers *et al.*, 2014). However, in our experimental conditions, I did not detect any increase in Ca^{2+} signals in the distal stumps of *Sarm1*^{-/-} axons after injury (Fig. 3.5A and B; Movie S2), which remained similar to the uninjured axons (Fig. 3.1F). In parallel, axonal integrity was preserved for the entire duration of the experiment (Fig. 3.5A and C). Importantly, in the absence of SARM1, NMN administration failed to induce Ca^{2+} rise in injured axons (Fig. 3.5A-C; Movie S2).

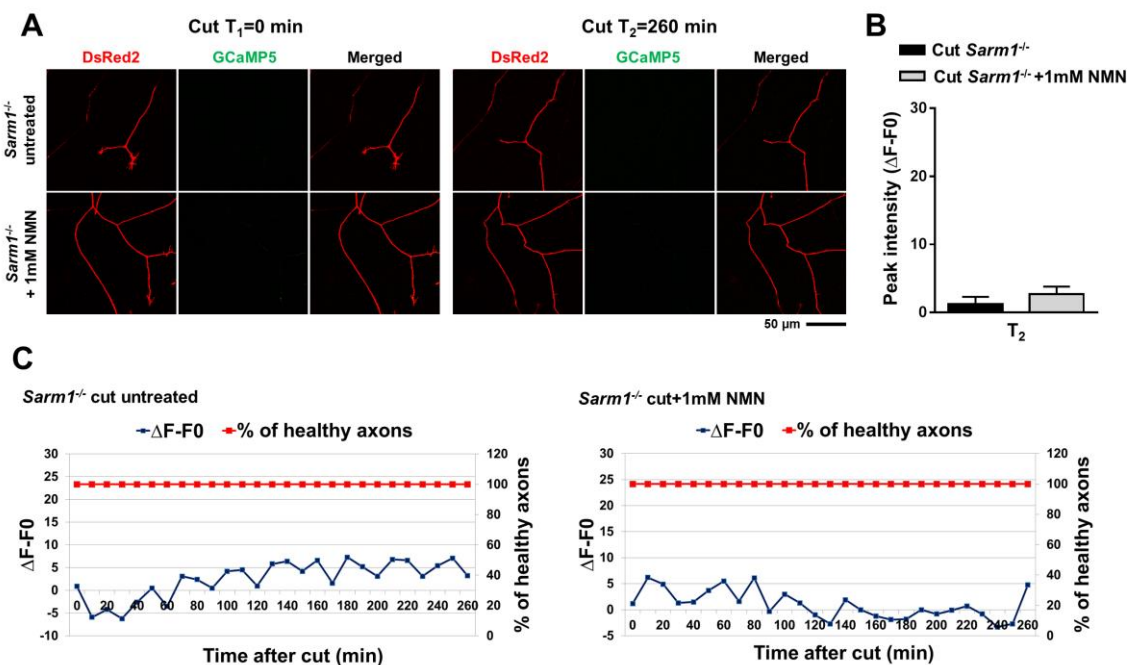


Figure 3.5 NMN requires SARM1 to induce Ca^{2+} increase after injury.

(A) Representative fluorescent images of *Sarm1*^{-/-} SCG transected axons expressing GCaMP5 and DsRed2, untreated or treated with NMN. **(B)** Quantification of Ca^{2+} peak intensities. All measurements were normalised to uncut controls (Mean $\pm$ SEM; n=6, 2 fields in 3 independent experiments; one-way ANOVA followed by Bonferroni post-hoc test). **(C)** Representative traces showing Ca^{2+} responses in *Sarm1*^{-/-} axons fragments distal to the site of injury over time left untreated or treated with NMN as shown, in correlation with the percentage of healthy axons.

As a control, I treated axons with the Ca^{2+} ionophore ionomycin at the end of each experiment. In all the experimental conditions where the rise in intra-axonal Ca^{2+} after injury was blocked, ionomycin addition still induced a marked Ca^{2+} elevation (Fig. 3.6A). This acted as a positive control to show that a rise of intra-axonal Ca^{2+} can be detected in these axons by the Ca^{2+} sensor.

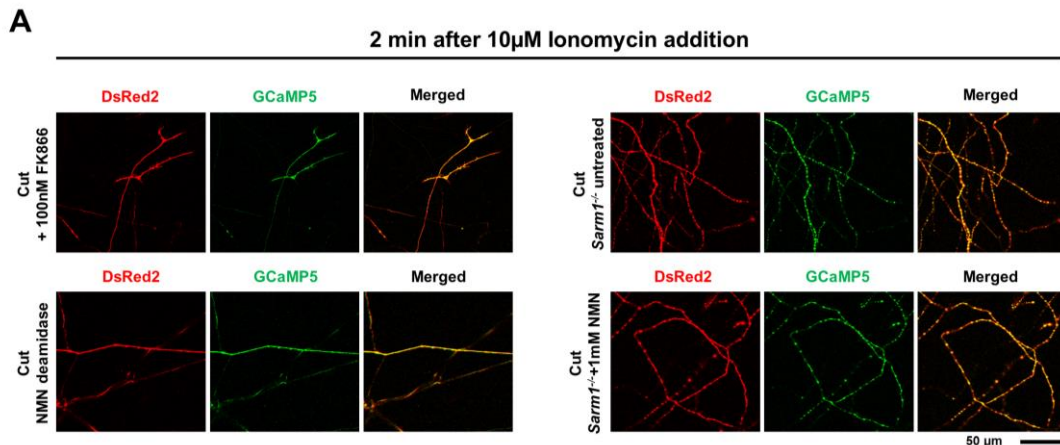


Figure 3.6 Ca^{2+} elevation in response to ionomycin treatment in FK866 treated, NMN deamidase expressing and *Sarm1*^{-/-} axons.

(A) Representative fluorescent images of wild-type and *Sarm1*^{-/-} SCG transected axons expressing GCaMP5 and DsRed2 treated as shown, 2 minutes after ionomycin addition.

These results indicate that NMN requires the presence of SARM1 to induce Wallerian degeneration and Ca^{2+} increase represents a common downstream step.

3.3.3 A rapid Ca^{2+} increase near the injury site is not abolished in *Sarm1*^{-/-}, NMN deamidase or FK866-treated axons

As previously mentioned, an increase in intra-axonal Ca^{2+} in close proximity to the cut rapidly follows the transection injury (Adalbert *et al.*, 2012). This immediate and localised Ca^{2+} rise leads to rapid degeneration of a short axonal segment in proximity to the site of cut, an event known as AAD (Kerschensteiner *et al.*, 2005). It is still debated whether this rapid degeneration event is simply the result of a Ca^{2+} influx from the cut site or if it follows a regulated mechanism, sharing features with Wallerian degeneration of the distal

stump. To address this in more detail, I tested whether the early Ca^{2+} rise and the following AAD was influenced in a number of conditions that protect against Wallerian degeneration. I found an early rise in Ca^{2+} levels in *Sarm1*^{-/-} axons near the cut site, followed by degeneration of short axonal segments. This was also observed in wild-type controls untreated or treated with FK866 or with FK866+NMN and in NMN deamidase expressing axons (Fig. 3.7A and B). These results suggests that *Sarm1*^{-/-} and NMN deamidase axons, as well as axons treated with FK866, retain the capability to degenerate in response to Ca^{2+} , but that this early, proximal Ca^{2+} rise is distinct from the delayed increase in the distal stump that occurs just before fragmentation (Fig. 3.2).

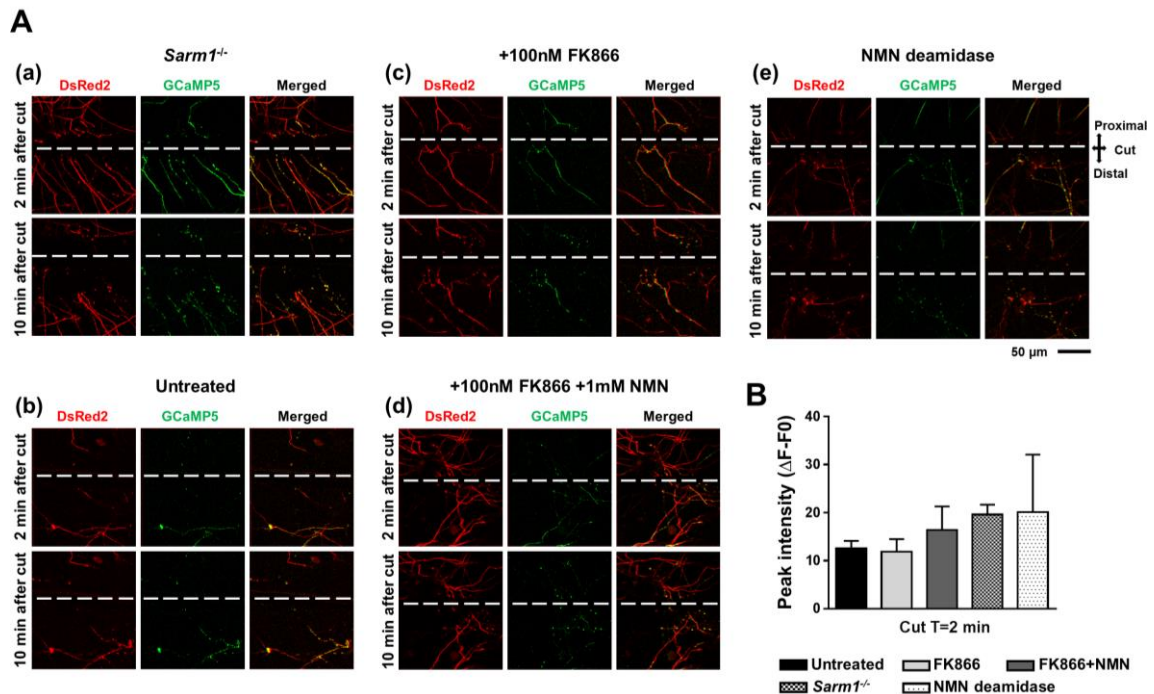


Figure 3.7 A rapid Ca^{2+} increase near the injury site is not abolished in *Sarm1*^{-/-}, NMN deamidase or FK866-treated axons.

(A) Representative fluorescent images showing a marked increase in Ca^{2+} levels at the site of cut at the indicated time points after axotomy in *Sarm1*^{-/-} (a), wild-type untreated (b), FK866-treated (c), FK866+NMN-treated (d) and NMN deamidase expressing (e) axons. The indicated treatments were added 30 minutes before axotomy. The increase in Ca^{2+} occurring within minutes after axotomy is rapidly followed by degeneration and retraction of short axonal segments adjacent the injury site (dotted white line). **(B)** Quantification of Ca^{2+} peak intensities adjacent to the cut site (distal area). All measurements were normalised to uncut controls. (Mean $\pm$ SEM; n=2-6, 1-2 fields in 2-3 independent experiments; one-way ANOVA followed by Bonferroni post-hoc test).

3.3.4 Inhibiting NMN synthesis with FK866 delays injury-induced intra-axonal Ca^{2+} increase *in vivo*

Next, I assessed the relationship between NMN and intra-axonal Ca^{2+} *in vivo*. To this purpose, I used zebrafish larvae 48–54 hours post fertilization (hpf), transiently expressing DsRed along with the Ca^{2+} sensor GCaMP-HS in trigeminal and Rohon–Beard somatosensory neuron (see Materials and Methods). These were treated with FK866 added in the water for 6 hours prior to two-photon laser axotomy (Fig. 3.8A and B). As we previously reported, FK866 strongly delays axon fragmentation in this model organism (Fig. 3.8B (m-o) and C) (Di Stefano *et al.*, 2015). Consistent with our *in vitro* observations, we detected a marked Ca^{2+} increase within axons distal to the site of injury (Fig. 3.8B (e-h) and D). The first signs of axon damage appeared as soon as 4 minutes after Ca^{2+} rise (Fig. 3.8B (a-d) and E; Movie S3). Remarkably, FK866 delayed this increase in intra-axonal Ca^{2+} (Fig. 3.8B (p-r), D and E; Movie S4) and the subsequent axon fragmentation (Fig. 3.8B (m-o), C and E).

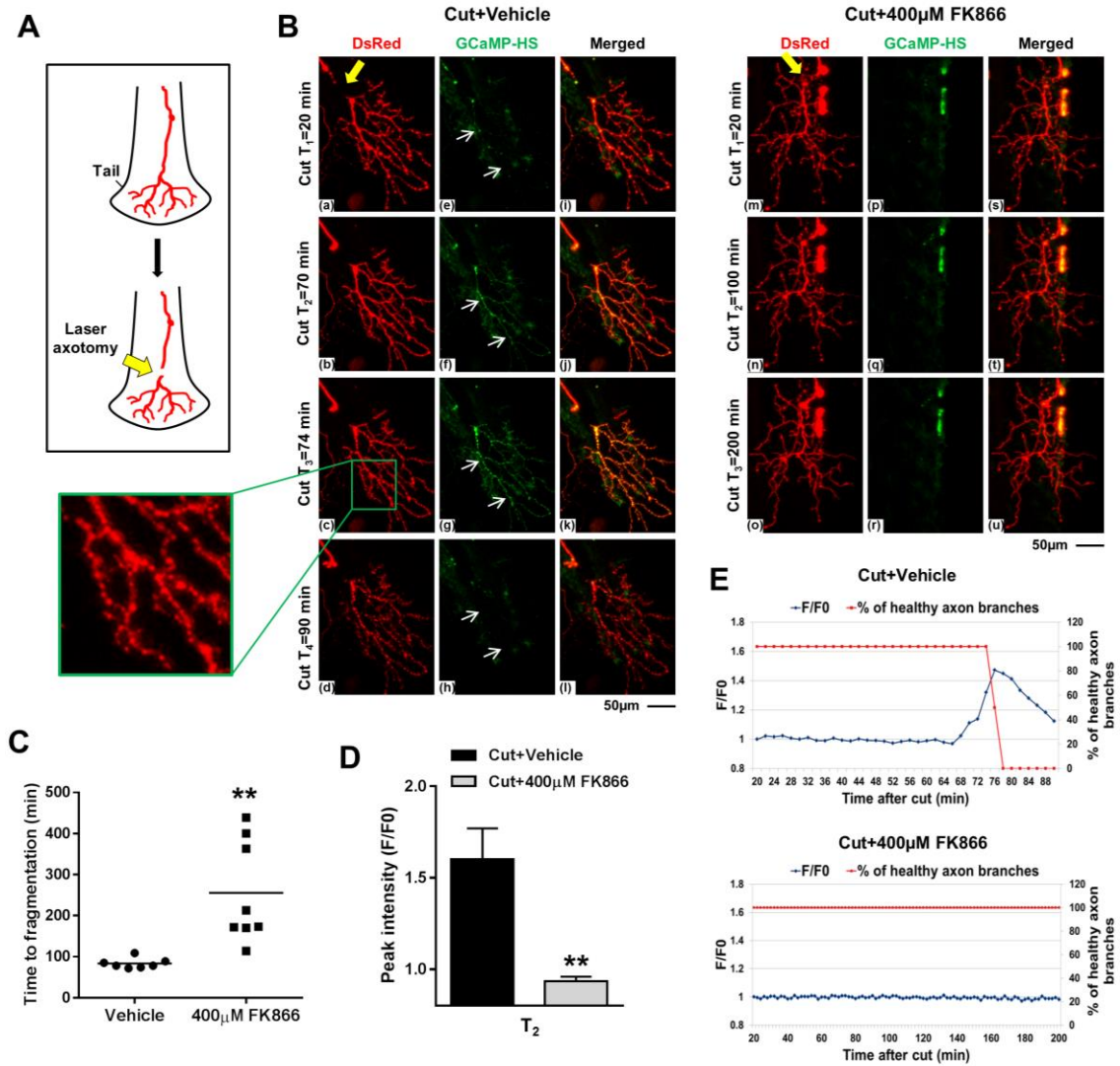


Figure 3.8 NMN-synthesis inhibitor FK866 delays injury-induced intra-axonal Ca^{2+} increase *in vivo*.

(A) Schematic representation of larval zebrafish tail indicating the position of the sensory (Rohon-Beard) axons and the site of axotomy. **(B)** Representative time-lapse fluorescent images of axons expressing DsRed and GCaMP-HS, treated with vehicle or FK866 as shown and then transected. The yellow arrow indicates the cut site. White arrows (e-h) show a clear Ca^{2+} increase immediately preceding the appearance of morphological damage. The enlargement represents the boxed area in panel (c). **(C)** Time to beginning of fragmentation following laser axotomy; each point represents one experiment; horizontal bar denotes average degeneration time (Vehicle $n=7$, FK866 $n=8$; Student's t test; **, $p<0.01$). **(D)** Quantification of Ca^{2+} peak intensities. F0 (first time point imaged) was set to 1 (Mean $\pm$ SEM; $n=7-8$; Student's t test; **, $p<0.01$). **(E)** Representative traces showing Ca^{2+} responses distal to the injury site over time in correlation with the percentage of healthy axonal branches.

Thus, NMN synthesis inhibitor FK866 blocks the increase in Ca^{2+} that immediately precedes axonal fragmentation *in vivo* in a vertebrate model organism.

3.3.5 The extracellular environment is the main source of NMN-induced intra-axonal Ca^{2+} rise

As mentioned in the introductory section of this chapter, neurons regulate intra-axonal/cytosolic Ca^{2+} levels by a complex regulation between extracellular Ca^{2+} influx and its storage in intracellular organelles with Ca^{2+} buffering activity. Since previously both extracellular influx of Ca^{2+} and its release from intracellular stores have been implicated in Wallerian degeneration (Zhai *et al.*, 2003; Barrientos *et al.*, 2011; Gerdts *et al.*, 2011; Wang *et al.*, 2012; Villegas *et al.*, 2014), I tried to identify the primary source of the Ca^{2+} rise induced by NMN. To this purpose, SCG explant axons were cut and immediately treated with FK866. In order to exclude intracellular conversion of NMN to NAD mediated by any residual axonal NMNAT2, I added NMN to FK866-protected axons 12 hours after cutting (Fig. 3.9A), a time when little or no NMNAT2 remains (Gilley & Coleman, 2010). In these conditions, NMN induced complete degeneration of FK866-protected axons within 3 hours of its addition (Fig. 3.9B (e-l) and C) (Di Stefano *et al.*, 2015). I used EGTA to chelate extracellular Ca^{2+} and saw a remarkable delay of axon degeneration, even after NMN addition (Fig. 3.9B (m-p) and C). Similar results were obtained with pharmacological inhibition of extracellular Ca^{2+} influx by targeting L-type Ca^{2+} channels present on the plasma membrane with verapamil (Fig. 3.9B (q-t) and C) and nifedipine (Fig. 3.10A (m-p) and B). In addition, KB-R7943, an inhibitor of the reverse $\text{Na}^+/\text{Ca}^{2+}$ exchanger present on the plasma membrane, also significantly delayed NMN-induced degeneration, although its effect was weaker (Fig. 3.9B (u-x) and C).

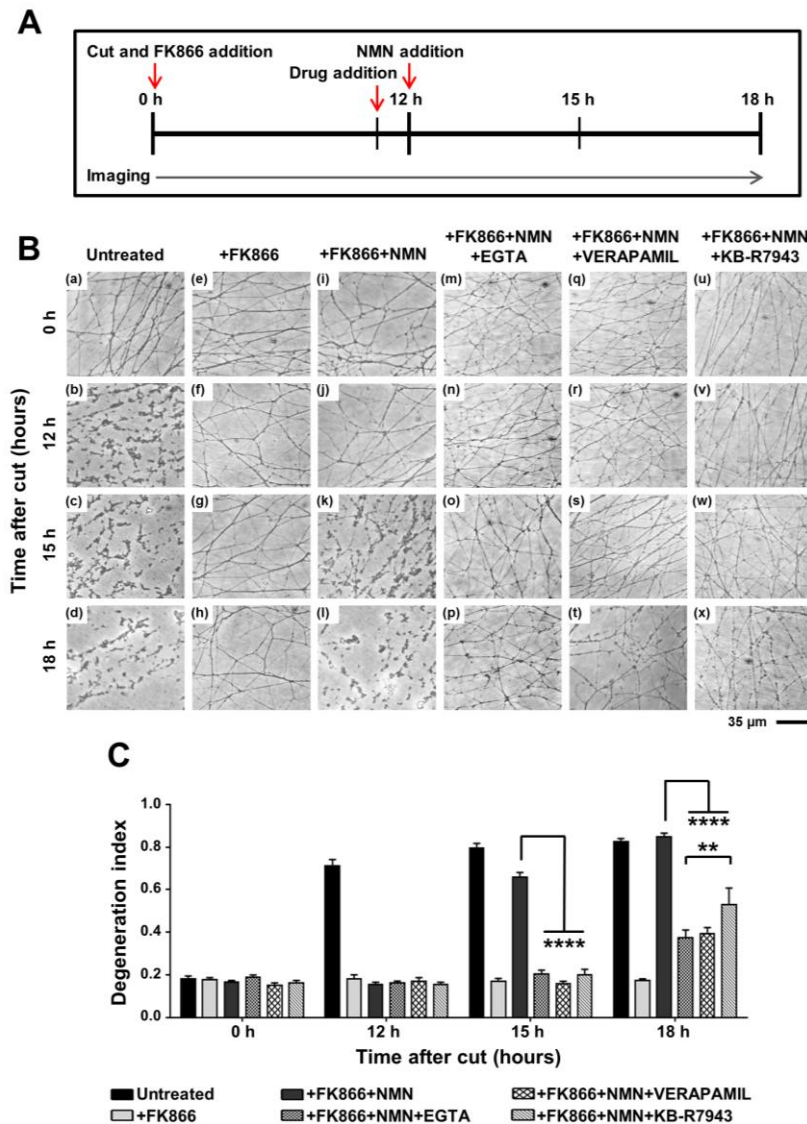


Figure 3.9 Blocking extracellular Ca^{2+} influx delays NMN-induced axon degeneration.

(A) Schematic representation of the experimental design. Wild-type SCG axons were cut and left untreated or treated with FK866 immediately after cut. NMN was added 12 hours after cut and caused degeneration within 3 hours. EGTA, verpamil and KB-R7943 were added 30 minutes before NMN administration. The following concentrations were used: 100nM FK866, 100 μ M NMN, 5mM EGTA, 10 μ M verpamil, 10 μ M KB-R7943. **(B)** Representative phase contrast images acquired at the indicated time points after cut. **(C)** Degeneration index was calculated from 3 fields per sample in 3 independent experiments (Mean $\pm$ SEM; n=3; two-way ANOVA followed by Bonferroni post-hoc test; ****, $p < 0.0001$; **, $p < 0.01$).

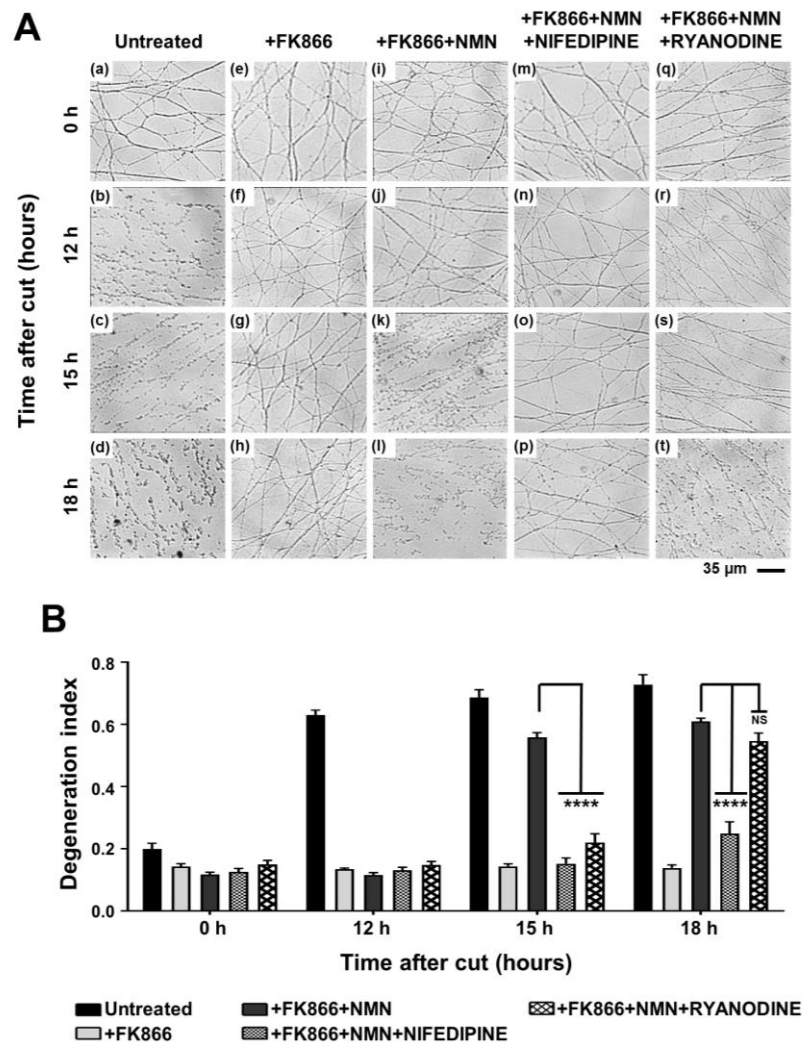


Figure 3.10 Ryanodine delays NMN-induced axon degeneration.

(A) Wild-type SCG axons were cut and then left untreated or treated with FK866 immediately after cut. NMN was added 12 hours after cut. Degeneration was completed within 3 hours after NMN addition. Nifedipine and ryanodine (panels i-p) were added 45 minutes before NMN administration. Representative phase contrast images acquired at the indicated time points after cut. **(B)** Degeneration index was calculated from 3 fields per sample in 3 independent experiments (Mean $\pm$ SEM; n=3; two-way ANOVA followed by Bonferroni post-hoc test; ****, $p < 0.0001$, **, $p < 0.01$; NS, non-significant). The following drug concentrations were used: 100nM FK866, 100 μ M NMN, 30 μ M nifedipine, 10 μ M ryanodine.

Next, I tested whether similar results were reproducible in DRG axons. Wild-type DRG axons normally degenerate slower than SCG axons after injury. In this model, FK866 was added immediately after cut and NMN was added 24

hours later (Fig. 3.11A). Similar to SCG, the degeneration was completed within 3 hours. Preliminary data showed that EGTA also delayed NMN-induced degeneration in DRG cultures (Fig. 3.11B and C), indicating that this pathway is activated in more than one neuronal type.

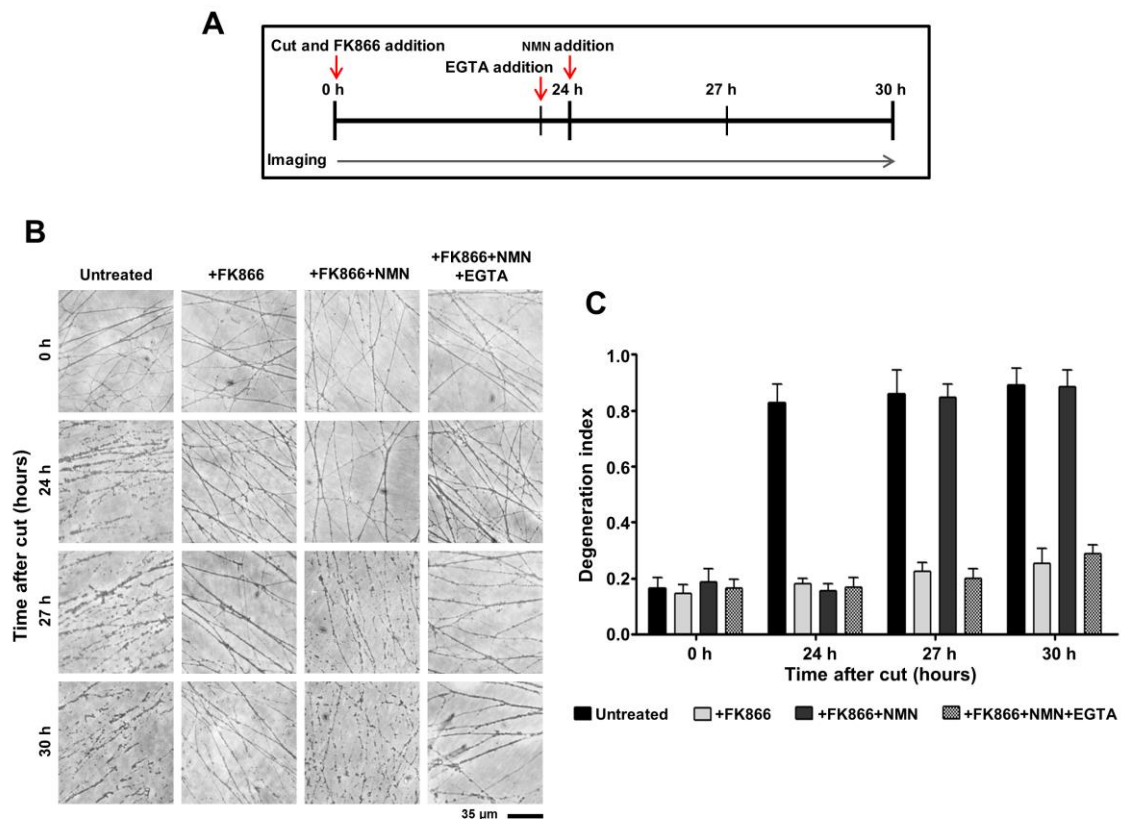


Figure 3.11 EGTA delays NMN-induced axon degeneration in cut DRG axons.

(A) Schematic drawing of the experimental design. Wild-type DRG axons were cut and then left untreated or treated with FK866 immediately after cut. NMN was added 24 hours after cut and degeneration was completed within 3 hours of NMN addition. EGTA was added 30 minutes before NMN administration. The following drug concentrations were used: 100nM FK866, 100 μM NMN, 5mM EGTA. **(B)** Representative phase contrast images of DRG axons cut and treated as shown acquired at the indicated time points. **(C)** Degeneration index was calculated from 3 fields per sample in 1 experiment (Mean $\pm$ SD).

A release of Ca^{2+} from axonal ER, leading to mPTP opening and release of intra-mitochondrial Ca^{2+} has been described to contribute to the execution phase of Wallerian degeneration (Villegas *et al.*, 2014). Therefore, I tested

whether this sequence of events is induced by NMN. I found that ryanodine receptor antagonist ryanodine significantly delayed NMN-induced degeneration, albeit its effect was weaker than that obtained with EGTA and the other Ca^{2+} -channel blockers used (Fig. 3.10A (q-t) and B).

As mentioned before, mitochondria are Ca^{2+} buffering organelles which have been proposed to release Ca^{2+} during Wallerian degeneration (Barrientos *et al.*, 2011; Avery *et al.*, 2012; Villegas *et al.*, 2014). To investigate the temporal relationship between Ca^{2+} influx and its internal release and to detect any Ca^{2+} increase in areas surrounding mitochondria, SCG neurons were microinjected with GCaMP5 and the mitochondrial marker TagRFP-mito. I then cut the axons and immediately added FK866 and NMN. As expected, NMN administration induced a diffuse intra-axonal Ca^{2+} increase in FK866-treated axons, rapidly followed by the formation of axonal swellings which started to appear as early as 10 min after Ca^{2+} rise. Interestingly, 80% of these swellings contained mitochondria (Fig. 3.12A (b and d)). EGTA addition drastically reduced NMN-induced Ca^{2+} rise (Fig. 3.12A (e) and B) and blocked axon degeneration (Fig. 3.9B (m-p) and C). However, I still observed a weak, sporadic increase in Ca^{2+} levels confined in some axonal areas where mitochondria were present (Fig. 3.12A (e) and B). This localised Ca^{2+} rise was considerably weaker than that observed following extracellular Ca^{2+} influx (Fig. 3.12B); small swellings also appeared within the same confined axonal regions; nevertheless, degeneration of the distal axon stump was delayed for many hours (Fig. 3.12A (g and h)).

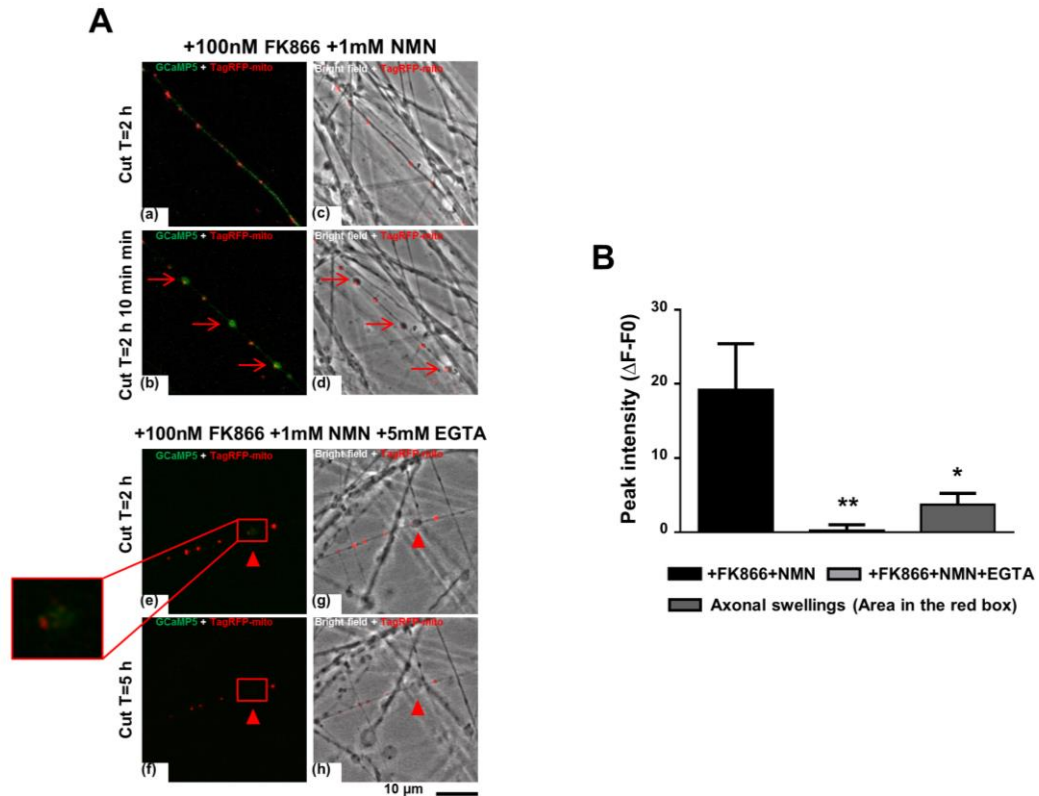


Figure 3.12 The extracellular environment is the main source of NMN-induced intra-axonal Ca^{2+} rise.

(A) Representative fluorescent and phase contrast images of wild-type SCG axons expressing GCaMP5 and TagRFP-mito. Axons were cut, treated with 100nM FK866+1mM NMN (a-d) or with 100nM FK866+1mM NMN+5mM EGTA (e-h) and imaged at the indicated time points. Arrows in (b) and (d) indicate mitochondria co-localising with axonal swellings. EGTA addition completely abolished the marked cytosolic Ca^{2+} rise induced by NMN addition. Sporadic localised increases in Ca^{2+} levels after EGTA treatment were still detectable confined in some of the axonal areas where mitochondria were present (red box and arrowheads in panels e-h). The enlargement represents the boxed area in panel (e). **(B)** Quantification of Ca^{2+} peak intensities (see Materials and Methods). All measurements were normalised to uncut controls (Mean $\pm$ SEM; n=6 axons, 3 independent experiments; one-way ANOVA followed by Bonferroni post-hoc test; *, p<0.05, **, p<0.01).

Together, these results indicate that NMN stimulates a late influx of Ca^{2+} from the extracellular environment. They suggest that a more modest Ca^{2+} release from intracellular stores also occurs, but this is not sufficient to trigger fragmentation. The role of mitochondria in this pathway will be addressed in more detail in chapter 4.

3.4 Discussion

Our proposed model of Wallerian degeneration predicts that axonal integrity is lost as a consequence of the accumulation of the pro-degenerative molecule NMN (Di Stefano *et al.*, 2015), whose levels in axons are normally limited by the NAD-biosynthetic enzyme NMNAT2 (Gilley & Coleman, 2010). In this chapter, I focused on trying to elucidate the role of Ca^{2+} in axon degeneration and more specifically its connection to regulators of Wallerian degeneration such as NMN and SARM1. To this purpose, I used pharmacological and genetic experimental approaches in *in vitro* mammalian primary neurons and in an *in vivo* vertebrate model organism. I found that NMN initiates a Ca^{2+} -mediated execution program of axon degeneration after injury, which requires the presence of the recently identified pro-axon death protein SARM1.

Intra-axonal Ca^{2+} increase after axotomy follows a biphasic pattern, with an initial rise soon after and near the site of injury being followed by a second, delayed increase along the whole axon length. However its temporal relationship with axonal fragmentation is still debated (Gerdtts *et al.*, 2011; Adalbert *et al.*, 2012; Avery *et al.*, 2012; Yang *et al.*, 2013; Villegas *et al.*, 2014). Our finding that NMN induces a late intra-axonal Ca^{2+} increase after injury, which is rapidly followed by axonal degeneration, is consistent with a model in which the late Ca^{2+} rise is the central and causative event marking the execution phase of Wallerian degeneration, and one on which several other mediators may converge. Consistent with a recent report that SARM1 is required for degeneration downstream of NMNAT2 loss (Gilley *et al.*, 2015), our data suggest that Ca^{2+} lies downstream of both NMN and SARM1 in a central, unique axon death pathway (Fig. 4.12) (Conforti *et al.*, 2014). A recent study proposed that SARM1 acts downstream of an early intra-axonal Ca^{2+} increase (Summers *et al.*, 2014). I also detect an early rise of Ca^{2+} after injury, but it only spreads across a short axonal stump in close proximity of the site of injury. Importantly, in the previous study a different method to measure Ca^{2+} has been used. However, the two Ca^{2+} sensors used (Fluo-4AM (Summers *et al.*, 2014) and GCaMP5) have a similar K_d for Ca^{2+} , therefore it is unlikely that the discrepancy between the two studies is due to a difference in the sensitivity or Ca^{2+} kinetics of the two methods (Akerboom *et al.*, 2012; Grienberger &

Konnerth, 2012). In another report, the early Ca^{2+} rise has been suggested as a causative step in Wallerian degeneration (Avery *et al.*, 2012). Our data, however, indicate that the early Ca^{2+} increase, and its associated acute degeneration, occur indistinguishably in wild-type rapidly degenerating axons as well as in *Sarm1*^{-/-}, NMN deamidase and FK866-treated axons, all conditions where Wallerian degeneration is dramatically delayed. Similarly, WLD^S does not prevent the early rise of Ca^{2+} near the cut site in mammalian cultures and in zebrafish (Adalbert *et al.*, 2012; Vargas *et al.*, 2015). Furthermore, Ca^{2+} blockers delay degeneration induced by NMN in axons injured as long as 12 hours earlier but maintained intact by FK866. Crucially, WLD^S-sensitive degeneration can be induced without injury *in vitro* or *in vivo* by NMNAT2 selective depletion and other non-injury methods (Gilley & Coleman, 2010; Hicks *et al.*, 2012; Gilley *et al.*, 2013) indicating that it does not require an influx of Ca^{2+} from the cut site to initiate it. All these observations dissociate initial Ca^{2+} influx from degeneration.

Two ways of preventing Ca^{2+} influx from the extracellular environment confer strong protection from NMN-induced axon degeneration and indicate a causative role of Ca^{2+} in inducing the degeneration: **i)** sequestering extracellular Ca^{2+} with EGTA; **ii)** blocking Ca^{2+} influx via Ca^{2+} channels. These data strongly implicate a causative role for extracellular Ca^{2+} influx in the late execution stages of Wallerian degeneration. Intriguingly, Ca^{2+} rise in zebrafish cut axons occurs surprisingly late, preceding the actual fragmentation by just a few minutes. Nevertheless, a recent report showed that chelating Ca^{2+} after axotomy also delays axonal fragmentation in zebrafish, suggesting a causative role for Ca^{2+} in this model organism too (Vargas *et al.*, 2015). However, the fact that Ca^{2+} increase only shortly precedes axonal fragmentation, as revealed by the precise time-course of Ca^{2+} dynamics after injury, suggests that the Ca^{2+} sensitive step could be at a point where morphologically protecting axons may not restore their function. Nevertheless, in a model of oxygen-glucose deprivation, Ca^{2+} chelation is effective to functionally preserve axons (Stys & Jiang, 2002). However, inhibiting Ca^{2+} -mediated activation of calpain does not maintain axonal function after injury (Ma *et al.*, 2013). Therefore, upstream

mediators of the axon death pathway may prove more useful therapeutic targets.

Extracellular Ca^{2+} influx may be due to a dysregulation of Ca^{2+} channels following a depolarization of the axonal membrane (Mishra *et al.*, 2013), which itself could be a consequence of the marked energy depletion detected in the axon after injury (Wang *et al.*, 2005). A recent study showed that Wallerian degeneration is regulated by the activation of a MAPK signalling pathway, which then leads to local energy depletion (Yang *et al.*, 2015). In addition, SARM1 action has been recently linked to a downstream effect on NAD levels (Gerdtts *et al.*, 2015). SARM1-induced NAD depletion is likely to affect energy production. Consistent with this, SARM1 deletion completely abolishes intra-axonal Ca^{2+} rise.

EGTA addition results in almost complete blockage of intraxonal- Ca^{2+} accumulation; nevertheless, NMN-induced degeneration can also be delayed by interfering with Ca^{2+} release from the ER, although to a lesser extent. We speculate that the increase of cytosolic Ca^{2+} mainly arising from the extracellular environment might stimulate a release of Ca^{2+} from intracellular stores, following a CICR mechanism. Of note, most axonal swellings forming soon after Ca^{2+} increases contain mitochondria, which could contribute to a further increase in cytosolic Ca^{2+} through the opening of mPTP. This hypothesis will be tested more directly in chapter 4. Importantly, the substantially weaker protection achieved by chelating extracellular Ca^{2+} compared to WLD^S/NMNATs implies the existence of other mechanisms. For instance, I also detected a sporadic increase in Ca^{2+} signals in areas surrounding mitochondria in axons treated with EGTA. This indicates that a release of Ca^{2+} from intracellular stores can also occur without stimulation from an influx of extracellular Ca^{2+} . Although this localised rise is insufficient to trigger degeneration in the time-scale of Wallerian degeneration, it might have a significant impact on axonal morphology at later stages. MORN4, another regulator of Wallerian degeneration, also seems to act at very late stages of the degeneration process. Knockdown of MORN4 (first identified in *Drosophila*, ortholog called retinophilin) blocks axonal fragmentation but not the appearance of axonal swellings. Interestingly, MORN4 is involved in regulation of cytoplasmic Ca^{2+} levels preceding

phagocytosis (Cuttell *et al.*, 2008; Bhattacharya *et al.*, 2012). Similarly, calpain inhibition confers a weaker protection compared to Ca²⁺ chelation and blocks fragmentation but not the appearance of axonal swellings (Zhai *et al.*, 2003; Gerdts *et al.*, 2011).

In summary, these data identify crucial steps of an axon death pathway initiated by NMN accumulation. We found that NMN requires SARM1 to activate a destruction process where extracellular Ca²⁺ influx is the main executor (Fig. 3.13). These data clarify some of the controversies in the field, identifying Ca²⁺ as a late and key player of the execution phase of Wallerian degeneration. In addition, they provide a chronological order of events regulating axon degeneration after injury.

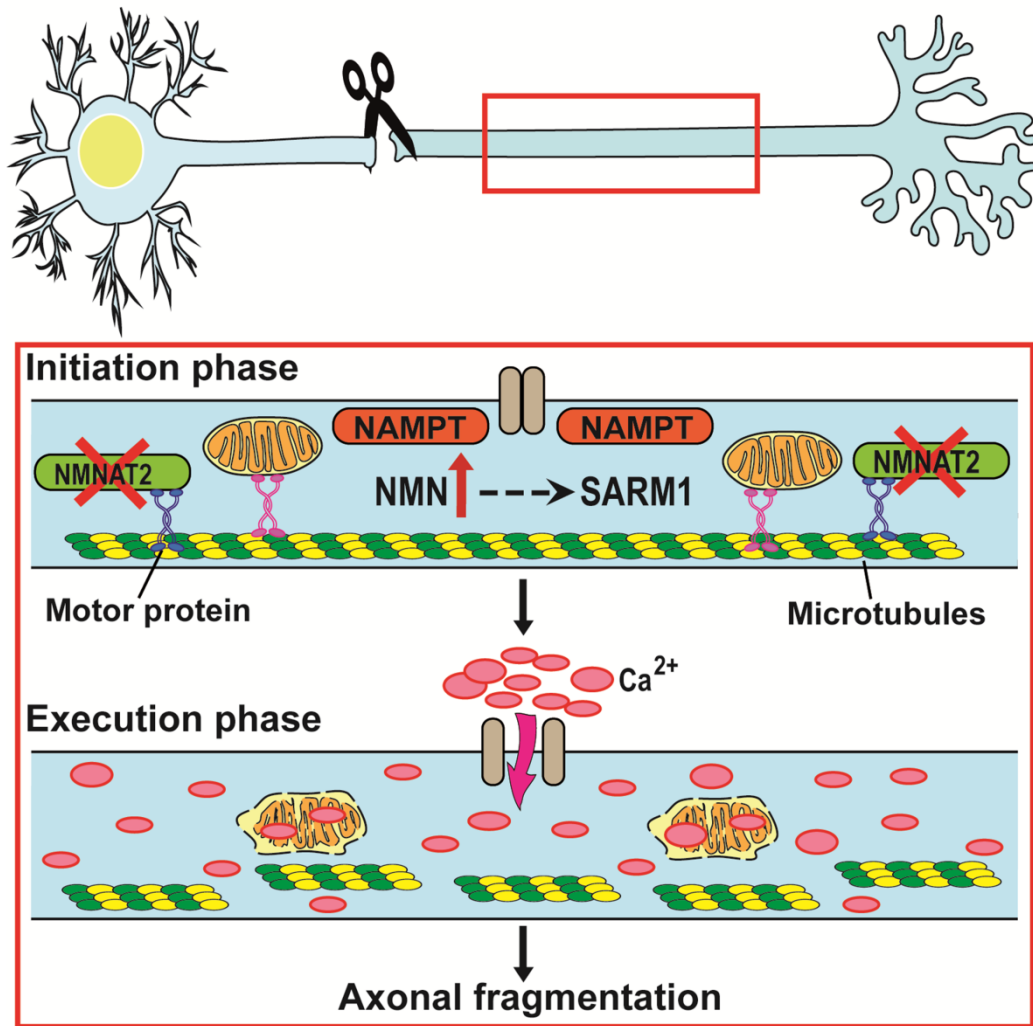


Figure 3.13 Graphical representation of the findings in this experimental chapter.

CHAPTER 4

Mitochondria play a late role in Wallerian degeneration

4.1 Introduction

(The basic principles described in this section are based on the relevant chapters of the book “Molecular biology of the cell” by Alberts, Johnson, Lewis, Raff, Roberts and Walter in addition to the cited primary publications).

Mitochondria regulate several intracellular processes ranging from ATP synthesis, regulation of intracellular Ca^{2+} levels, ROS generation and release of pro-apoptotic signals. Dysfunctional mitochondria lead to a dysregulation of some or all these processes, which ultimately affect cell fate (Ernster & Schatz, 1981). Neuronal survival profoundly depends on the correct functioning of these organelles and several neurodegenerative disorders are characterized/caused by mitochondrial dysfunction (Lin & Beal, 2006; Court & Coleman, 2012).

In this chapter, I will focus on the role of mitochondrial dysfunction in Wallerian degeneration and the relationship with NMN and SARM1. First, I will give a brief overview of the functions of mitochondria in the cell and what is known to date about their role in Wallerian degeneration. The wider link between mitochondrial dysfunction and neurodegenerative disorders will be addressed in chapter 5.

4.1.1 Mitochondrial functions: from ATP production to regulation of cell death and ROS production

Mitochondria are double membrane organelles which possess their own DNA (mtDNA) containing 37 genes. These genes code for 22 tRNAs and 13 proteins (core subunits of the mitochondrial electron transport chain complexes) which are directly translated inside the mitochondria through mitochondrial ribosomes (Friedman & Nunnari, 2014; Amunts *et al.*, 2015). However, the vast majority of the mitochondrial proteins are encoded by nuclear genes, translated in the cytosol and then imported into the mitochondria. Mitochondria cannot be synthesized *de novo*; their biogenesis can be described as the growth and division of pre-existing mitochondria (Jornayvaz & Shulman, 2010). Mitochondria are mainly known for their role in the production of ATP, the main source of energy used by the cell. Briefly, mitochondria generate ATP via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. The full set of

reactions leading to ATP synthesis is generally referred to as mitochondrial respiration. During the TCA cycle, acetyl-CoA, derived from glucose metabolism, amino acids metabolism and fatty acid degradation, is oxidised to generate electron donor molecules, such as NADH and FADH₂. These molecules transfer the electrons to molecular oxygen through four multi-subunit complexes localised in the mitochondrial inner membrane, known as complexes I, II, III and IV, and two electron carriers ubiquinone/coenzyme Q and cytochrome c. All together, these complexes are known as the electron transport chain. This process is coupled to the transfer of protons across the mitochondrial inner membrane leading to the generation of a proton electrochemical gradient. This supplies the energy to phosphorylate ADP to ATP by the ATP-synthase. Thus, loss of mitochondrial membrane potential severely impairs ATP production. The process in which ATP is formed as a result of the transfer of electrons starting from NADH or FADH₂ is known as oxidative phosphorylation.

The role of mitochondria in apoptosis in mammals is also well established. There are several regulators of apoptosis, but the activation of the BCL-2 family proteins (which includes several pro and anti- apoptotic proteins) and their role in the release of cytochrome c from mitochondria with the consequent activation of caspases (Liu *et al.*, 1996; Kluck *et al.*, 1997) are considered early and central events in the cell death program (Wang & Youle, 2009; Czabotar *et al.*, 2014).

Finally, mitochondria are also an important source of intracellular ROS (Murphy, 2009). ROS have a number of physiological functions as signalling molecules inside the cell (Sena & Chandel, 2012), but they are also known to cause damage to proteins, lipids and DNA, a process termed “oxidative stress”, which is involved in the pathogenesis of neurodegenerative diseases (Federico *et al.*, 2012).

4.1.2 Ca²⁺ buffering activity and mitochondrial permeability transition

Mitochondria were the first intracellular organelles to be associated to Ca²⁺ handling (Rizzuto *et al.*, 2012). Ca²⁺ enters into the mitochondrial matrix mainly through the mitochondrial Ca²⁺ uniporter (MCU) (Kirichok *et al.*, 2004).

Mitochondrial uptake of Ca^{2+} is important to control intracellular Ca^{2+} signalling, cell survival, cell metabolism and other cell-type specific functions by buffering cytosolic Ca^{2+} levels (Marchi & Pinton, 2014). However, an overload of Ca^{2+} has been described as one of the main inducers of mitochondrial permeability transition (mPT) (Baumgartner *et al.*, 2009; Bernardi & Di Lisa, 2015). mPT is defined as the increase in the permeability of the inner mitochondrial membrane (normally highly impermeable) to solutes with a molecular mass of less than 1500 Da. It is thought to be caused by the opening of a large channel known as the mitochondrial permeability transition pore (mPTP) (Rao *et al.*, 2014; Bernardi & Di Lisa, 2015). Although the structure of these channels is not fully characterized, three major proteins are proposed to comprise the mPTP: the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane, the adenine nucleotide translocator (ANT) located in the inner membrane and the modulator (but not structural component) cyclophilin D (CypD) in the matrix. Under normal conditions, CypD resides in the mitochondrial matrix and the mPTP remains closed. In the presence of signals acting as permeability transition inducers, CypD becomes associated with the inner membrane and stimulates the opening of the mPTP (Rao *et al.*, 2014). A prolonged opening of mPTP leads to a series of toxic events such as Ca^{2+} release, mitochondrial depolarization, block of ATP synthesis, inhibition of respiration and matrix swelling, mobilization of cytochrome c and release of other pro-apoptotic signals and outer mitochondrial membrane rupture (Petronilli *et al.*, 2001; Rao *et al.*, 2014; Bernardi & Di Lisa, 2015). Therefore, mPTP opening has been traditionally linked to mitochondrial dysfunction, cell death and it is regarded as a potential therapeutic target for neurodegenerative disorders. However, short-term openings may be involved in physiological regulation of Ca^{2+} and ROS homeostasis, and provide mitochondria with a fast mechanism for Ca^{2+} release (Ichas *et al.*, 1997; Barsukova *et al.*, 2011; Bernardi & Di Lisa, 2015). The role of mPTP opening in Wallerian degeneration is discussed below.

4.1.3 Mitochondrial trafficking in neurons

A recent study determined that a resting cortical neuron in human brain consumes ~4.7 billion ATP molecules per second (Zhu *et al.*, 2012). Due to their complex morphology, maintaining energy homeostasis in neurons is

challenging. Most cells are measured in micrometers; neurons, instead, possess exceptionally long axonal projections which can extend up to a meter in humans. Therefore, a correct distribution of mitochondria is an essential and complicated task to achieve. Although not fully understood, mitochondrial trafficking is critical for the correct functioning of the neuron and its different cellular compartments. For example, some areas of a neuron do have a higher energy and Ca^{2+} buffering demand. Synapses are an example, where a high number of mitochondria is present (Palay, 1956). Another crucial role for mitochondrial transport is to maintain a healthy peripheral mitochondrial pool. In fact, motile mitochondria are involved processes like fusion/fission, which permits an exchange and a refreshment of mitochondrial material (Liu *et al.*, 2009; Schwarz, 2013).

Mitochondrial transport in neurons (and possibly in most animal cells) is mainly mediated by a motor/adaptor complex formed by a motor protein (a kinesin for anterograde transport and a dynein for retrograde transport) which binds to an adaptor protein called Milton, which in turn binds to Miro, a protein anchored to the outer membrane of the mitochondrion (Stowers *et al.*, 2002; Guo *et al.*, 2005; Glater *et al.*, 2006; Schwarz, 2013). This complex requires ATP to exert its function. The ATP that fuels mitochondrial transport mainly derives from mitochondrial respiration, in contrast to vesicular transport which mainly utilizes ATP produced through glycolysis (Zala *et al.*, 2013). One well established regulator of mitochondrial movement is cytosolic Ca^{2+} . An increase in cytosolic Ca^{2+} causes an arrest of both anterograde and retrograde transport of mitochondria (Wang & Schwarz, 2009; Schwarz, 2013). The role of this regulation is not well established, but is widely believed to be a homeostatic response to local Ca^{2+} influx. The presence of mitochondria in areas with increased Ca^{2+} levels will provide Ca^{2+} buffering capacity. In addition, subcellular areas with inadequate cytosolic ATP are likely to have local elevations of Ca^{2+} , and directing mitochondria to such areas will correct the lack of ATP (Wang & Schwarz, 2009; Schwarz, 2013).

4.1.4 Mitochondria and Wallerian degeneration

The importance of mitochondria in cell death pathways, the link between mitochondrial dysfunction and several neurodegenerative disorders (see

chapter 5), in addition to studies reporting mitochondrial damage in injured axons, have led to the concept that this organelle play an active role in Wallerian degeneration. However, despite this widespread belief, conclusive evidence in support of this, including at which stage in the degeneration pathway mitochondria come into play, is still lacking. For clarification purposes, the data reported in this paragraph refer to changes in mitochondrial homeostasis in the distal segment of injured axons. Morphological analysis of mitochondria following axonal transection revealed signs of damage after injury (Vial, 1958; Webster, 1962). In addition, loss of mitochondrial membrane potential and ATP depletion has been documented in injured axons (Sievers *et al.*, 2003; Wang *et al.*, 2005; Barrientos *et al.*, 2011; Yang *et al.*, 2015). However, it is still unclear whether these changes play a causative role or are simply a consequence of the activation of earlier steps in the pathway. Overexpression of NMNAT3, the mitochondrial NMNAT isoform, confers strong axonal protection (comparable to WLD^S) after injury both in *Drosophila* (Avery *et al.*, 2009) and mouse (Yahata *et al.*, 2009). Superficially, NMNAT3 localisation to mitochondria seems to imply a direct involvement of these organelles in the degeneration program, and WLD^S partial co-localisation with mitochondria supports this hypothesis (Beirowski *et al.*, 2009; Yahata *et al.*, 2009; Babetto *et al.*, 2010). However, as a consequence of overexpression, proteins may assume ectopic locations (Coleman & Freeman, 2010). Indeed, when overexpressed in *Drosophila*, NMNAT3 localises to the cytoplasm (Avery *et al.*, 2009). Similarly, overexpressed NMNAT3 in mice is also detectable outside mitochondria (Yahata *et al.*, 2009). As NMNAT2 and 3 share the same enzymatic activity, with the mitochondrial isoform being more stable (Gilley & Coleman, 2010), even a small quantity of NMNAT3 in the cytoplasm could be sufficient to substitute for NMNAT2 loss, especially in the light of the robust protection exerted by low levels of expression of WLD^S (Beirowski *et al.*, 2009) or axonally-targeted NMNAT1 (Babetto *et al.*, 2010). This would imply that NMNAT3 protects by mimicking WLD^S action rather than through a direct effect on mitochondria. Noteworthy, whether NMNAT3 is actually present inside mitochondria is still debated (Felici *et al.*, 2013; Yamamoto *et al.*, 2016) and, despite the abundance of mitochondria, its expression and activity in the brain is low (Conforti *et al.*, 2011; Orsomando *et al.*, 2012). If there were a strict

endogenous mitochondrial localisation this could actually limit its protective effect, as suggested by a protective variant of NMNAT2 which loses its efficacy once targeted to the mitochondria (Milde *et al.*, 2013c). Of note, in contrast to observations in NMNAT2 null mice, NMNAT3 deficient mice do not show any clear neuronal damage (Yamamoto *et al.*, 2016). Finally, wild-type axons should, at least in theory, express NMNAT3. Nevertheless, they undergo rapid Wallerian degeneration.

Axonal injury has been shown to cause a reduction in mitochondrial axonal transport (Avery *et al.*, 2012; O'Donnell *et al.*, 2013). In 2012, Avery *et al.* suggested that WLD^S confers protection by maintaining mitochondrial motility in the distal stump of injured axons and enhanced Ca²⁺ buffering capacity. They find that decreasing mitochondrial axonal transport reduces the protective effect of WLD^S in *Drosophila* (Avery *et al.*, 2012). In contrast, WLD^S expression in zebrafish axons only temporally rescues the reduction in mitochondrial motility after injury, despite conferring strong protection (O'Donnell *et al.*, 2013), and in mammalian primary culture it does not appear to boost Ca²⁺ buffering (Adalbert *et al.*, 2012). SCG10, another short-lived protein that gets rapidly depleted in axons after injury, has been implicated in the maintenance of mitochondrial transport. However, delaying SCG10 loss, despite rescuing the defects in mitochondrial axonal transport only confers very weak protection against Wallerian degeneration, and its knock down is not sufficient to trigger axon degeneration (Shin *et al.*, 2012). In 2011, Barrientos *et al.* showed that the opening of mPTP is involved at later stages of Wallerian degeneration, downstream of WLD^S (Barrientos *et al.*, 2011). Mitochondria are also an important source of ROS (Turrens, 2003), whose production might be enhanced via mPTP opening (Batandier *et al.*, 2004). Of note, ROS accumulation has also been observed in Wallerian degeneration and WLD^S is able to prevent it (O'Donnell *et al.*, 2013).

Recent findings from Kitay *et al.* show that mitochondria are dispensable for Wallerian degeneration to occur in *Drosophila* motor neurons. In fact, Wallerian degeneration is still occurring in axons deprived of mitochondria (with a modest delay in the degeneration timing) and, most importantly, WLD^S is still protective (Kitay *et al.*, 2013). These data strongly argue against mitochondria as a

primary site of action for WLD^s and add further controversy to the field. Wallerian degeneration also occurs in the absence of mitochondria in *C. elegans* (Rawson *et al.*, 2014). Nonetheless, axon degeneration after injury in *C. elegans* does not follow a WLD^s-sensitive pathway, highlighting the existence of different mechanisms of axon death (Nichols *et al.*, 2016).

Mitochondria have also been called into question as a possible site of action for SARM1, which possesses a mitochondrial-targeting sequence (Panneerselvam *et al.*, 2012; Panneerselvam *et al.*, 2013). Intriguingly, SARM1 abundance increases in the mitochondrial fraction in a model of viral encephalitis caused by Bunyavirus infection, where it induces mitochondrial damage and neuronal cell death (Mukherjee *et al.*, 2013). However, SARM1 has been detected both in mitochondria and cytosol, and disrupting its mitochondrial localisation sequence does not affect its pro-degenerative function (Osterloh *et al.*, 2012; Gerdtz *et al.*, 2013). Of note, although mitochondrial localisation has been reported for overexpressed SARM1-GFP, endogenous Sarm1 shows a broad localisation to axons (Osterloh *et al.*, 2012), suggesting the exclusive mitochondrial localisation of SARM1-GFP is likely an artefact of the GFP tag.

4.2 Hypothesis and aims of this chapter

The discussion above shows that the contribution of mitochondria to the mechanisms regulating Wallerian degeneration is still not fully understood. Whilst numerous studies report changes in mitochondrial homeostasis in axons following an injury, recent findings point to a mechanism of axon death independent of axonal mitochondria. Therefore, it is still unclear whether these changes are simply a marker or a causative step in the degeneration process (Fig. 1.7). In this chapter, I will attempt to clarify the role of mitochondria in Wallerian degeneration and in our model of NMN-induced axon degeneration, testing the hypothesis that mitochondrial dysfunction plays a late and marginal role in the degeneration of injured axons. Hence, the specific aims of this chapter are as follows:

- To determine whether mitochondria contribute to the injury-induced Ca^{2+} rise through the opening of mPTP.
- To determine whether changes in mitochondrial shape and membrane potential are early or late steps of the degeneration process.
- To test whether FK866 and SARM1 deletion delay Wallerian degeneration through preserving mitochondrial transport.
- To assess whether pharmacological impairment of mitochondrial function followed by axotomy has an impact on the rate of Wallerian degeneration and on the protective effect of FK866 and SARM1 deletion.

4.3 Results

These data are reported in a manuscript produced as a result of this work; (Loreto et al., 2015) Wallerian degeneration is executed by an NMN-SARM1-dependent late Ca^{2+} Influx but only modestly influenced by mitochondria. Cell reports, 13, 2539-2552.

4.3.1 The roles of mitochondrial Ca^{2+} and mPTP in NMN-induced axon degeneration

As mentioned above, mitochondria are one of the most relevant sources of intracellular Ca^{2+} . In chapter 3, I found that mitochondria were present in the vast majority of axonal swellings that appear following NMN addition (Fig. 3.12) and preceding full axonal fragmentation. Of note, EGTA treatment abolished the main Ca^{2+} rise triggered by NMN addition (Fig. 3.12), indicating the extracellular environment as the main source of intra-axonal Ca^{2+} following NMN administration. We hypothesized that, in response to the robust Ca^{2+} increase arising from the extracellular environment, mitochondria would act to buffer this rise, but their buffering capacity eventually would be exceeded, triggering the release of Ca^{2+} into the cytosol as a consequence of mPTP opening. Consistent with this hypothesis, interfering with the opening of mPTP has been shown to delay Wallerian degeneration (Barrientos et al., 2011; Villegas et al., 2014). To test whether this is also the case in our experimental setting, I first compared the relative intensity of the Ca^{2+} signal inside axonal swellings (where mitochondria accumulate following NMN administration) compared to adjacent axonal segments. I found that the signal was significantly higher inside the swellings (Fig. 4.1A and B).

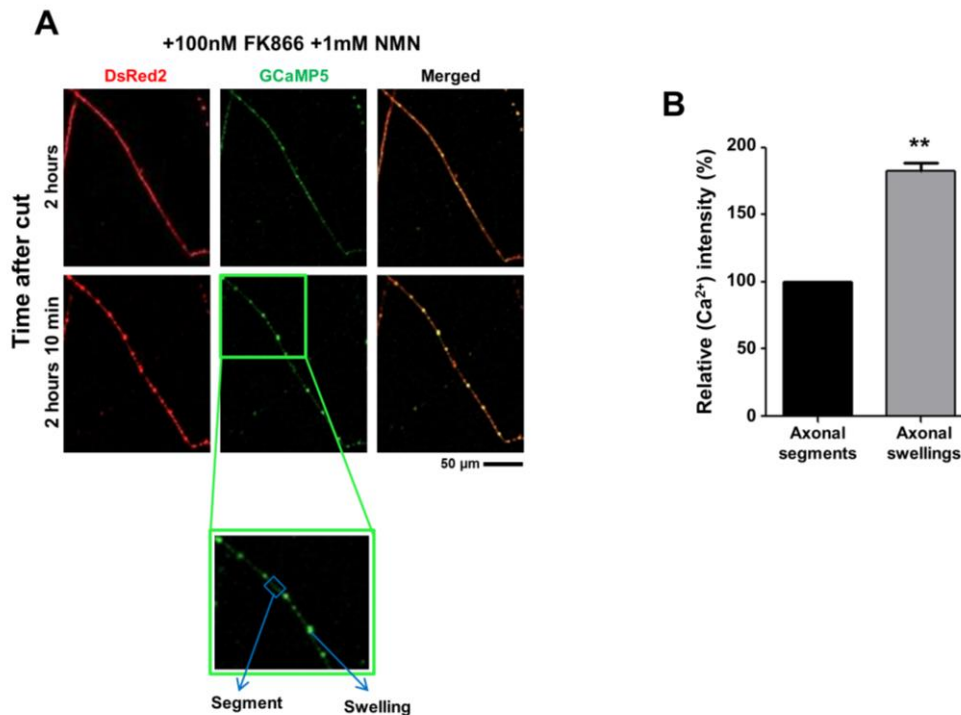


Figure 4.1 Ca^{2+} accumulates inside axonal swellings following NMN administration.

(A) Wild-type SCG axons expressing GCaMP5 and DsRed2 were subjected to axotomy and treated as indicated. Representative fluorescent images of distal axons acquired at the time points shown. The enlarged image from the corresponding boxed area shows the regions used for quantitative analysis. **(B)** Quantification of relative Ca^{2+} intensity inside axonal swellings normalised to the adjacent axonal segments signals as described in “Materials and methods” (Mean $\pm$ SEM; n=10 axons; 3 independent experiments; Student’s t test; **, $p<0.01$).

I then tested whether pharmacological inhibition of mPTP opening was sufficient to delay axonal degeneration induced by NMN addition. To this purpose, I used two different pharmacological agents that were shown to delay Wallerian degeneration in nerve explants: cyclosporin A and ruthenium red. (Barrientos *et al.*, 2011). The first targets cyclophilin D, a modulator of the mPTP, and therefore directly interferes with mPTP opening. The latter, instead, blocks Ca^{2+} uptake into the mitochondria and indirectly interferes with mPTP opening by preventing a Ca^{2+} overload into the mitochondria. Surprisingly, cyclosporin A had a weak protective effect against NMN-induced degeneration. Ruthenium red administration, instead, did not confer any protection (Fig. 4.2A-C).

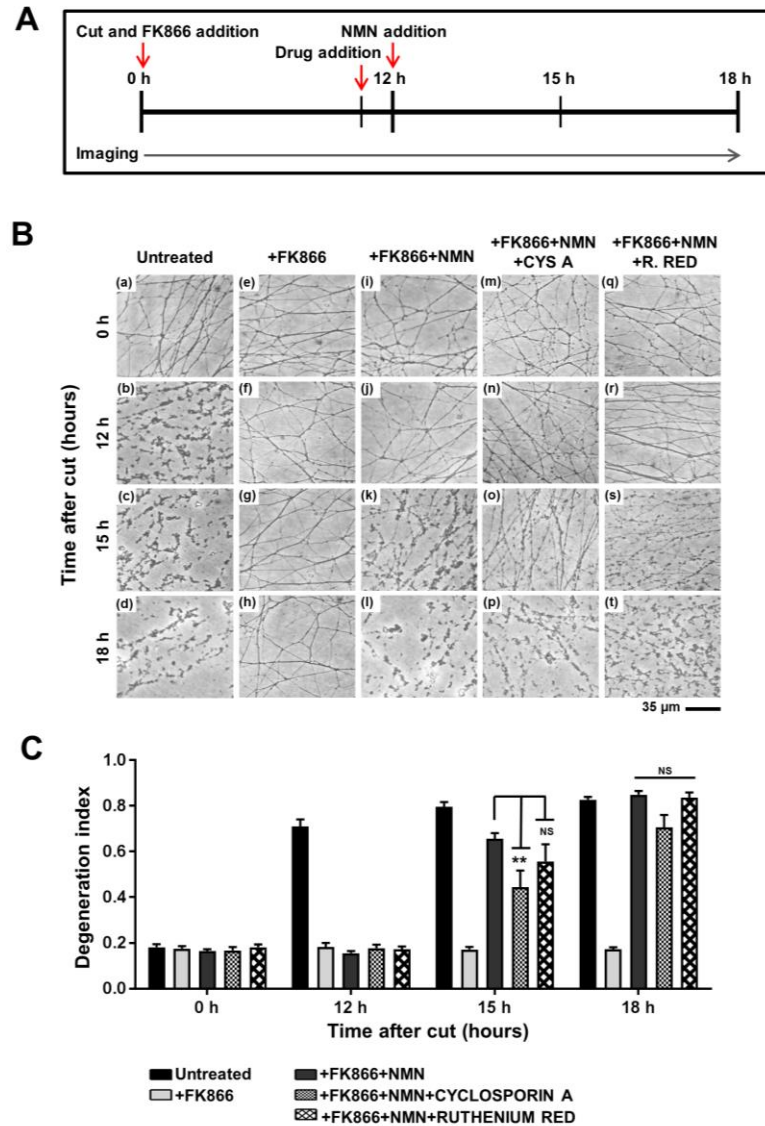


Figure 4.2 Pharmacological inhibition of mPTP opening modestly delays NMN-induced axon degeneration.

(A) Schematic representation of the experimental design. Wild-type SCG axons were cut and then treated with FK866 immediately after cut. NMN was added 12 hours after cut. Degeneration was completed within 3 hours after NMN addition. Cyclosporin A (CYSA) and ruthenium red (R. RED) (**B**, panels m-t) were added 30 minutes before NMN administration. **(B)** Representative phase contrast images acquired at the indicated time points after cut. **(C)** Degeneration index was calculated from 3 fields per sample in 3 independent experiments (Mean±SEM; n=3; two-way ANOVA followed by Bonferroni post-hoc test; **, p<0.01; NS, non-significant). The following drug concentrations were used: 100nM FK866, 100μM NMN, 20μM cyclosporin A, 50μM ruthenium red.

Taken together, these results suggest a minor involvement of mPTP in NMN-induced axon degeneration.

4.3.2 Changes in mitochondrial membrane potential and morphology mark different stages of axonal degeneration

As mentioned in the introductory section of this chapter, changes in mitochondrial function and structure have been previously shown to occur after injury (Sievers *et al.*, 2003; Villegas *et al.*, 2014). However, it is still unclear whether mitochondrial dysfunction has a key role or is simply a marker of the degeneration process, resulting from previous toxic events upstream in the pathway. To clarify this, I monitored a number of parameters of mitochondrial function in axons at different time points after injury to understand whether mitochondrial impairment occurs early in the degeneration process. Importantly, in our explant cultures, degeneration after injury is slower compared to what is observed in dissociated cultures (see Materials and Methods), with axons appearing morphologically intact up to 4 hours after injury (or NMN addition) and signs of degeneration followed by full fragmentation appearing between 6 to 8 hours after injury. I therefore used 4 hours as a cut off to determine changes occurring before any sign of damage to axons. First, I labelled mitochondria with the membrane potential indicator tetramethylrhodamine methyl ester perchlorate (TMRM). TMRM is readily sequestered by active mitochondria and a reduction in the intensity of TMRM fluorescence reflects the depolarization of mitochondrial membrane potential. I found that mitochondrial membrane potential remained stable for at least 4 hours after axotomy. Similar results were observed after FK866 treatment and when NMN was re-added to FK866-treated axons (Fig. 4.3A and B).

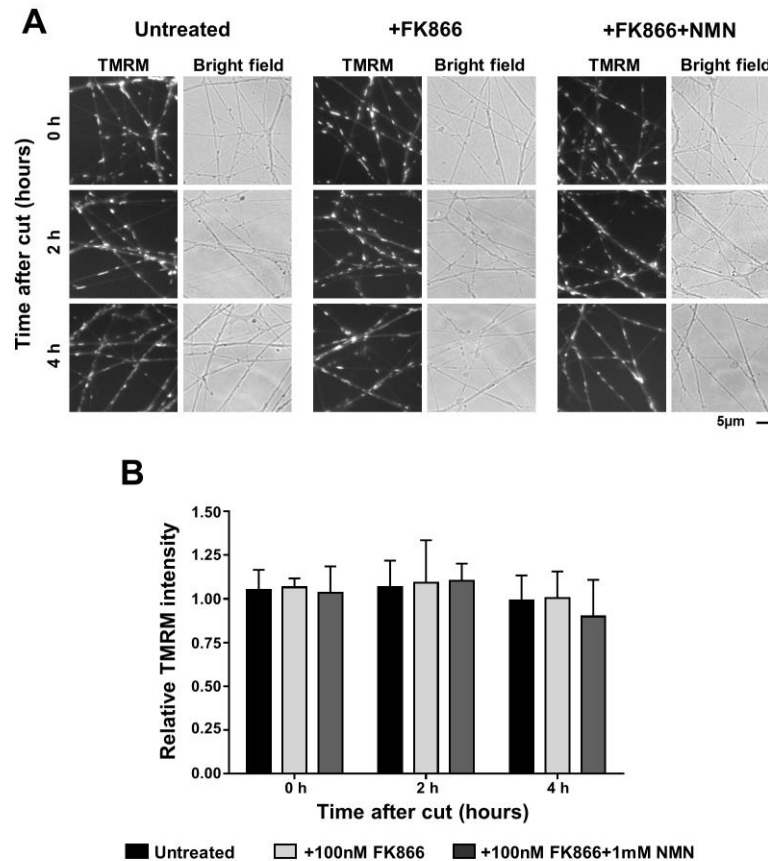


Figure 4.3 Mitochondrial membrane potential remains stable up to 4 hours after injury and NMN administration.

(A) Representative fluorescent and bright field images of wild-type SCG axons, labelled with the mitochondrial membrane potential indicator TMRM, at the indicated time points after cut. Where indicated, FK866 and NMN were added immediately after cut. **(B)** Quantification of relative TMRM intensity; all measurements were normalised to uncut controls to which an arbitrary value of 1 was assigned (Mean±SEM; n=3, two-way ANOVA followed by Bonferroni post-hoc test).

A visible decrease in TMRM signal only appeared 6 hours after injury. FK866 prevented the drop in TMRM signal visible at this time point, which was then re-established by NMN addition. Of note, at this stage clear signs of degeneration were already present in the untreated sample and following FK866 and NMN administration (Fig. 4.4A and B). We also found that SARM1 deletion remarkably delayed the drop in TMRM signal after injury, which remained stable up to 32 hours after axotomy (Fig. 4.4C and D).

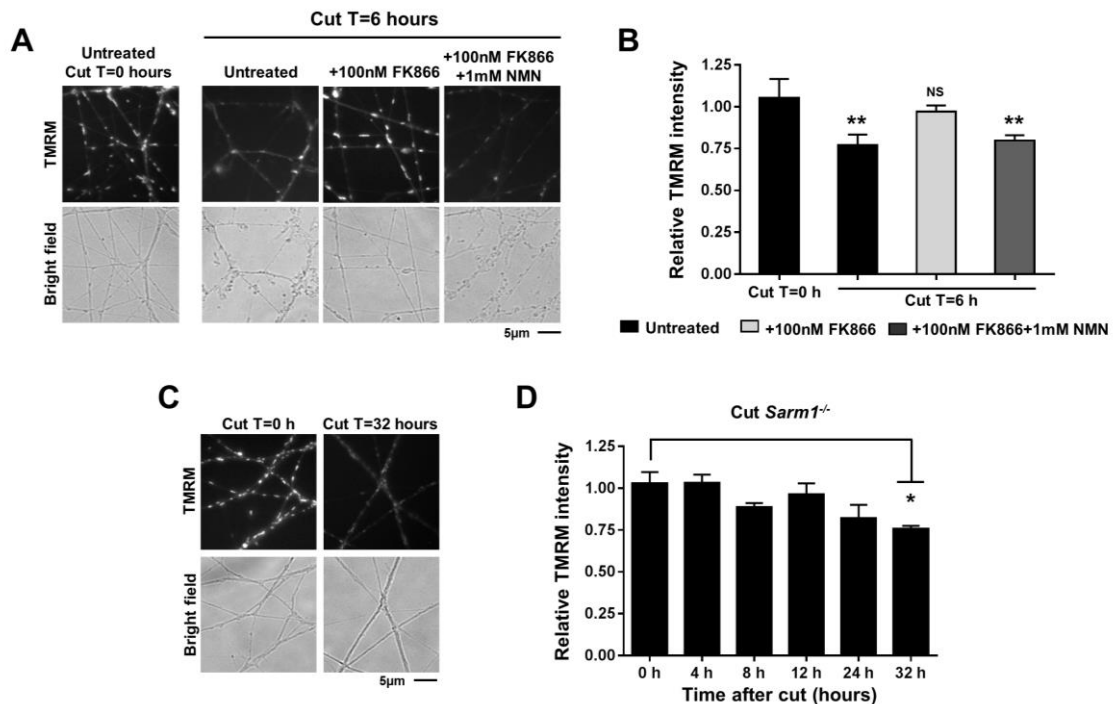


Figure 4.4 Late loss of mitochondrial membrane potential in cut axons is rescued by FK866 administration and SARM1 deletion.

(A-C) Wild-type and *Sarm1*^{-/-} SCG explants were labelled with TMRM, subject to axotomy and treated as indicated. Representative fluorescent and bright field images of distal axons acquired at the indicated time points after axotomy. (B-D) Relative TMRM intensity was quantified in wild-type SCG distal axons treated as shown, and in distal axons of *Sarm1*^{-/-} SCG explants at the indicated times after axotomy. All measurements were normalised to uncut controls to which an arbitrary value of 1 was assigned (Mean±SD; n=3 (D) and n=5 (E), one-way ANOVA followed by Bonferroni post-hoc test; *, p<0.05; **, p<0.01, NS, non-significant).

Mitochondrial morphological changes have been reported to be among the earliest detectable alterations during Wallerian degeneration (Vial, 1958; Webster, 1962). We thus tested whether NMN accumulation after injury could influence mitochondria morphology. Mitochondria were labelled with the mitochondrial marker mitotracker red. No significant change was observed among the different treatment groups in the size distribution of mitochondria immediately after cut (Fig. 4.5A and B). I found a small but significant increase in the number of shorter mitochondria 4 hours after injury only in the sample treated both with FK866 and NMN (p=0.0028). However, no significant change was detected in the untreated sample, or the sample treated with FK866 alone (Fig. 4.5A and B).

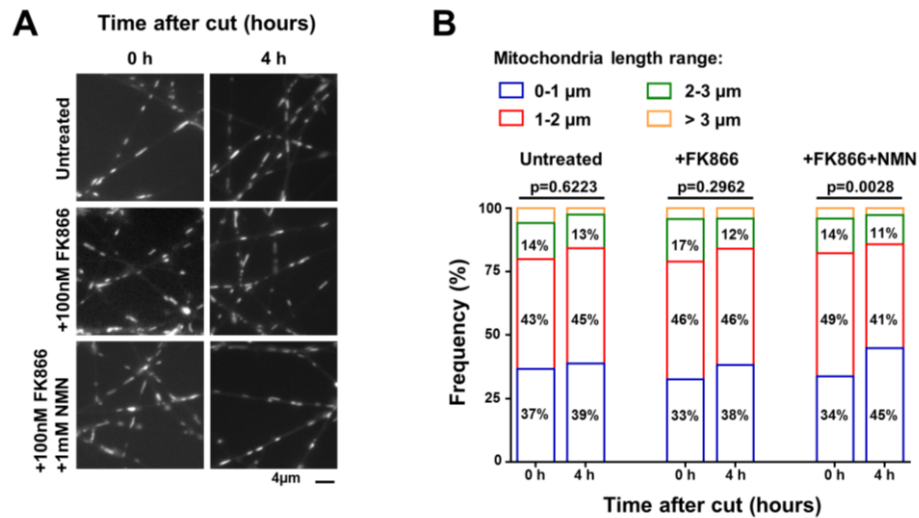


Figure 4.5 Changes in mitochondrial morphology in wild-type axons following injury and NMN administration.

(A) Representative fluorescent images of wild-type SCG axons, labelled with the mitochondrial marker mitotracker red, at the indicated time points after injury. Where indicated, FK866 and NMN were added immediately after cut. **(B)** Quantification of mitochondria length in wild-type SCG axons after cut. The results are expressed as the percentage of mitochondria that fall within the indicated range of length (Total number of mitochondria analysed per condition: Cut untreated, 0h n=359, 4h n=350; Cut+FK866, 0h n=410, 4h n=445; Cut+FK866+NMN, 0h n=362, 4h n=395; Kolmogorov-smirnov test).

Conversely, we observed a dramatic reduction in mitochondrial length in *Sarm1*^{-/-} axons 48 hours after injury (Fig. 4.6A and B), long before clear fragmentation occurs (Fig. 3.4A and B). Interestingly, we also found a significant difference (K-S p=0.0005) in the size distribution of mitochondria between *Sarm1*^{-/-} and wild-type axons immediately after cut (compare Fig. 4.5B and 4.6B), perhaps reflecting a direct influence of SARM1 on the maintenance of normal mitochondrial shape.

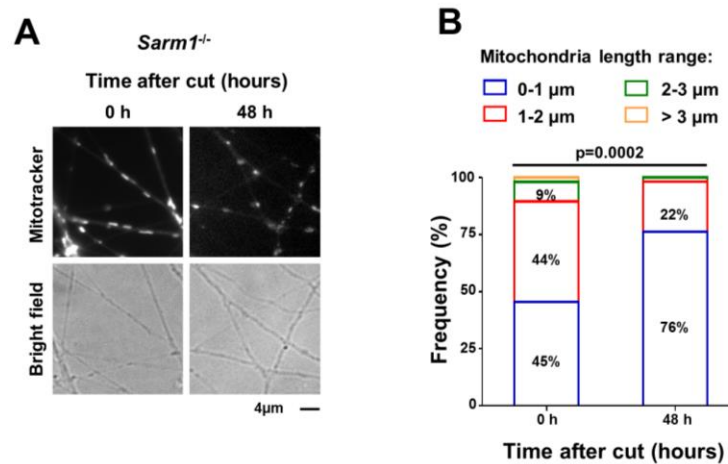


Figure 4.6 Changes in mitochondrial morphology in *Sarm1*^{-/-} axons.

(A) Representative fluorescent images of *Sarm1*^{-/-} SCG axons, labelled with the mitochondrial marker mitotracker red, at the indicated time points after injury. **(B)** Quantification of mitochondria length in *Sarm1*^{-/-} SCG axons after cut. The results are expressed as detailed above (Total number of mitochondria analysed per condition: 0h n=418, 48h n=223; Kolmogorov-smirnov test).

Thus, in this experimental system, a decrease in mitochondrial membrane potential marks the late stages of degeneration. Furthermore, while mitochondrial size is subject to modifications following an injury, these changes do not appear to correlate with the extent of degeneration.

4.3.3 FK866 and SARM1 deletion do not prevent early changes in mitochondrial motility after cut

WLD^S was suggested to confer axon protection after injury by maintaining mitochondrial axonal transport, although this is a modest and short-lived effect in some studies (Avery *et al.*, 2012; O'Donnell *et al.*, 2013). Therefore, I next investigated whether a reduction in mitochondrial motility could underline NMN-induced axon degeneration. To this purpose, mitochondria were labelled with mitotracker red (Fig. 4.7A). Consistent with previous reports (Avery *et al.*, 2012; O'Donnell *et al.*, 2013), axotomy caused a striking reduction in the percentage of axonal motile mitochondria 4 hours after cut in SCG explant cultures (Fig. 4.7B; Movie S5). Surprisingly, I also observed a drop in the number of motile mitochondria in cut axons treated with FK866, similar to that in untreated cut axons (Fig. 4.7B; Movie S5), despite FK866 conferring protection during this

time (Fig. 4.7C), and for at least another 30 hours (Di Stefano *et al.*, 2015). A similar reduction in mitochondrial motility was also observed in axons treated with both FK866 and NMN (Fig. 4.7B; Movie S5). Interestingly, FK866-treated axons showed an almost complete arrest of mitochondrial transport 24 hours after axotomy, a time point when they still have overtly normal morphology (Movie S6).

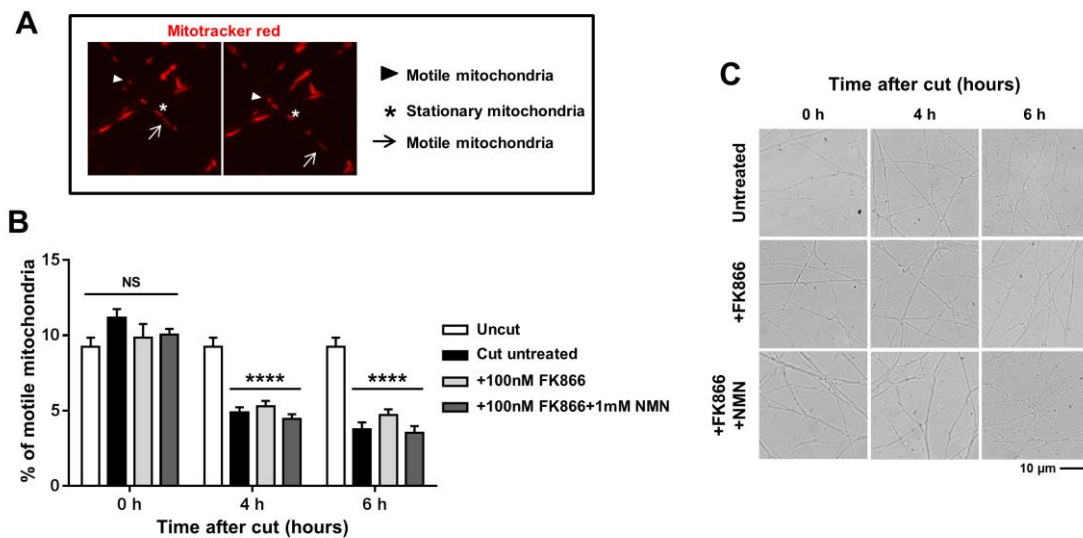


Figure 4.7 FK866 does not prevent early changes in mitochondrial motility after cut.

(A) Representative consecutive fluorescent images of SCG axons labelled with the mitochondrial marker mitotracker Red. The arrowheads and the arrow indicate two motile mitochondria relative to a stationary mitochondria (asterisk). **(B)** Quantification of mitochondria motility in wild-type SCG axons uncut or cut, treated as indicated and imaged at the time points shown. Where indicated, FK866 and NMN were added immediately after cut. % of motile mitochondria was calculated from 2 fields per sample in 4 different experiments (Mean $\pm$ SEM; n=4; two-way ANOVA followed by Bonferroni post-hoc test; ****, p<0.0001; NS, non-significant). **(C)** Representative bright field images of wild-type SCG axons showing axonal morphology at the indicated time points after cut.

I then also analysed mitochondrial transport in *Sarm1*^{-/-} SCG axons, where protection against Wallerian degeneration is even stronger than that achieved with FK866. Remarkably, a substantial reduction in mitochondrial motility was also detected as early as 4 hours after injury, when *Sarm1*^{-/-} axons are intact

(Fig. 4.8A and B; Movie S7); mitochondrial transport completely stopped 48 hours after injury (Movie S8), long before *Sarm1*^{-/-} axons fragment (Fig. 3.4A and B).

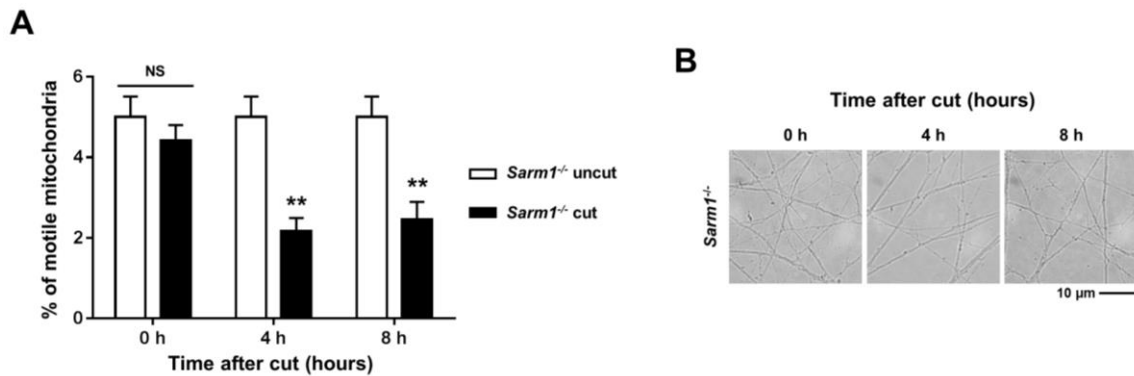


Figure 4.8 SARM1 deletion does not prevent early changes in mitochondrial motility after cut.

(A) Quantification of mitochondria motility in *Sarm1*^{-/-} SCG axons uncut or cut and imaged at the indicated time points. % of motile mitochondria was calculated from 2 fields per sample in 4 different experiments (Mean±SEM; n=4; two-way ANOVA followed by Bonferroni post-hoc test; **, p<0.01; NS, non-significant). **(B)** Representative bright field images of *Sarm1*^{-/-} SCG axons showing axonal morphology at the indicated time points after cut.

Thus, axon protection by FK866 and SARM1 deletion is not mediated by an effect on mitochondrial dynamics.

4.3.4 Mitochondrial depolarization does not alter the rate of Wallerian degeneration

The results above show that axotomy induces changes in mitochondrial membrane potential, shape and motility. However, these changes either occur at late stages in the degeneration process (e.g. mitochondrial membrane potential loss) downstream of NMN rise and SARM1, or they also manifest when axons are protected either by pharmacological or genetic interventions (e.g. mitochondrial transport and shape). This makes it difficult to understand whether they are active players of the axon-death pathway regulating Wallerian degeneration. The only way to understand this is to induce these changes directly before axotomy and test whether they affect the rate of Wallerian

degeneration. To this purpose, I treated axons with the protonophore Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which uncouples the proton gradient and causes an immediate disruption of mitochondrial membrane potential (Ly *et al.*, 2003). This treatment has also been shown to profoundly affect mitochondrial axonal transport and morphology (Summers *et al.*, 2014), replicating the above observations after injury.

When exposed to 50 μ M CCCP, mitochondria in axons rapidly depolarize, as shown by a remarkable and rapid decrease in TMRM fluorescent signal (Fig. 4.9A). Despite this, first signs of degeneration and full axonal fragmentation only occurred 24-32 hours after CCCP addition (Fig. 4.9A and B). This is a much slower time course than that of Wallerian degeneration, which in our experimental system is completed within 8 hours.

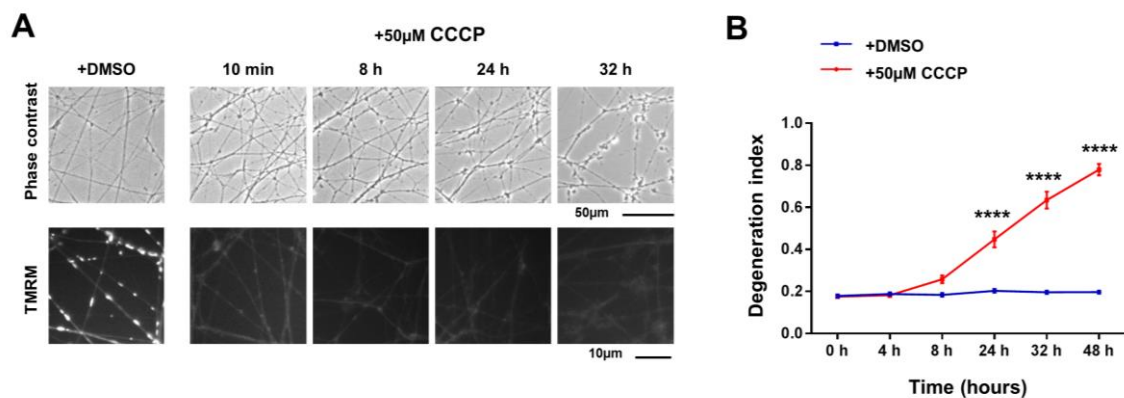


Figure 4.9 Mitochondria depolarization causes degeneration of uninjured axons.

(A) Uninjured wild-type SCG explants were treated with 50 μ M CCCP or vehicle (DMSO). 50nM TMRM was added to confirm full depolarization of axonal mitochondria following CCCP addition. Representative phase contrast and fluorescent images of wild-type SCG axons acquired at the indicated time points after CCCP addition. **(B)** Degeneration index was calculated from 3 fields per sample in 6 independent experiments (Mean $\pm$ SEM; n=6; two-way ANOVA followed by Bonferroni post-hoc test; ****, $p < 0.0001$).

This remarkable time difference enabled us to test whether mitochondrial depolarization affects the rate of Wallerian degeneration. We added CCCP to SCG explants immediately prior to axotomy (Fig. 4.10A) and then followed the

time course of axon degeneration. Despite the much earlier, almost instantaneous loss of TMRM signal, cut axons treated with CCCP underwent Wallerian degeneration at the same rate as cut, vehicle-treated axons (Fig. 4.10B (a) and C). Furthermore, CCCP treatment did not affect axon protection conferred by FK866 or the reversion of this protection by NMN administration after axotomy (Fig. 4.10B (b) and (c), D and E).

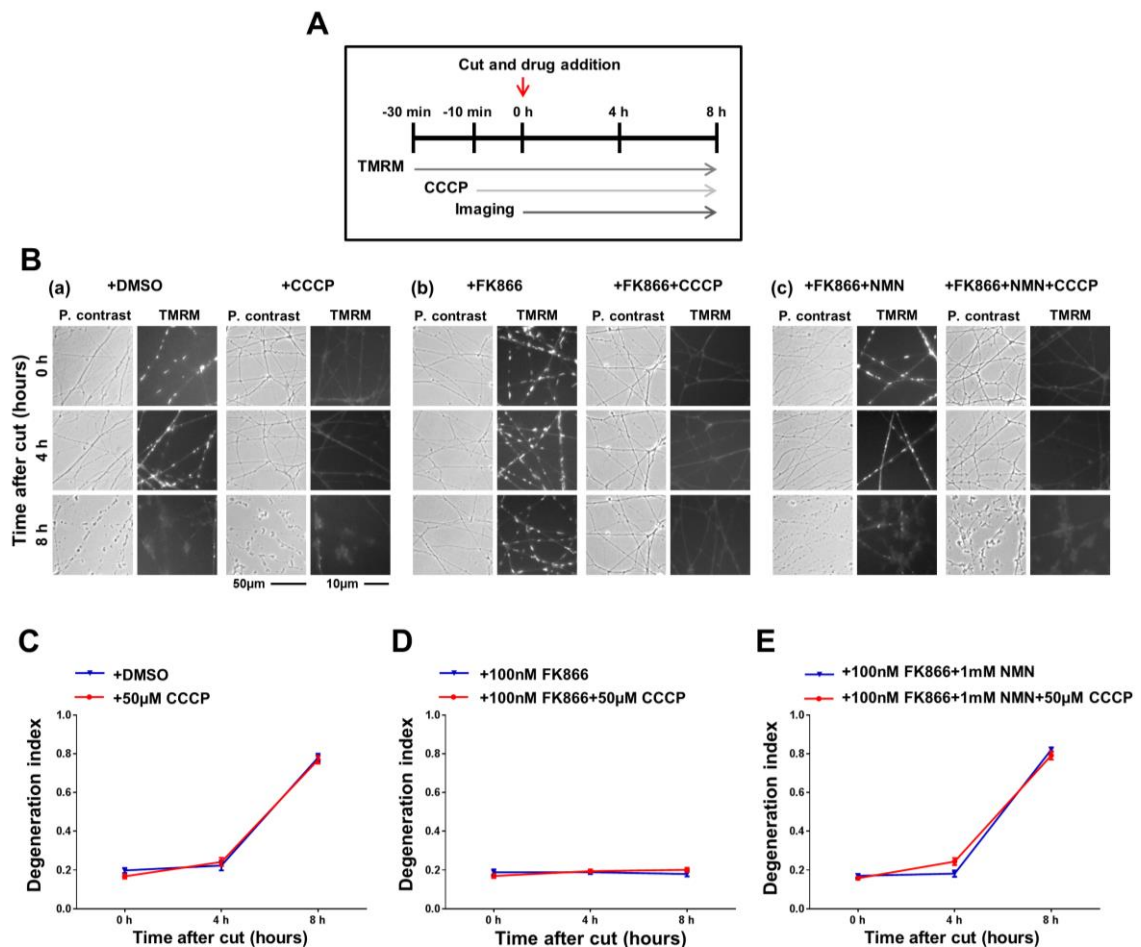


Figure 4.10 Mitochondria depolarization does not alter the rate of Wallerian degeneration.

(A) Schematic representation of the experimental design. Wild-type SCG axons were incubated with 50nM TMRM to monitor mitochondria membrane potential and treated with CCCP (added 10 minutes before axotomy) or DMSO. (B) Representative phase contrast (P. contrast) and fluorescent images of transected wild-type SCG axons treated with CCCP or vehicle (DMSO) (a-c) and with FK866 (b) or with FK866+NMN (c). The dramatic loss of TMRM signal following CCCP addition confirmed full depolarization of axonal mitochondria. (C-E) Degeneration index was calculated from 3 fields per sample in 3 independent experiments (Mean±SEM; n=3; two-way ANOVA followed by Bonferroni post-hoc test).

Similarly, the rate of axon degeneration in transected *Sarm1*^{-/-} axons treated with CCCP did not differ from that of the untreated axons, despite the rapid depolarization of *Sarm1*^{-/-} mitochondria upon CCCP addition (Fig. 4.11A and B).

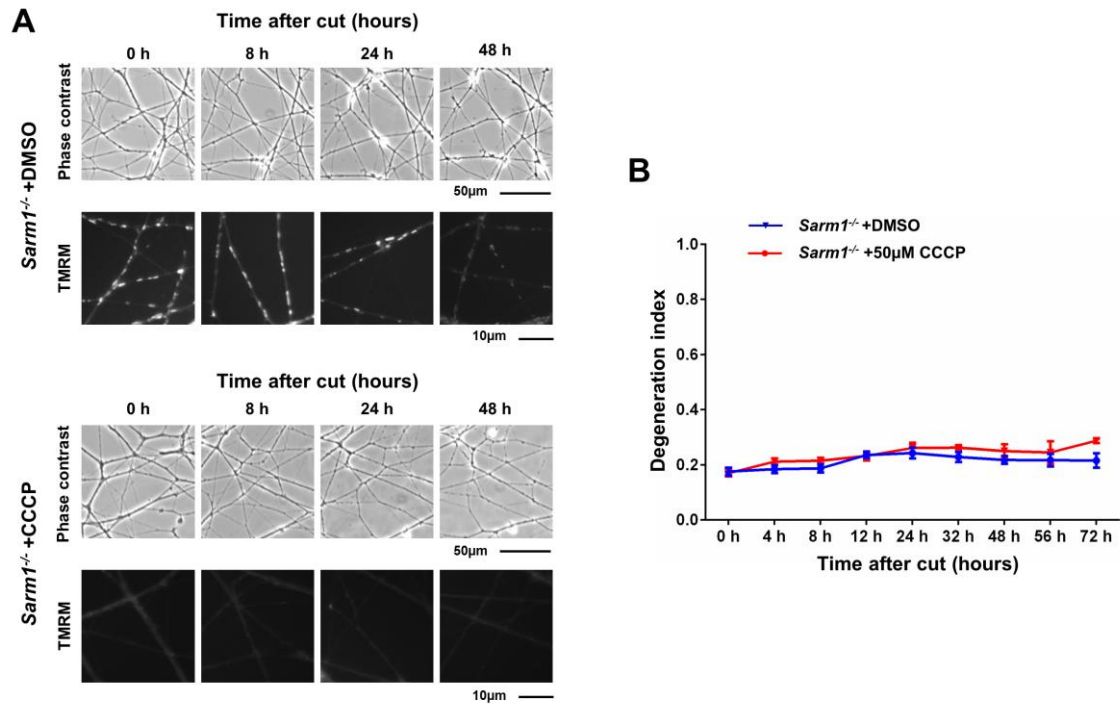


Figure 4.11 CCCP treatment in *Sarm1*^{-/-} transected axons.

(A) *Sarm1*^{-/-} SCG explants were treated with CCCP or vehicle (DMSO) and then cut. Representative phase contrast and fluorescent images acquired at the indicated time points after axotomy. The dramatic loss of TMRM signal following CCCP addition confirms full depolarization of axonal mitochondria. **(B)** Degeneration index was calculated from 3 fields per sample in 4 independent experiments (Mean±SEM; n=4; two-way ANOVA followed by Bonferroni post-hoc test).

Together, these results dissociate the loss of mitochondrial function from the onset of Wallerian degeneration.

4.4 Discussion

Whether mitochondria play a central role in Wallerian degeneration and whether the reported changes in mitochondrial shape, dynamics and membrane potential (Sievers *et al.*, 2003; Barrientos *et al.*, 2011; Avery *et al.*, 2012; Villegas *et al.*, 2014) are a cause of degeneration or general markers of compromised axons is unclear. In this chapter, I addressed these open questions. First, I have studied changes in a number of mitochondrial parameters changing in injured axons and tested how they correlate with the onset of degeneration and the effects of the NMN rise and presence or absence of SARM1. Next, I have compromised mitochondrial function before axotomy and studied the impact of this on the rate of Wallerian degeneration and the ability of FK866 and SARM1 deletion to confer protection.

As mentioned above, mitochondria are one of the major sources of intracellular Ca^{2+} and possess Ca^{2+} buffering activity. However, a Ca^{2+} overload into the mitochondrial matrix could eventually result in a release of Ca^{2+} into the cytosol through the opening of mPTP. Importantly, a short but significant delay in Wallerian degeneration has been observed in axons devoid of mitochondria (Kitay *et al.*, 2013), which might result from the lack of the additional Ca^{2+} release from mitochondria. Whilst we find that the Ca^{2+} signal inside axonal swellings is significantly higher, interfering with mPTP opening had only a modest protective effect in our model of NMN-induced degeneration. The discrepancies in the strength of protection compared to previous reports might be ascribed to the difference in the experimental settings. Of note, previous studies were conducted in nerve explants (Barrientos *et al.*, 2011), where Schwann cells and, more specifically, the effect of the pharmacological treatments on mitochondria in Schwann cells, could have a stronger influence on axonal survival.

Our data show that mitochondrial membrane potential drops after cut, but only when morphological damage to axons is already present. Whilst FK866 administration and SARM1 loss preserve mitochondrial membrane potential after injury, depolarizing mitochondria before axotomy does not alter the protective effect of FK866 and SARM1 deletion. This implies that the ability of

FK866 and SARM1 deletion to protect axons is independent of their effect on mitochondrial membrane potential, which is likely secondary to their ability to preserve axonal integrity or an independent consequence of FK866 administration and SARM1 deletion. The loss of mitochondrial membrane potential is therefore a late step in the degeneration process, possibly a consequence of the prolonged rise in Ca^{2+} described in chapter 3.

Increased axonal transport of mitochondria has recently been linked to Wallerian degeneration and WLD^S mechanism of protection (Avery *et al.*, 2012). I also find a reduction in the percentage of motile mitochondria in axons after injury; however, both FK866 and SARM1 deletion are unable to rescue this defect. This shows that maintenance of mitochondrial axonal transport is not necessary to protect axons after injury. The depletion of other axonal proteins may explain the effects on mitochondrial homeostasis. For instance, SCG10, which is involved in the regulation of mitochondrial dynamics, is also rapidly lost after injury (Shin *et al.*, 2012), raising the possibility that changes in mitochondrial axonal transport may follow the depletion of such a regulator. Consistent with our results, delaying SCG10 depletion in axons after injury only confers very weak protection, despite rescuing the defects in mitochondrial axonal transport (Shin *et al.*, 2012). Of note, local variations in Ca^{2+} levels might explain mitochondrial arrest (Wang & Schwarz, 2009). I also observed changes in mitochondrial shape after injury, which appeared to be unrelated to the onset of Wallerian degeneration. Intriguingly, axonal mitochondria are shorter and more static in the absence of SARM1. While supporting that Wallerian degeneration is independent of mitochondria shape and motility, these observations raise a series of questions on the role of SARM1 in mitochondria. Indeed, SARM1 contains an N-terminal mitochondrial localisation signal and is, at least in part, associated with mitochondria (Osterloh *et al.*, 2012). SARM1 mitochondrial localisation may be dispensable for the execution of Wallerian degeneration (Gerds *et al.*, 2013), however it is required for viral-induced cell death (Mukherjee *et al.*, 2013). The relevance of mitochondrial homeostasis in SARM1 biological function therefore needs to be further explored.

CCCP administration causes immediate mitochondrial depolarization; moreover, CCCP has been shown to impair mitochondrial axonal transport and

shape (Summers *et al.*, 2014). Whilst CCCP treatment replicates all the changes in mitochondrial homeostasis observed during Wallerian degeneration, its addition before axotomy does not alter the rate of Wallerian degeneration and, most importantly, it does not alter the ability of FK866 and SARM1 deletion to protect axons. Our results, therefore, dissociate the changes observed in mitochondria parameters after injury from the mechanisms regulating Wallerian degeneration and are consistent with a recent study showing that Wallerian degeneration occurs also in axons devoid of mitochondria and WLD^s protection is independent of axonal mitochondria (Kitay *et al.*, 2013). A previous study showed that CCCP toxicity in uninjured axons was insensitive to WLD^s (Ikegami & Koike, 2003), which may appear in contrast to our data. However, I used CCCP at a lower concentration and, most importantly, in the context of axotomy where CCCP action could differ.

Whilst the changes in mitochondrial homeostasis caused by axotomy are not central to the mechanisms of Wallerian degeneration, how they occur is still unclear. As mentioned before, steps in the pathway that eventually lead to the degeneration of injured axons could also be toxic for mitochondria. In addition, mitochondria depend on the integrity of a nuclear-mitochondrial communication pathway regulated by NAD metabolism (Gomes *et al.*, 2013; Fang *et al.*, 2014), and they could be affected when this control is lost as consequence of the injury. However, whether the loss of the nuclear-mitochondrial communication can affect mitochondrial homeostasis in the relatively short time of Wallerian degeneration is still uncertain.

In our hands treatment of axons with CCCP does not affect the rate of Wallerian degeneration, but it does initiate a slower axonal degeneration process even in uninjured neurons. Intriguingly, other reports show that mitochondrial dysfunctions activate a pathway of degeneration sensitive to regulators of Wallerian degeneration (Press & Milbrandt, 2008; Antenor-Dorsey & O'Malley, 2012; Summers *et al.*, 2014). Explaining the mechanistic links between mitochondrial dysfunction-induced axon degeneration and Wallerian degeneration will be important in view of the causative role of mitochondrial dysfunction in a variety of neurodegenerative disorders characterized by axonal degeneration (Conforti *et al.*, 2014). Our data suggest that mitochondrial

dysfunction does not have a central role in Wallerian degeneration, where the physical injury itself causes the activation of the axon-death pathway. However, compromising mitochondrial function may be sufficient to initiate an axon-death pathway in uninjured axons similar to Wallerian degeneration. This hypothesis will be tested in chapter 5.

In summary, our data support a model where regulation of Wallerian degeneration is independent of axonal mitochondria. Finally, based on the data presented in chapters 3 and 4 of this thesis, we propose a sequence of events leading to axon degeneration after injury (Fig. 4.12).

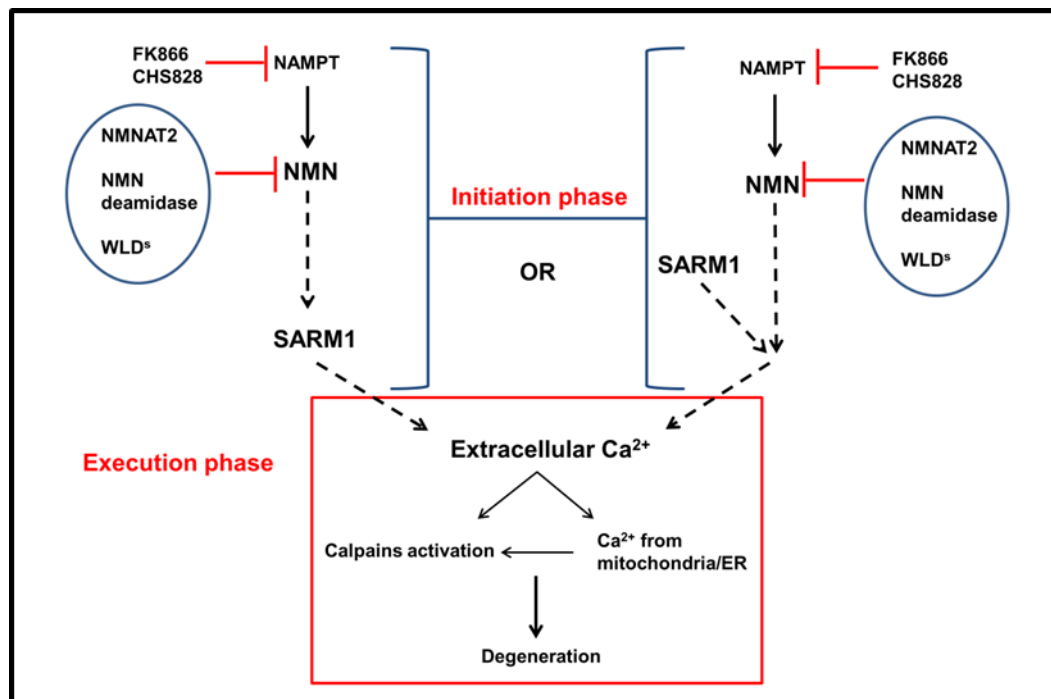


Figure 4.12 Proposed sequence of events leading to axon degeneration after injury.

NMNAT2 depletion after injury (Gilley & Coleman, 2010; Gilley *et al.*, 2015) could remove an inhibitory control on NMN levels, which could rise and lead to the activation of an axon-death pathway (Di Stefano *et al.*, 2015) dependent on SARM1 (Gilley *et al.*, 2015). WLD^s could limit NMN accumulation by substituting for NMNAT2 (Di Stefano *et al.*, 2015). Our data are consistent with a model where SARM1 acts directly downstream of NMN in a linear pathway or converges on common downstream steps. The combined action of NMN and SARM1 triggers an axon-death program where extracellular Ca²⁺ influx is the main executor.

CHAPTER 5

Regulators of Wallerian degeneration control axonal degeneration caused by mitochondrial toxins

5.1 Introduction

Studies of axon degeneration following an injury and of the axon-protective protein WLD^s have led to the discovery of critical endogenous regulators of the mechanisms resulting in axon destruction. As mentioned before, one of the main goals in the Wallerian degeneration field is to test whether the pathway regulating axon degeneration following an injury is also accountable for the degeneration of uninjured axons in response to toxic stimuli (a critical feature of several neurodegenerative disorders). In chapter 4, we propose mitochondria as late players of Wallerian degeneration, where the injury itself is responsible for the activation of the axon death pathway. Nevertheless, mitochondrial depolarization per se can lead to degeneration of axons in the absence of a cut (Fig. 4.9). Therefore, it remains possible that mitochondrial dysfunction in uninjured axons, a common feature of several neurodegenerative disorders characterized by axon loss (see below) (Court & Coleman, 2012), acts as an upstream signal triggering the activation of the same pathway of axon death controlling Wallerian degeneration. In the final experimental chapter of this thesis, I will explore this possibility. First, I will give a brief introduction of the role of mitochondrial dysfunction in neurodegenerative disorders.

5.1.1 Mitochondrial dysfunction in neurodegenerative disorders

Impairment of mitochondrial function is a common theme in a wide group of neurodegenerative disorders with different aetiology. Among these, some conditions are directly caused by mutations in mitochondrial proteins, including cases of Parkinson's disease, Charcot-Marie-Tooth and Friedreich's ataxia (Lin & Beal, 2006; Neusch *et al.*, 2007; Gonzalez-Cabo & Palau, 2013). For other pathologies, including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, multiple sclerosis and many others, an association with mitochondrial dysfunction has been found, but it is uncertain whether it plays a causative role (Johri & Beal, 2012). Aging is by far the major risk factor for the development of these diseases and mitochondrial dysfunction is believed to profoundly contribute to the aging process. There are a number of reasons why mitochondria become dysfunctional with aging. First, the accumulation of mutations in mtDNA, which ultimately impairs several mitochondrial functions

(Lin & Beal, 2006). Aging also affects the nuclear-mitochondrial communication pathway (Gomes *et al.*, 2013). In addition to aging, mutations in nuclear genes encoding proteins that directly or indirectly control mitochondrial function have been identified as a cause of familial forms of neurodegenerative diseases or associated with an increased risk of developing these disorders (Lin & Beal, 2006; Court & Coleman, 2012). Of note, mitochondrial dysfunction is present in diseases in which axon degeneration is the predominant and/or an early step (Court & Coleman, 2012). Among these, the link between multiple sclerosis and mitochondrial defects is becoming increasingly recognized (Mahad *et al.*, 2009; Mao & Reddy, 2010; Campbell *et al.*, 2011; Witte *et al.*, 2014). Mitochondria and mtDNA are vulnerable to oxidative injury. The current model predicts that inflammatory products and chronic oxidative stress occurring in multiple sclerosis eventually lead to damage to mitochondria, resulting in energy deficiency. mtDNA deletions and mitochondrial abnormalities are seen both in the white and grey matter. Over time, the accumulation of abnormal mitochondria in neurons could lead to axonal degeneration and cell death (Campbell *et al.*, 2012; Campbell & Mahad, 2012; Witte *et al.*, 2013; Mahad *et al.*, 2015). Importantly, whether mitochondrial quality control, (involved in the clearance of unhealthy mitochondria) is impaired in multiple sclerosis is unknown. Defects in mitochondrial quality control would strongly support the model above and offer a convincing explanation for the accumulation of damaged mitochondria in multiple sclerosis over time.

Parkinson's disease (PD) is another neurodegenerative disorder that is unquestionably linked to mitochondrial dysfunction. PD is characterized clinically by progressive rigidity, bradykinesia and tremor. Loss of neurons in the substantia nigra and the presence of Lewy bodies, distinctive cytoplasmic inclusions containing alpha-synuclein (Spillantini *et al.*, 1997; Lin & Beal, 2006), are pathophysiological hallmarks of the disease. Mitochondria were first implicated in PD after the discovery that 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (more details can be found in the section below), an inhibitor of mitochondrial complex 1 (Mizuno *et al.*, 1988), causes Parkinsonism. Mutations in several genes affecting mitochondrial function are now known to cause familial forms of PD (10-15% of disease cases), and the same genes and

proteins, in combination with environmental factors, are also possibly involved in the remaining 85-90% cases of sporadic PD (Klein & Westenberger, 2012; Ryan *et al.*, 2015). Among these, mutations in α -synuclein, parkin, DJ-1, PINK1, leucine-rich-repeat kinase 2 (LRRK2) are all associated with mitochondrial, and often complex-1, impairment (Lin & Beal, 2006; Ryan *et al.*, 2015).

In recent years, there has been a growing emphasis on axon loss in PD, due to be a major feature and possibly the earliest and causative step of the pathology. Therefore, protecting axons as well as the soma could be the most effective therapeutic strategy (Tagliaferro & Burke, 2016).

5.1.2 Mitochondrial toxins: relevance to neurodegenerative disorders

The use of mitochondrial toxins to impair mitochondrial function has been instrumental in studying important physiological and pathological processes taking place in mitochondria and to identify proteins and molecules involved in their regulation. Here, I will focus on inhibitors of mitochondrial complex-1 and mitochondrial uncouplers as they are more relevant to this chapter.

In 1982, a group of drug users in California was hospitalized showing all the classic signs and symptoms of PD. Langston and colleagues then identified MPTP, present in the drug the patients consumed, as the molecule accountable for the symptoms observed (Langston *et al.*, 1983; Langston, 2017). The discovery of MPTP (and the toxicity of its derivative MPP⁺) was the first evidence of mitochondrial involvement in PD. These findings opened years of research in the PD field on complex-1 deficiency and mitochondrial dysfunction (Schapira *et al.*, 1989; Schapira *et al.*, 1990; Winklhofer & Haass, 2010). The discovery of MPTP supports the “environmental hypothesis” which proposes that sporadic PD is caused by exposure to environmental mitochondrial toxins, possibly with similar toxicological profiles to MPTP (Goldman, 2014). Another example is the pesticide rotenone, a widely studied inhibitor of mitochondrial complex-1 that also causes Parkinsonism and selective degeneration of nigral dopaminergic neurons (Cannon *et al.*, 2009). In reality, an interaction between genetic and environmental factors is likely to be responsible for the development and progression of the disease, with the genes and proteins

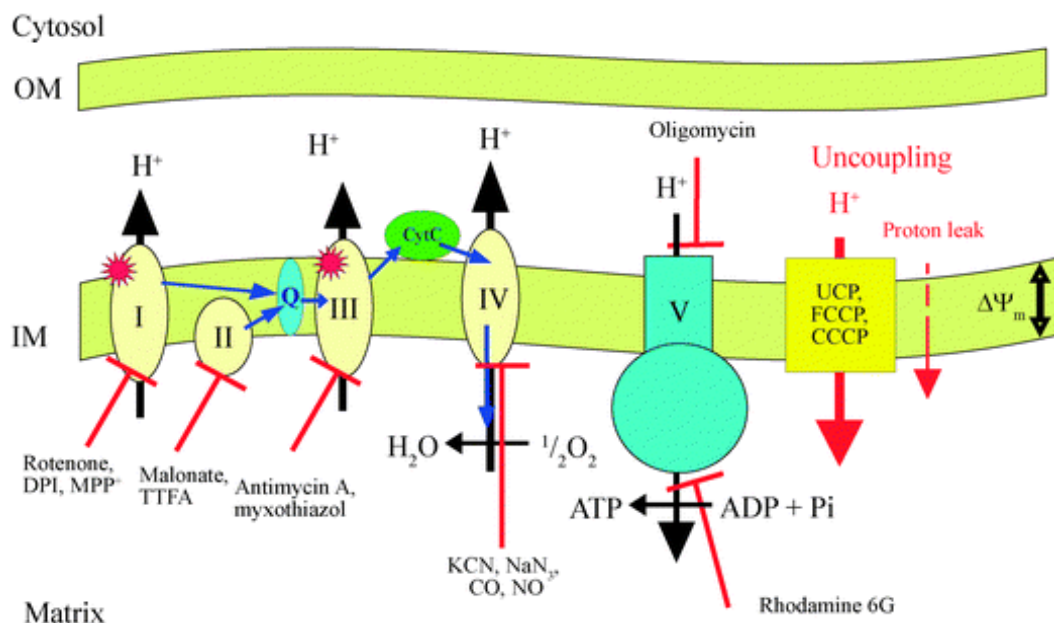
involved in familial cases possibly playing a role also in sporadic PD. Importantly, many converge on mitochondria dysfunction (Ryan *et al.*, 2015).

Also mitochondrial uncouplers such as CCCP have proven really useful to identify important mechanisms regulating mitochondrial homeostasis. For instance, the use of CCCP helped in the identification of the role of PINK1 and parkin in mitophagy and mitochondrial quality control (Narendra *et al.*, 2008; Narendra *et al.*, 2010; Ashrafi & Schwarz, 2013). As mentioned above, mutations in both PINK1 and parkin cause familial Parkinsonism.

An important limitation that has to be taken into account when utilising these toxins is off-target effects. This makes it difficult to define whether the observed phenotype is related to mitochondrial toxicity or their off-target actions, or a combination of both. For instance, recent studies suggest that dopaminergic neuron death caused by rotenone might be independent of mitochondrial complex-1 inhibition (Choi *et al.*, 2008; Choi *et al.*, 2011). Moreover, CCCP has been shown to interfere with lysosomal function in autophagy (Weinert *et al.*, 2010; Padman *et al.*, 2013). In addition, acute models of PD caused by MPTP and rotenone administration are often criticized because they do not fully reproduce the signs and the slow progression of the pathology seen in humans. Nevertheless, there are several lines of evidence confirming the importance that studies on these toxins have. First, it was recently confirmed that exposure to rotenone increases the risk of developing PD in humans (Tanner *et al.*, 2011). Of note, a recent report shows that changes in the transcriptome in iPSC-derived human dopaminergic neurons from PD patients are similar to those found in post-mortem samples and those induced by rotenone treatment (Sandor *et al.*, 2017). Finally, researchers started working intensively on the link between PD and mitochondria after the MPTP discovery. After many years of research, the role of mitochondrial dysfunction in PD is widely accepted.

Despite years of research, there are still some important unanswered questions on the mechanism of action of these toxins. For instance, why dopaminergic neurons are more sensitive to rotenone is still unclear. For MPTP this is easily explained by its conversion into its toxic form MPP⁺ by glial cells (Langston *et al.*, 1984; Markey *et al.*, 1984) and its transport into dopaminergic neurons via

the dopamine transporter (Javitch *et al.*, 1985). However, rotenone is a lipophilic molecule that can cross the plasma membrane and enter in other cells other than dopaminergic neurons. Therefore, further investigations on the mechanism of action of these molecules could provide important clues on what makes dopaminergic neurons so sensitive to mitochondrial impairment.



Graphical representation of the mitochondrial electron transport chain; the sites of action of mitochondrial toxins are shown.

5.1.3 Wallerian-like degeneration and mitochondrial toxins

Studies *in vitro* and *in vivo* have shown that regulators of Wallerian degeneration can protect against toxicity caused by mitochondrial toxins. For instance, WLD^S mice are protected against nigrostriatal degeneration after acute administration of MPTP (Hasbani & O'Malley, 2006). Similar results are seen after injection of 6-hydroxydopamine (6-OHDA) (Sajadi *et al.*, 2004; Cheng & Burke, 2010). In primary dopaminergic neuronal cultures treated with MPP⁺, WLD^S preserves neurites and promotes neuronal survival (Antenor-Dorsey & O'Malley, 2012). Overexpression of NMNAT1 and 3 and deletion of SARM1 delay axonal degeneration caused by rotenone in DRG neurons (Press & Milbrandt, 2008; Summers *et al.*, 2014). SARM1 deletion has been shown to be

protective also against CCCP toxicity (Summers *et al.*, 2014). Finally, zebrafish expressing human wild-type α -synuclein show preservation of axons by WLD^s (O'Donnell *et al.*, 2013). As α -synuclein is required for MPTP toxicity in dopaminergic neurons (Dauer *et al.*, 2002; Thomas *et al.*, 2011), this suggests common pathway activated in both models.

5.2 Hypothesis and aims of this chapter

As stated in the introduction of this thesis, the term Wallerian-like degeneration refers to axon degeneration occurring in the absence of a cut which can be delayed by WLD^s or other regulators of Wallerian degeneration. We have now identified several regulators of Wallerian degeneration and data in chapter 3 and 4 of this thesis, in addition to recent studies, suggest that they cooperate in a core pathway regulating axon death (Conforti *et al.*, 2014; Gilley *et al.*, 2015; Gerdtz *et al.*, 2016). Therefore, we can now go further and test the activation of the pathway (and the chronological order of events) rather than focusing on testing the protective abilities of single genetic modifications or pharmacological treatments. This is important since it enables us to determine more accurately which insults or diseases share mechanistic features with Wallerian degeneration. In this chapter, I will test the hypothesis that mitochondrial dysfunction caused by CCCP and rotenone initiates an axon death pathway in uninjured axons similar to Wallerian degeneration. The aims of this chapter are as follows:

- To determine whether pharmacological and genetic interventions previously shown to delay Wallerian degeneration are also protective against axonal degeneration caused by mitochondrial toxins.
- To test whether CCCP and rotenone treatment lead to depletion of axonal NMNAT2.
- To assess whether reduced levels of NMNAT2 makes axons more vulnerable to these toxic insults.

5.3 Results

5.3.1 CCCP and rotenone cause degeneration of uninjured axons

As mentioned in the introduction of this chapter, CCCP and rotenone are two extensively studied toxins used to impair mitochondrial function. Previous studies showed that they both induce axonal degeneration in primary neuronal cultures (Press & Milbrandt, 2008; Summers *et al.*, 2014). First, I performed a dose-response experiment to identify the optimal concentration for each drug to trigger axonal degeneration in SCG explant cultures. Consistent with previous reports (Summers *et al.*, 2014), CCCP and rotenone caused “dying-back” axon degeneration (Fig. 5.1). CCCP exhibited a clear dose-dependent effect, with the lowest concentration tested (25 μ M) causing modest toxicity at 24 hours and the highest (100 μ M) causing axonal degeneration as soon as 4 hours after administration (Fig. 5.1A and B). 50 μ M CCCP (same concentration used in chapter 4 of this thesis to induce full mitochondrial depolarization) consistently promoted axonal degeneration 24 hours after administration (Fig. 5.1A and B). Rotenone displayed a similar dose-dependent effect (Fig. 5.1C and D), with 10 μ M rotenone leading to full axonal fragmentation at 24 hours (Fig. 5.1 C and D). However, in contrast to CCCP, increasing concentrations of rotenone did not further accelerate the degeneration process (Fig. 5.1C and D). For the continuation of the study, I have selected the concentrations that reproducibly caused axon degeneration 24 hours after treatment (50 μ M CCCP and 10 μ M rotenone).

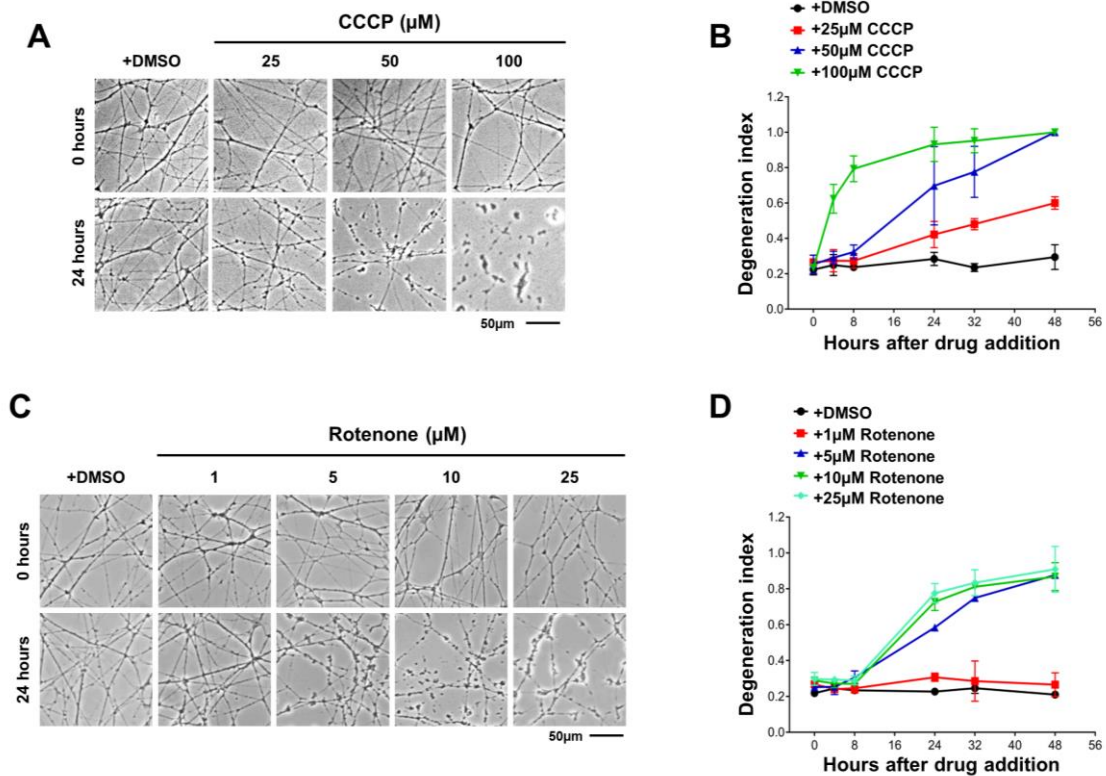


Figure 5.1 CCCP and rotenone cause axon degeneration in uninjured axons; dose-response curves.

(A-C) Wild-type SCG neurons were treated with increasing concentration of CCCP and rotenone. Representative phase contrast images acquired at the indicated time points after drug addition. **(B-D)** Degeneration index was calculated from 3 fields per sample in 2 independent experiments (Mean $\pm$ SD; n=2).

5.3.2 Interventions that delay Wallerian degeneration also strongly delay axonal degeneration caused by mitochondrial toxins

Next, I tested whether the degeneration caused by CCCP and rotenone administration follows a “Wallerian-like” mechanism. For this purpose, I used a number of pharmacological and genetic interventions that have been shown to delay axon degeneration following an injury. First, I blocked NMN synthesis with NAMPT inhibitor FK866. As it is the case in Wallerian degeneration ([Sasaki et al., 2009b](#); [Di Stefano et al., 2015](#)), FK866 treatment strongly delayed axonal degeneration following CCCP and rotenone administration in uninjured SCG neurons. Of note, co-administration of exogenous NMN reverted FK866

protection (Fig. 5.2A-D) in the same way that it reverts FK866-mediated protection of severed axons (Di Stefano *et al.*, 2015).

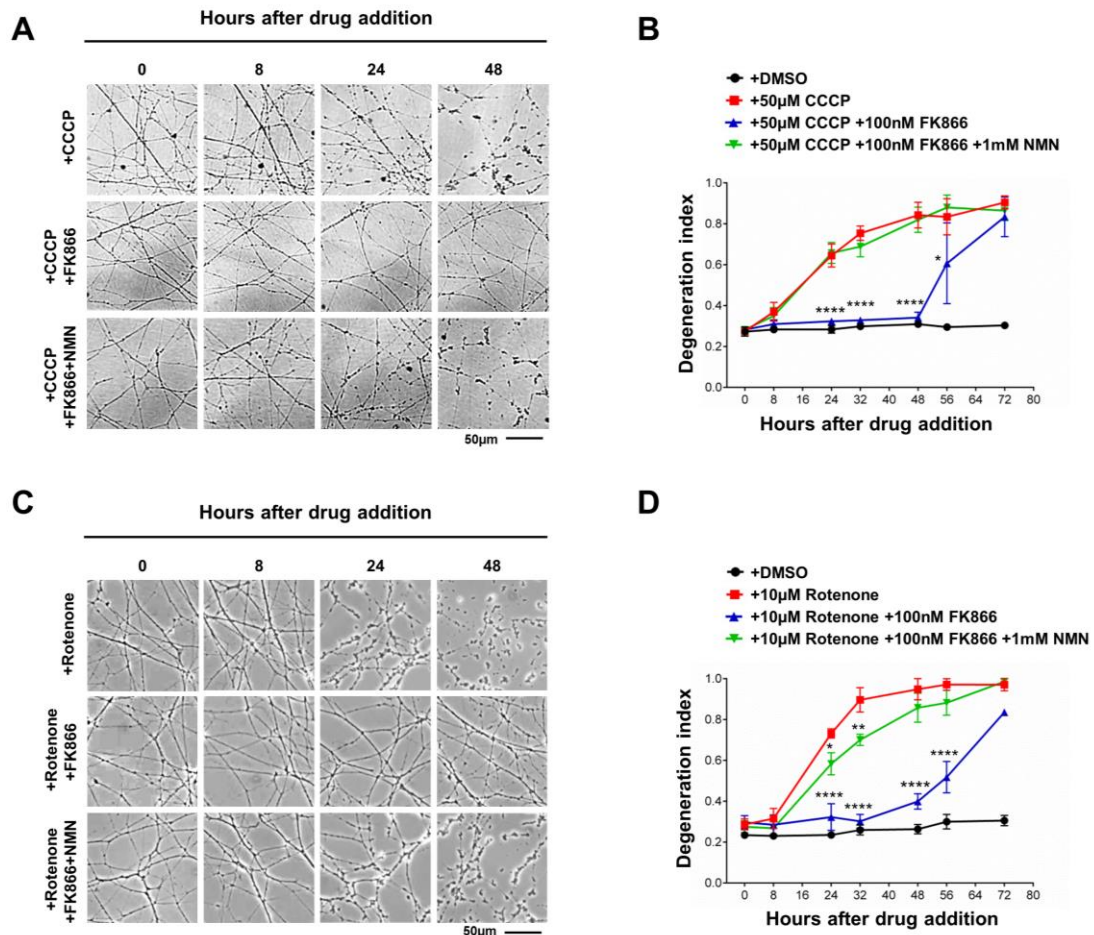


Figure 5.2 NMN accumulation promotes axon degeneration caused by CCCP and rotenone.

(A-C) Wild-type SCG neurons were treated with CCCP and rotenone. Where indicated, FK866 and NMN were added at the same of CCCP and rotenone. Representative phase contrast images acquired at the indicated time points after drug addition. (B-D) Degeneration index was calculated from 3 fields per sample in 4 independent experiments (Mean±SEM; n=4; two-way ANOVA followed by Bonferroni post-hoc test; ****, p<0.0001; **, p<0.01; *, p<0.05. Statistical significance shown relative to +50μM CCCP and +10μM rotenone).

Next, I tested whether SCG neurons of *Wld^S* and *Sarm1^{-/-}* mice are protected against mitochondrial dysfunction-induced axonal degeneration. As previously reported, overexpression of NMNAT1 and 3 conferred protection against

rotenone-induced axon degeneration (Press & Milbrandt, 2008). Similarly, SARM1 deletion has been shown to delay degeneration of DRG axons following CCCP and rotenone treatment (Summers *et al.*, 2014). Consistent with these studies, WLD^S expression and SARM1 deletion conferred remarkable protection against CCCP toxicity in SCG neurons (Fig. 5.3A-D). The strength of protection exceeded that of FK866. A previous study showed that CCCP toxicity in uninjured axons could not be rescued by WLD^S (Ikegami & Koike, 2003). However, in our hands the concentration used in their study (100 μ M CCCP) was extremely toxic (Fig. 5.1A and B).

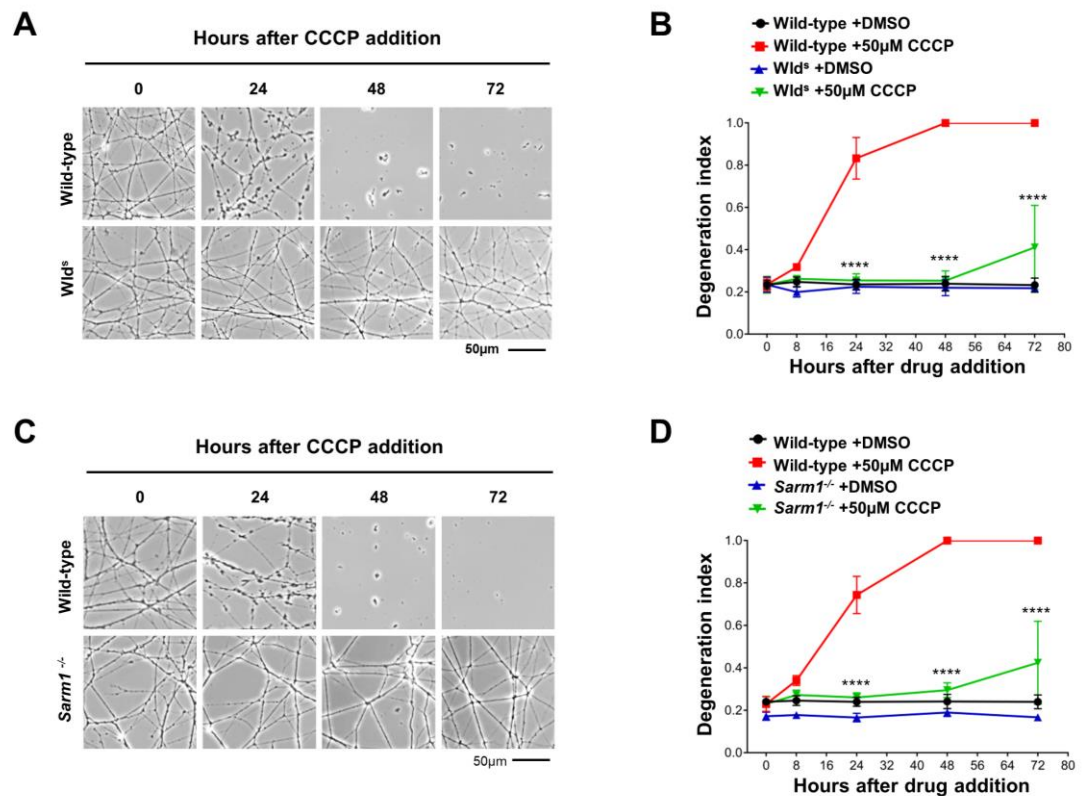


Figure 5.3 WLD^S expression and SARM1 deletion strongly protect against CCCP toxicity. (A-C) Wld^S and Sarm1^{-/-} SCG neurons were treated with CCCP. Representative phase contrast images acquired at the indicated time points after drug addition. (B-D) Degeneration index was calculated from 3 fields per sample in 4 independent experiments (Mean $\pm$ SEM; n=4; two-way ANOVA followed by Bonferroni post-hoc test; ****, p<0.0001. Statistical significance shown relative to wild-type +50 μ M CCCP).

Surprisingly, preliminary experiments suggested that WLD^S conferred only weak protection against rotenone toxicity, whereas SARM1 deletion was still remarkably protective (Fig. 5.4A-D).

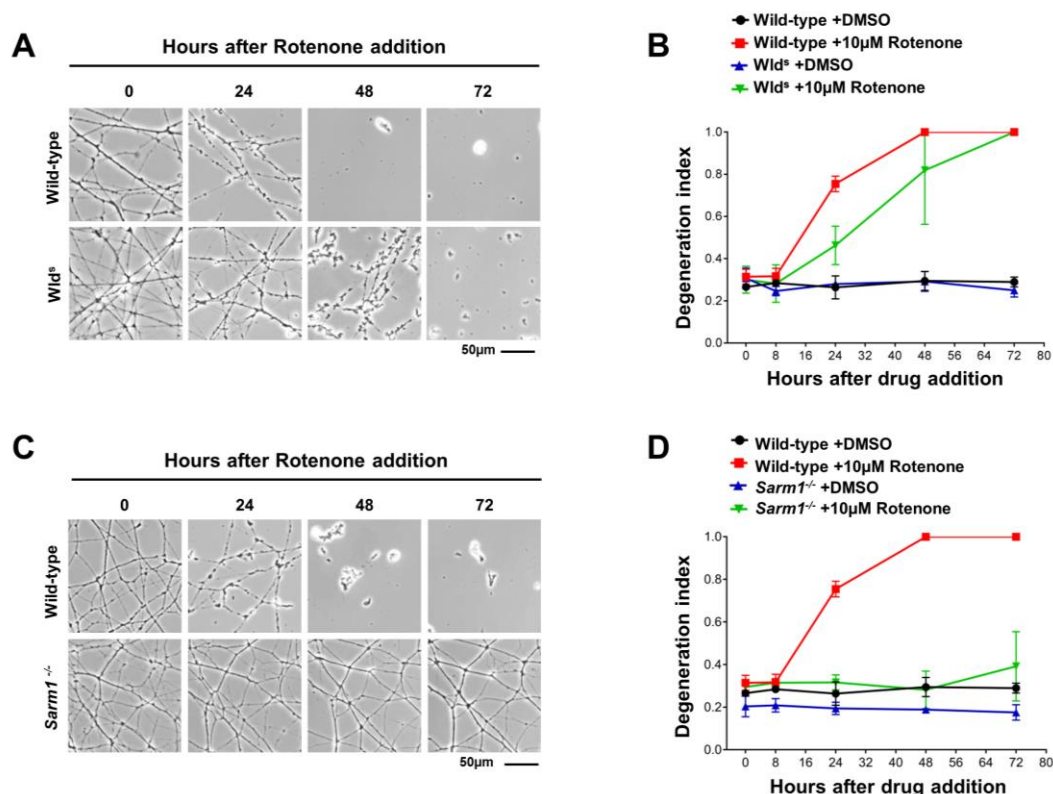


Figure 5.4 Different strength of protection against rotenone toxicity achieved with WLD^S expression and SARM1 deletion.

(A-C) Wld^S and *Sarm1*^{-/-} SCG neurons were treated with rotenone. Representative phase contrast images acquired at the indicated time points after drug addition. **(B-D)** Degeneration index was calculated from 3 fields per sample in 2 independent experiments (Mean±SD; n=2).

Taken together, these data suggest that the degeneration of axons following mitochondrial impairment can be delayed by regulators of Wallerian degeneration and indicate a toxic role of NMN accumulation also in these experimental models. They also reveal a difference between the treatment with rotenone and CCCP which will be further explored.

5.3.3 Mitochondrial depolarization leads to depletion of axonal NMNAT2

Our model predicts that axonal integrity after injury is lost as a consequence of NMNAT2 depletion in the axon, followed by accumulation of NMN and the activation of downstream steps in the pathway (Fig. 4.12) (Gilley & Coleman, 2010; Di Stefano *et al.*, 2015). I therefore tested whether NMNAT2 depletion also occurs following CCCP treatment in uninjured axons. I found that axonal levels of NMNAT2 rapidly decline starting 2 hours after CCCP addition (Fig. 5.5A). Importantly, loss of NMNAT2 occurs long before the appearance of visible morphological damage to axons (Fig. 5.5C). Preliminary data showed that NMNAT2 levels dropped also in CCCP-treated axons protected with FK866 (Fig. 5.5D). This is consistent with the predicted protective action of FK866 being that of limiting NMN accumulation downstream of NMNAT2 depletion (Di Stefano *et al.*, 2015). I also found a reduction in SCG10 levels (Fig. 5.5B), another short-lived protein involved in Wallerian degeneration (Shin *et al.*, 2012) (see chapter 4). In contrast, the levels of the NMN-synthesizing enzyme NAMPT remained stable up to at least 8 hours after CCCP addition (Fig. 5.5B). This is important, since NAMPT expression is required to synthesize and lead to the toxic accumulation of NMN following NMNAT2 loss.

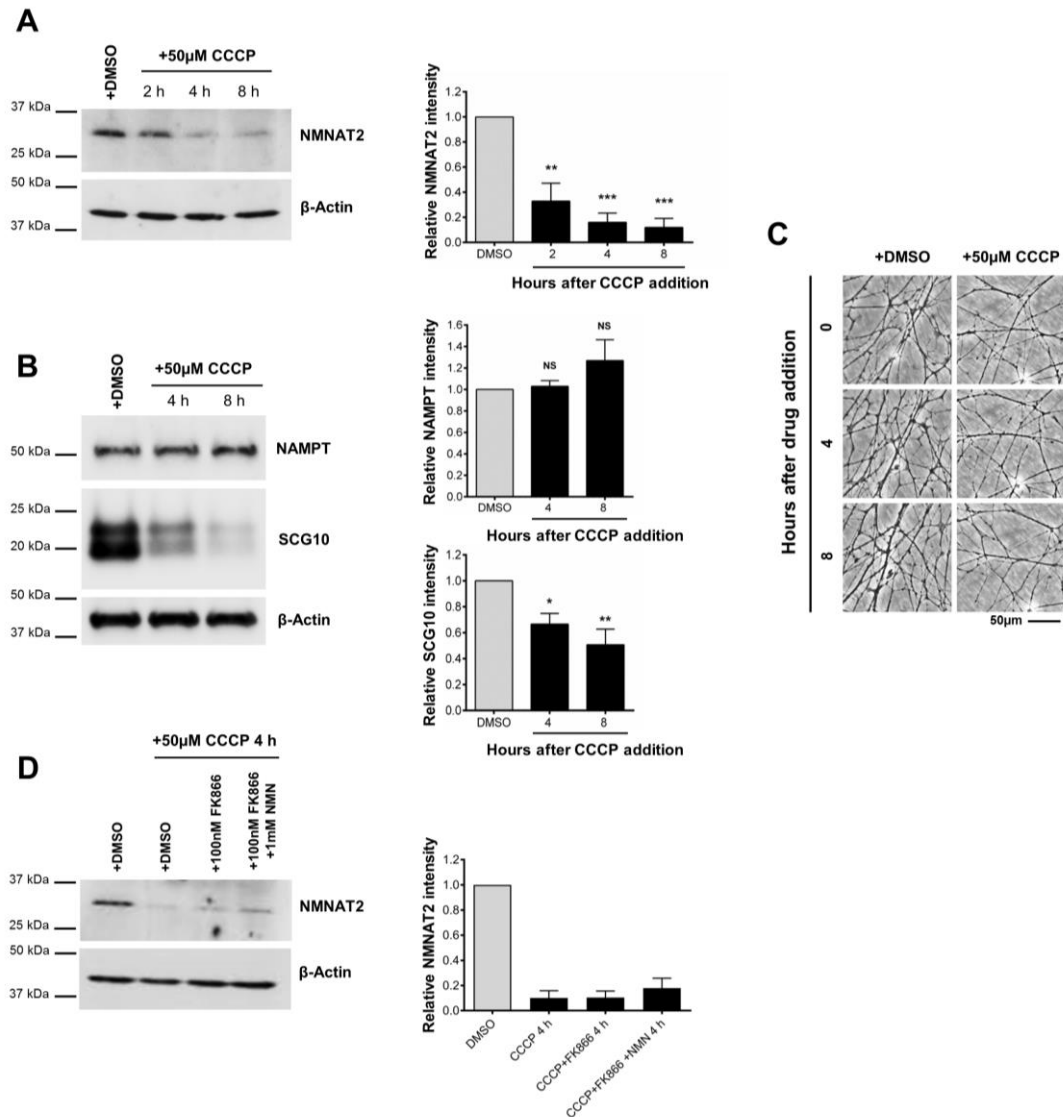


Figure 5.5 CCCP administration leads to NMNAT2 depletion in axons.

(A) Representative immunoblot of wild-type SCG axonal extracts after treatment with CCCP at the indicated time points probed for NMNAT2 and β -actin (loading control). Quantification of normalised NMNAT2 levels (to β -actin) is shown on the right, with data presented relative to uncut (set at 1) (Mean $\pm$ SEM; n=3; one-way ANOVA followed by Bonferroni post-hoc test; ***, $p < 0.001$, **, $p < 0.01$). **(B)** Representative immunoblot of wild-type SCG axonal extracts after treatment with CCCP at the indicated time points probed for NAMPT, SCG10 and β -actin (loading control). Quantification of normalised NAMPT and SCG10 levels (to β -actin) is shown on the right, with data presented relative to uncut (set at 1) (Mean $\pm$ SEM; n=4; one-way ANOVA followed by Bonferroni post-hoc test; ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, NS, non-significant). **(C)** Representative phase contrast images showing morphologically intact axons at the time points used for immunoblot. **(D)** Representative immunoblot of wild-type SCG axonal extracts 4 hours after treatment with CCCP and, where indicated, with FK866 and NMN, probed for NMNAT2 and β -actin (loading control). Quantification of normalised NMNAT2 levels (to β -actin) is shown on the right, with data presented relative to uncut (set at 1) (Mean $\pm$ SD; n=2).

As mentioned in the introduction of this thesis, knocking out NMNAT2 in mice leads to severe axon growth defect and perinatal lethality. However, mice expressing NMNAT2 protein at ~25% of wild-type levels are born and develop healthy (Gilley *et al.*, 2013), although they display an age-related locomotor defect and a reduced number of axons in peripheral nerves (Gilley *et al.* unpublished). We recently hypothesized that continuous depletion of NMNAT2 would eventually lead to its levels dropping below a critical threshold with consequent activation the degeneration pathway (Gilley & Coleman, 2010; Di Stefano *et al.*, 2015). If this is true, axons expressing lower levels of NMNAT2 might be more vulnerable to toxic insults leading to axon degeneration. To test this, I treated SCG neurons expressing levels of NMNAT2 down to 50% ($Nmnat2^{+/gtE}$) and 25% ($Nmnat2^{gtBay/gtE}$) of wild-type levels (Gilley *et al.*, 2013) with CCCP. Remarkably, I found a significant, dose-dependent acceleration of the degeneration process, with the first signs of morphological damage appearing as early as 4 hours in axons with a 4-fold reduction in NMNAT2 levels (compared to 12 hours in wild-type axons) (Fig. 5.6A and B).

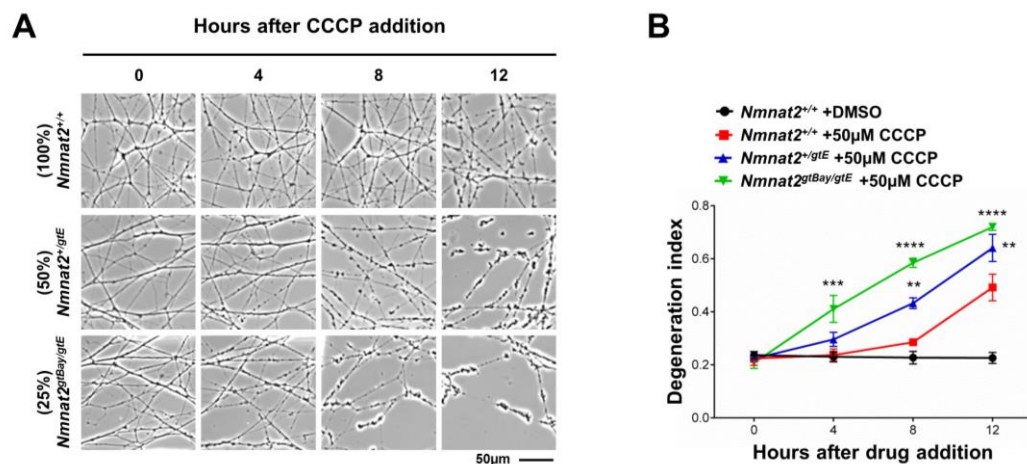


Figure 5.6 Axons expressing lower levels of NMNAT2 are more vulnerable to CCCP toxicity.

(A) SCG neurons of mice expressing lower levels of NMNAT2 compared to wild-type were treated with CCCP. Representative phase contrast images acquired at the indicated time points after drug addition. (wild-type- $Nmnat2^{+/+}$ -100% expression, $Nmnat2^{+/gtE}$ -50% expression, $Nmnat2^{gtBay/gtE}$ -25% expression). **(B)** Degeneration index was calculated from 3 fields per sample in 4 independent experiments (Mean±SEM; n=4; two-way ANOVA followed by Bonferroni post-hoc test; ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$. Statistical significance shown relative to $Nmnat2^{+/+}$ + 50μM CCCP).

Finally, to understand when NMNAT2 levels drop below a critical threshold incompatible with axonal survival, I added FK866 at 4, 8 and 12 hours after CCCP addition. Preliminary data show that treatment with FK866 up to 8 hours after CCCP addition was still sufficient to confer full protection, suggesting that, despite the detected clear reduction of NMNAT2 (Fig. 5.5A), enough protein is present to limit the toxic accumulation of NMN (Fig. 5.7A and B). Surprisingly, FK866 addition 12 hours after CCCP, a time point where axons already showed morphological damage, arrested the degeneration from progressing further (Fig. 5.7A and B).

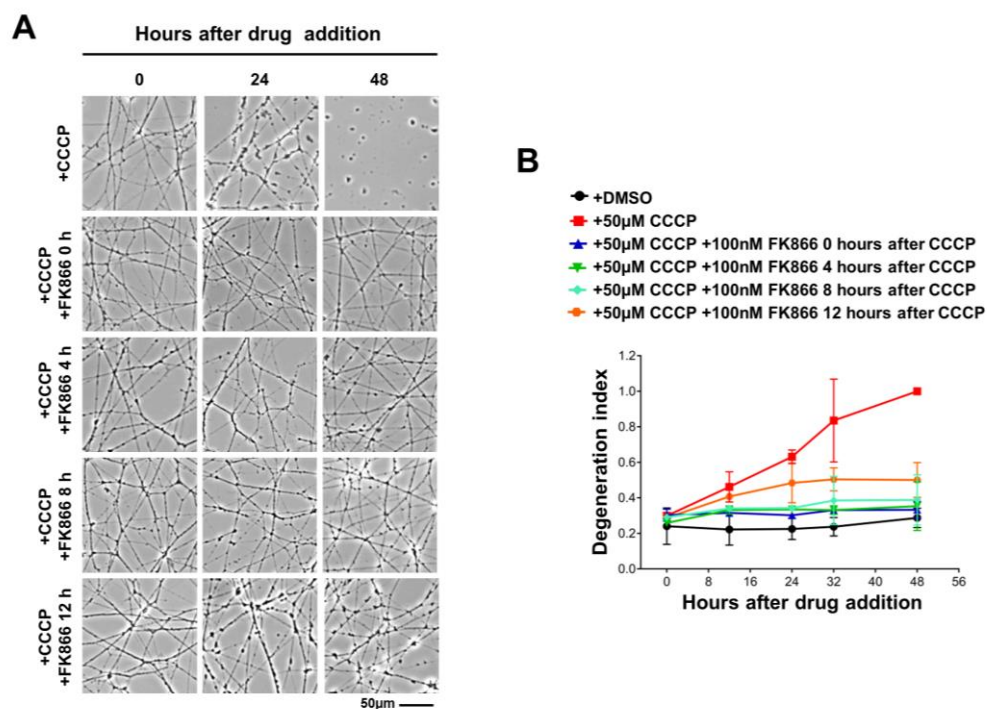


Figure 5.7 FK866 confers protection when added up to 12 hours after CCCP addition.

(A) Wild-type SCG neurons were treated with CCCP. Where indicated, FK866 was added at the specified time points after CCCP addition. Representative phase contrast images acquired at the indicated time points after drug addition. **(B-D)** Degeneration index was calculated from 3 fields per sample in 2 independent experiments (Mean±SEM; n=2).

Taken together, these data suggest that NMNAT2 depletion and the activation of the Wallerian degeneration pathway regulate axon death following mitochondrial depolarization.

5.3.4 Rotenone might cause late depletion of NMNAT2 in axons.

Whilst both CCCP and rotenone affect mitochondrial functionality and lead to axonal degeneration, the data above reveal differences in the mode of action of the two toxins. More specifically, the strength of WLD^S protection in the two experimental models is markedly different (Fig. 5.3 and 5.4). In addition, also the dose-response experiments show differences in the time course of the degeneration induced by CCCP and rotenone (Fig. 5.1). To further explore this, I tested whether rotenone promotes loss of axonal NMNAT2. Surprisingly, preliminary experiments showed that NMNAT2 levels remained relatively stable up to 12 hours following rotenone treatment (Fig. 5.8A). NMNAT2 levels might be reduced around 16 hours after rotenone addition (Fig. 5.8A). However, at this stage axons already showed signs of morphological damage (Fig. 5.8B). This might affect the outcome of the experiment and cause unequal loading of proteins on the gel.

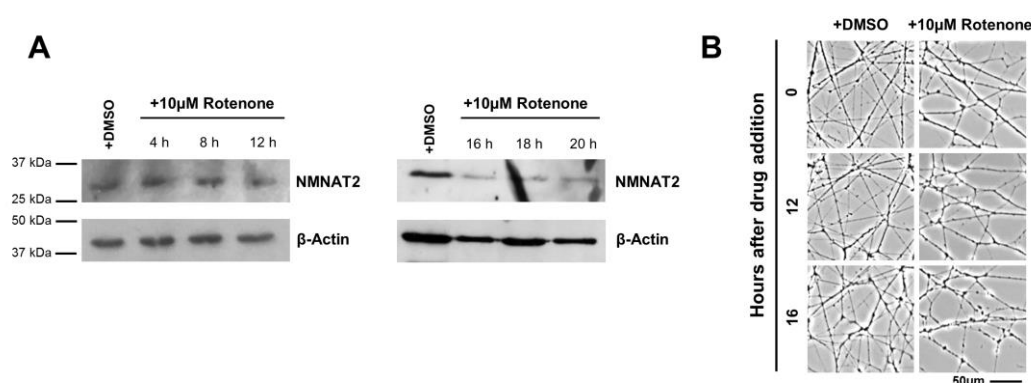


Figure 5.8 Rotenone might cause late depletion of NMNAT2 in axons.

(A) Representative immunoblot of wild-type SCG axonal extracts after treatment with rotenone at the indicated time points probed for NMNAT2 and β-actin (loading control). **(B)** Representative phase contrast images showing that axons appear morphologically intact at the time points used for Western blot.

I then tested whether reduced levels of NMNAT2 could accelerate degeneration also after rotenone treatment. Consistent with the western blot data, a preliminary experiment showed no differences at 12 hours after rotenone

addition (no changes in NMNAT2 levels at this time point), when axons were still intact (Fig. 5.9A and B). I did detect a modest difference at 16-20 hours, where axons with lower levels of NMNAT2 appeared more damaged (Fig. 5.9A and B).

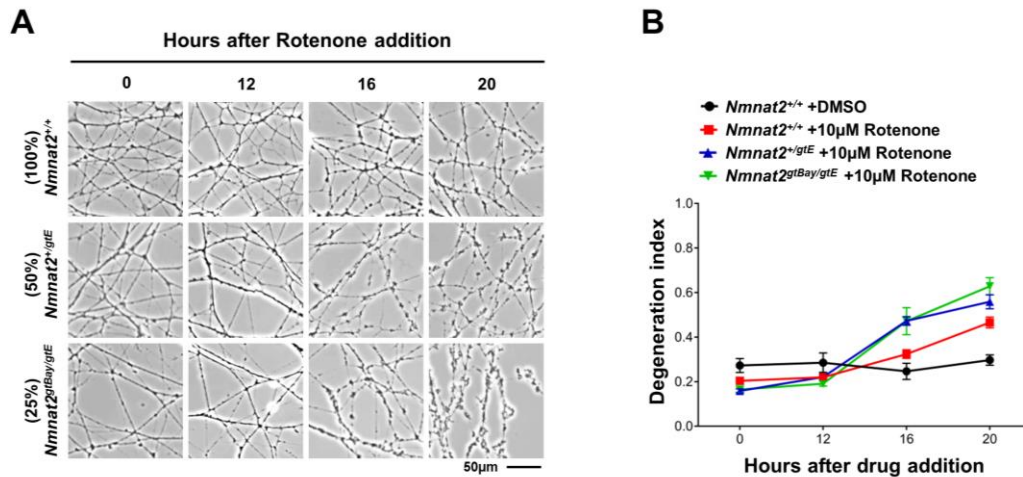


Figure 5.9 Axons expressing lower levels of NMNAT2 are marginally more vulnerable to rotenone toxicity.

(A) SCG neurons of mice expressing lower levels of NMNAT2 compared to wild-type were treated with rotenone. Representative phase contrast images acquired at the indicated time points after drug addition. (wild-type-*Nmnat2*^{+/+}-100% expression, *Nmnat2*^{+/gtE}-50% expression, *Nmnat2*^{gtBay/gtE}-25% expression). **(B)** Degeneration index was calculated from 3 fields per sample in 1 experiment (Mean±SD; n=1).

The data above suggests that rotenone might cause intracellular changes that activate the axon death pathway around 16 hours after treatment. Consistent with this and the western blot data, preliminary data showed that FK866 addition up to 12 hours after rotenone was still able to confer complete protection against axonal degeneration. As previously seen with CCCP, FK866 addition at 16 and 20 hours, when axons are already compromised, blocked the further progression to full fragmentation (Fig. 5.10A and B).

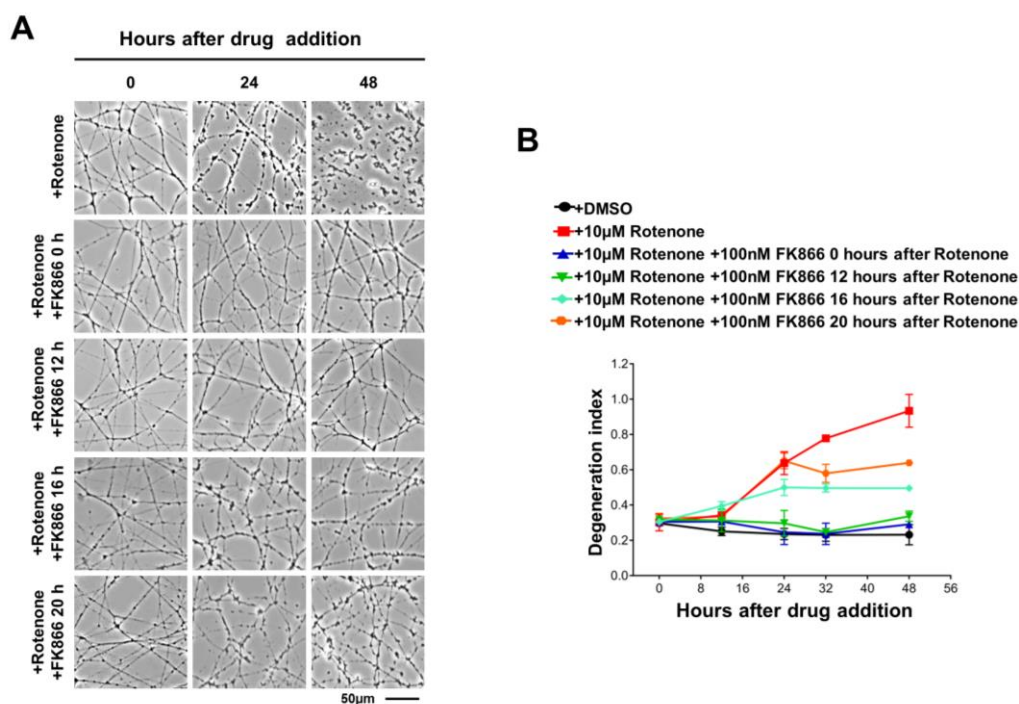


Figure 5.10 FK866 confers protection when added up to 20 hours after rotenone addition.

(A) Wild-type SCG neurons were treated with rotenone. Where indicated, FK866 was added at the specified time points after rotenone addition. Representative phase contrast images acquired at the indicated time points after drug addition. **(B)** Degeneration index was calculated from 3 fields per sample in 2 independent experiments (Mean±SEM; n=2).

Thus, although these data revealed some differences in the mode of action of rotenone and CCCP, axonal degeneration caused by these two mitochondrial toxins still converge on the activation of the Wallerian degeneration pathway.

5.3.5 Towards *in vivo* models of axonal disorders caused by mitochondrial dysfunction

The last experiments presented in this section are planned to be a step towards future *in vivo* studies of mammalian CNS neurons and of zebrafish to further investigate the link between mitochondrial dysfunction and Wallerian degeneration. A first, necessary step is to confirm that NMNAT2 loss and the consequent NMN rise are involved in the degeneration of CNS and zebrafish axons. In view of the data presented above, it will then be important to test whether mice expressing lower levels of NMNAT2 are more vulnerable to mitochondrial toxins or genetic mutations impairing mitochondrial function.

As a first approach to test whether the same mechanisms have a role in CNS neurons, I performed axotomy on primary cortical neurons. This is important since there are differences in the degeneration of CNS and PNS axons (as stated in chapter 1 of this thesis). Therefore, the molecular mechanisms leading to degeneration of PNS axons might differ from that of CNS axons. Of note, I found that FK866 administration also delayed degeneration of injured cortical neuron axons, and NMN addition reverted this protection (Fig. 5.11A and B), confirming that similar mechanisms control the degeneration of axons in the CNS, which are often primarily affected in several *in vivo* models of neurodegenerative disorders caused by mitochondrial dysfunction.

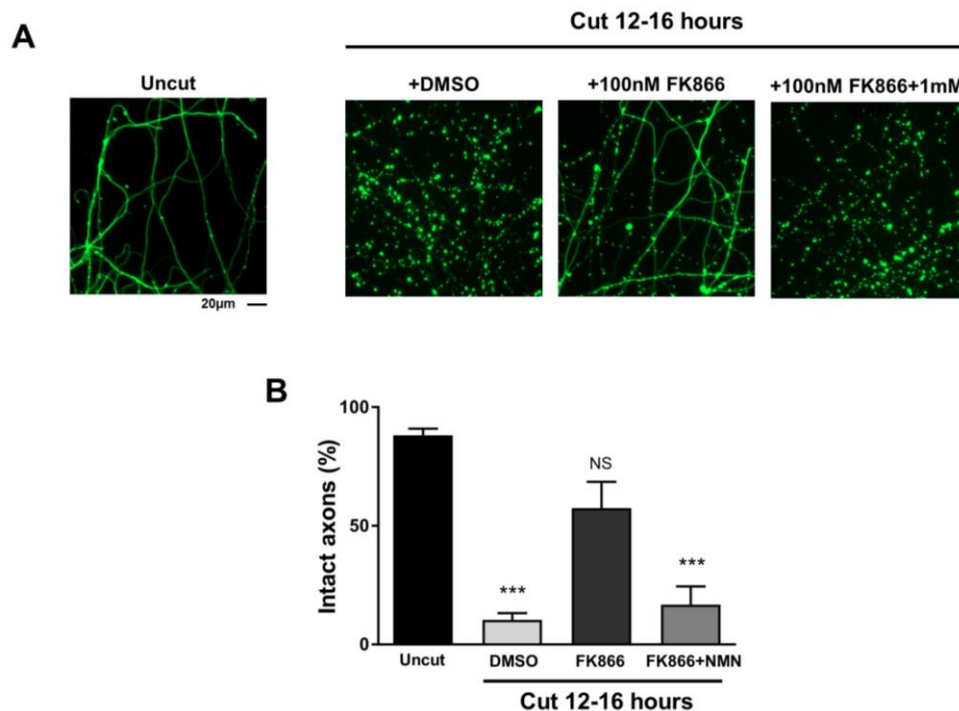


Figure 5.11 FK866 delays injury-induced axon degeneration in CNS axons.

(A) Representative fluorescent images of cortical neuron axons uncut or 12-16 hours after cut, treated as indicated. FK866 and NMN were added immediately after axotomy. Axons are stained for β -tubulin III. **(B)** The % of intact axons was calculated from 15 random fields per sample in 3 independent experiments. (Mean $\pm$ SEM; n=3; one-way ANOVA followed by Bonferroni post-hoc test; ***, p<0.001; NS, non-significant. Statistical significance shown relative to uncut).

In addition to mice, zebrafish can also be used to study the effects of mitochondrial dysfunction on axon degeneration *in vivo*. As mentioned in chapter 3, MPTP and rotenone treatment in zebrafish cause loss of dopaminergic neurons, decline in dopamine levels and locomotor defects (Bretaud *et al.*, 2004; Lam *et al.*, 2005; McKinley *et al.*, 2005; Wen *et al.*, 2008; Sallinen *et al.*, 2009; Wang *et al.*, 2017). In addition, knockdown of parkin in zebrafish is associated with dopaminergic neuronal cell loss and reduction in mitochondrial complex 1 activity (Flinn *et al.*, 2009). Impairment of dopaminergic neuron development and motor function is also present in PINK1 knockdown (Xi *et al.*, 2010) and PINK1 deficient zebrafish are more susceptible to MPTP toxicity (Sallinen *et al.*, 2010).

We previously showed that FK866 delays Wallerian degeneration in zebrafish larvae, likely through inhibition of NMN accumulation and a subsequent rise in Ca^{2+} (Fig. 3.8). To confirm the role of NMN accumulation *in vivo* also in this experimental model, the bacterial enzyme NMN deamidase was transiently expressed (along with DsRed) in trigeminal and Rohon-beard somatosensory neurons. Axons were cut 48–54 hours post-fertilization using two-photon laser axotomy (Fig. 5.12A). Control larvae displayed a normal rate of axon degeneration after injury (expressed as lag phase (Martin *et al.*, 2010)), usually completed within 2 hours under these experimental conditions (Fig. 5.12B and C; Movie S9). In contrast, NMN deamidase blocked injury-induced axon degeneration up to at least 12 hours, similar to the previously reported effects of WLD^s (Martin *et al.*, 2010). A few axons (n=3) were followed up to 24 hours and were found to remain intact (Movie S10). Axon regrowth from the site of injury confirmed successful axon transection and that the zebrafish larvae were viable for the duration of the experiment.

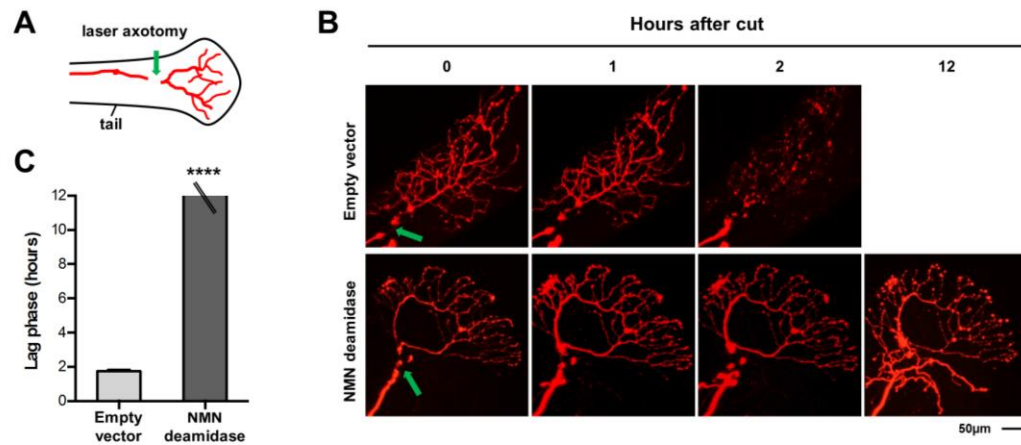


Figure 5.12 NMN deamidase blocks Wallerian degeneration in zebrafish larvae.

(A) Schematic representation of larval zebrafish tail showing the location of the sensory (Rohon-Beard) axon and the site of injury (green arrow). **(B)** One-cell-stage embryos were injected with plasmids for DsRed and NMN deamidase or empty vector. Representative time-lapse fluorescent images show DsRed-expressing axons in empty vector or NMN deamidase embryos after cut (green arrow shows the location of cut site). **(C)** Length of the lag phase (time between the cut and the first sign of degeneration) in control or NMN deamidase-expressing axons (Mean $\pm$ SEM; empty vector $n = 9$, NMN deamidase $n = 8$; Student's t test, **** $p < 0.0001$).

Drug screenings and mechanistic studies can be carried out in zebrafish embryos, since their aqueous environment facilitates drug delivery. Moreover, rapid genetic manipulations are possible by microinjecting expression constructs or morpholinos into single cell embryos. Finally, zebrafish can be made transparent to carry out live imaging. Therefore, zebrafish are potentially a good model that we could adopt for further *in vivo* investigations.

5.4 Discussion

In chapter 4, we suggest that mitochondrial contribution to axon degeneration after axotomy is confined to late stages of the degeneration process. The data in this chapter suggest, instead, that the mitochondrial toxins CCCP and rotenone cause axon degeneration in uninjured neurons through the activation of the same pathway controlling Wallerian degeneration. Whilst these data may seem contradictory, results in this chapter essentially reinforce the conclusion in chapter 4. In fact, our data indicate that mitochondrial dysfunction is an alternative insult to injury to activate the axon death pathway upstream of NMNAT2 (Fig. 5.13).

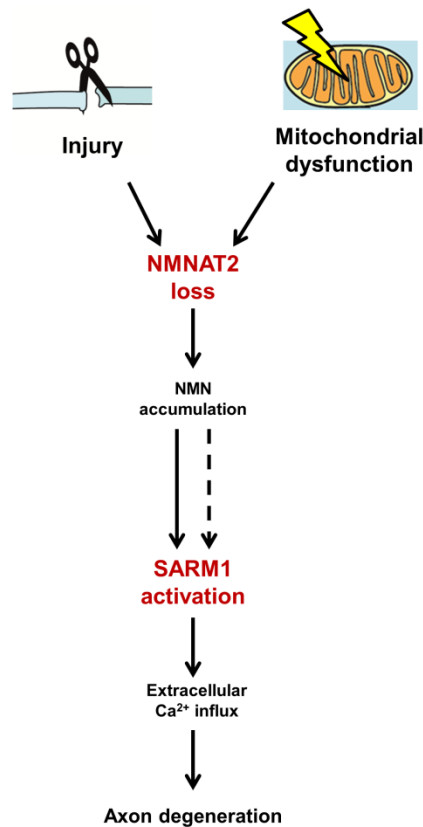


Figure 5.13 Mitochondrial dysfunction activates the same pathway controlling Wallerian degeneration upstream of NMNAT2.

Most regulators of Wallerian degeneration (and the one tested in this thesis) act downstream of NMNAT2 depletion. Therefore, impairment of mitochondrial function during Wallerian degeneration, where the pathway is already activated by injury itself, does not affect their protective functions.

Whilst these results suggest that axonal degeneration after mitochondrial impairment follows a Wallerian-like mechanism, they also reveal important differences on the mechanism of action of the two mitochondrial toxins used. CCCP administration replicates all the steps leading to axon degeneration after injury, from early depletion of NMNAT2 to sensitivity to FK866, WLD^S and SARM1 deletion. In addition to NMNAT2 loss, also SCG10 is rapidly lost after mitochondrial depolarization. Intriguingly, both SCG10 and NMNAT2 partially localise to Golgi/Golgi-derived vesicles via palmitoylation (Di Paolo *et al.*, 1997; Lutjens *et al.*, 2000), suggesting a similar degradation mechanism (Milde *et al.*, 2013b). In contrast, rotenone does not promote a rapid loss of NMNAT2. NMNAT2 depletion might still occur at later stages, just prior to fragmentation, making it difficult to understand whether it is simply a consequence of the ongoing degeneration process. Since NMNAT2 depletion still occurs after injury in *Sarm1*^{-/-} and FK866 treated axons (Di Stefano *et al.*, 2015; Gilley *et al.*, 2015), it will be important to repeat these experiments in the context of rotenone toxicity to assess whether NMNAT2 depletion still occurs around 16 hours also in protected axons.

To our surprise, we found that WLD^S confers weaker protection compared to SARM1 deletion against rotenone toxicity. To our knowledge, this is the first toxic condition where SARM1 deletion and WLD^S effect is so strikingly different. Whilst this might indicate a context-specific mechanism of action of the two proteins, with rotenone initiating an alternative pathway that converge of SARM1 independently of NMNAT2 and NMN, the fact that also FK866 protects is puzzling. FK866 is predicted to act by mimicking WLD^S action and limiting NMN accumulation (Di Stefano *et al.*, 2015). Moreover, the opposite result would have been easier to explain (weaker protection of SARM1 deletion) since the presence of WLD^S in the nucleus might impact on pathways unrelated to Wallerian degeneration and SARM1. This raises the possibility that rotenone affects NMNAT enzymatic activity in both isoforms rather than the levels of

NMNAT2. This could explain the weaker protection achieved with WLD^S compare to FK866 administration and SARM1 deletion. Rotenone is also well known to induce an increase in the production of ROS, which could lead to direct damage to protein with several different mechanisms (Birben *et al.*, 2012). Of note, increasing concentrations of rotenone do not further accelerate the degeneration process. This unusual dose-response supports an effect of rotenone on NMNAT enzymatic activity. This hypothesis will be tested in future studies. Previous reports showed that over-expression of NMNAT1 and 3 delays rotenone-induced toxicity. The discrepancies with our results might be related to the levels of expression of the protective proteins, which is likely to be much higher after viral transfection (Press & Milbrandt, 2008). Importantly, the number for some of the experiments with rotenone is low (1-2), therefore some of the observation need to be confirmed before any definitive conclusion can be drawn.

Further experiments are also needed to clarify how CCCP leads to NMNAT2 depletion. Blockage of axonal transport, reduced synthesis and increased degradation of NMNAT2 or combinations of the three are most obvious possibilities to explain the observed loss. ATP depletion could affect each of the three processes described above (Hackney, 1994; Lecker *et al.*, 2006; Proud, 2007; Zala *et al.*, 2013; Maday *et al.*, 2014). In addition, ATP is also needed for NMNATs enzymatic activity (Di Stefano & Conforti, 2013). Both CCCP and rotenone are well known to reduce ATP production and have been described to decrease ATP in primary neurons (Summers *et al.*, 2014; Yang *et al.*, 2015). However, despite the common action on ATP levels, the different impact of rotenone and CCCP treatment on NMNAT2 levels argues against ATP depletion as the first cause of NMNAT2 loss. Nevertheless, ATP levels have not been measured directly in our study, and differences to the extent of ATP reduction between the two treatments could account for some of the dissimilarities.

Whilst both rotenone and CCCP promote Wallerian-like degeneration, the differences on how they lead to degeneration raise the questions of whether their effects on mitochondria are critical to induce axon degeneration. Moreover, in a different study, axon degeneration induced by depletion of axonal

mitochondria (Fang *et al.*, 2012; Rawson *et al.*, 2014) is in contrast insensitive to NMNAT/WLD^s pathway (Fang *et al.*, 2012). Since there are several different methods to impair mitochondrial function, it is still possible that only specific types of insult impairing mitochondrial function will converge on the Wallerian degeneration pathway, with CCCP and rotenone falling among them. The findings that exposure to rotenone is associated with a higher risk to develop Parkinson's disease (Tanner *et al.*, 2011) makes our findings exciting, since the activation of the axon death pathway might explain axon loss occurring in some PD patients. Understanding whether the increased sensitivity and the death of dopaminergic neurons following rotenone administration is related to regulators of Wallerian degeneration is the next question.

A widespread concept in the Wallerian degeneration field is that, during the latent phase, degeneration can be delayed till axons reach a step where they commit to degenerate prior to fragmentation (Conforti *et al.*, 2014). Whilst this is probably true (or difficult to test correctly) for Wallerian degeneration *in vitro*, where axons degenerate rapidly, it might not be the case in the presence of insults that promote a slower degeneration process. In support of this, we find that FK866 blocks further degeneration when added to axons that are already damaged both after CCCP and rotenone administration. This is important, since it suggests that steps leading to axon degeneration might be reversible (e.g. SARM1 activation). In the context of a disease this could be of critical importance, since the activation of the Wallerian degeneration pathway might be slower than after injury. Of note, FK866 confers complete protection also when added when levels of NMNAT2 are really low. This might indicate that the levels of NMNAT2 present are still sufficient to limit the toxic accumulation of NMN and block the activation of the axon death pathway. This is consistent with previous observations that mice with a 4-fold reduction of NMNAT2 levels are born healthy (Gilley *et al.*, 2013) and suggests that only very low levels of NMNAT2 are required to limit NMN accumulation. However, an alternative, feasible explanation is that the pathway is already active when NMNAT2 levels are reduced (e.g. 8 hours after CCCP addition), but the addition of FK866 reverses the process.

The finding that axons expressing lower levels of NMNAT2 are more susceptible to toxic insults is also important and might have significant implications in disease development and progression. A recent study showed that the levels of NMNAT2 mRNA in post-mortem brain samples vary hugely between individuals in the human population (up to 50-fold changes) (Ali *et al.*, 2016). If this is correct, it is possible, even likely, that some people might be at a higher risk of developing axonal disorders. Future studies *in vivo* in mice expressing lower levels of NMNAT2 might provide helpful insights on this.

In conclusion, the results in this chapter support the importance of NMNAT2 and NMN in controlling axonal health which goes beyond the simple regulation of Wallerian degeneration and potentially into mitochondrial disorders such as Parkinson's disease, Friedreich's ataxia and Charcot-Marie-Tooth type 2A. Future experiments are important to address some open questions. Among these, measuring changes in NMN and NAD levels in axons following mitochondrial impairment is a priority. Understanding whether rotenone is affecting NMNATs enzymatic activity is also important, as well as confirming these results in CNS neurons. Finally, developing a genetic method to specifically impair mitochondrial function (e.g. mitochondrial targeted endonucleases) could help clarifying whether the activation of the Wallerian degeneration pathway is specifically caused by mitochondrial dysfunction. If this is the case, the potential impact on several neurodegenerative disorders characterized by axonal loss and mitochondrial dysfunction could be significant.

CHAPTER 6

General discussion and future directions

6.1 NAD depletion and NMN accumulation: a deadly combination leading to axon degeneration? Latest developments in the Wallerian degeneration field

The data presented in this thesis, in addition to previous studies from our and other groups (Di Stefano *et al.*, 2015; Gilley *et al.*, 2015; Di Stefano *et al.*, 2017), provide additional evidence in support of a pro-degenerative role for NMN in axons. We originally proposed this model in response to two observations that contradicted the view that maintaining NAD levels is instead critical for axon survival. First, inhibition of NAMPT with FK866 delays Wallerian degeneration despite causing a concurrent decline in NAD levels. Second, expression of the bacterial NMN deamidase in primary neuronal cultures, *in vivo* in mice and, as reported in this thesis, in zebrafish phenocopy WLD^s after injury (Di Stefano *et al.*, 2015; Di Stefano *et al.*, 2017), despite there being no clear route for making NAD from its product, NaMN, once NMNAT2 depletion has occurred. The simplest explanation that can link these observations to the protective effect of NMNATs (which do maintain NAD levels) is through a shared ability to limit the accumulation of NMN (Fig. 6.1). However, a recent study from Sasaki, Milbrandt and colleagues showed that genetic and pharmacological manipulation of the NAD biosynthetic pathway in DRG cultures resulting in a rise of NMN levels delayed degeneration in injured axons and did not promote degeneration in uninjured axons. Whilst these data seem inconsistent with the NMN model, it is notable that all instances where raised NMN correlates with axonal preservation also involve considerable increases in NAD (Sasaki *et al.*, 2016). The authors of the study proposed that changes in other, as yet unknown, metabolites might cause axon degeneration (Sasaki *et al.*, 2016). However, the common protective mechanism of FK866, bacterial NMN deamidase, and mammalian NMNATs is more likely to be their known, shared influence on NMN than similar effects on another metabolite that has not yet been identified or shown to be similarly affected by all three manipulations. Moreover, often overlooked are genetic data indicating that FK866 protection is specifically associated to its inhibition of NAMPT (using FK866-resistant NAMPT mutants) and that exogenous addition of NMN itself initiates

degeneration when NMNAT2 is depleted (Di Stefano *et al.*, 2015). The results in this thesis further support previous conclusions. In fact, they show that exogenous addition of NMN triggers axonal degeneration through a SARM1 dependent Ca^{2+} rise, therefore successfully replicating the steps leading to degeneration of cut axons.

Until recently, SARM1 was believed to lack enzymatic activity, mainly because of the presence in its structure of the TIR domain, previously described to only mediate protein-protein interactions (Ve *et al.*, 2015). However, an elegant, recent study showed that SARM1-TIR does possess enzymatic activity, being a NADase that degrades NAD into ADP-ribose (ADPR) and cyclic-ADPR (Essuman *et al.*, 2017). This unexpected discovery makes SARM1 even more attractive as a therapeutic target, since the substrate-binding pocket of enzymes is generally regarded as easier to target with low molecular weight inhibitors than large protein-binding surfaces, and might explain why data on the role of NAD in Wallerian degeneration were often controversial and inconsistent. In fact, whilst these new findings suggest a direct involvement of NAD depletion in the degeneration pathway, they also show that SARM1-dependent NAD degradation is very rapid (Gerdtts *et al.*, 2015; Essuman *et al.*, 2017). Therefore, boosting NAD levels downstream of SARM1 activation is unlikely to be the most effective strategy to confer long lasting protection after injury (Fig. 6.1). Of note, ADPR and cyclic-ADPR are involved in the regulation of Ca^{2+} homeostasis (Fliegert *et al.*, 2007) and could play a role in the increase in intracellular Ca^{2+} downstream of NMN and SARM1 described in chapter 3 of this thesis.

Does NMNAT2 loss signal to activate SARM1 in a linear pathway or they converge on common downstream steps? A widely held view in the field is that full-length SARM1 does not possess constitutive activity, but gets activated in response to a signal after injury to promote axon degeneration. Whilst there is no direct proof of this yet, there are lines of evidence that support this view. First, no increase in NAD is detected in *Sarm1*^{-/-} tissues (Gerdtts *et al.*, 2015; Gilley *et al.*, 2015). Moreover, SARM1-dependent NAD degradation is only seen after injury and can be blocked (Sasaki *et al.*, 2016). The alternative possibility predicts that SARM1 is constitutively active (or gets activated by an NMNAT2-independent signal after injury), but the presence of NAD synthesizing enzymes

(e.g. NMNAT2 in uninjured axons or WLD^S/NMNATs in cut axons) could counteract NAD depletion downstream of SARM1 and act as an inhibitory signal on a convergent pathway (Fig. 6.1). However, the fact that the expression of an artificial active form of SARM1 is sufficient on its own to induce degeneration in uninjured axons (where NMNAT2 is still present) (Gerdts *et al.*, 2015), in addition to the strong protective effect of NMN deamidase and FK866 (both unable to maintain NAD levels high) (Di Stefano *et al.*, 2015; Di Stefano *et al.*, 2017) argues against this alternative model. Therefore, based on current data, we suggest the linear pathway, with NMNAT2 loss upstream of SARM1 activation, as the most likely (Fig. 6.1).

The next crucial step is identifying the signal that triggers SARM1 activation. NMN is a candidate, supported by data in this thesis showing that exogenous NMN addition fails to induce degeneration in the absence of SARM1. However, given the new reports showing that NMN accumulation alone is not sufficient to initiate degeneration (Sasaki *et al.*, 2016), we suggest that the best fit for all currently available data is a model involving both NMN and NAD. For example, the effects of accumulated NMN may be evident only in the context of physiological or declining NAD levels, as occurs in axons in the first few hours after injury subsequent to NMNAT2 loss (Sasaki *et al.*, 2016). Given their structural relatedness, high NAD could inhibit the pro-degenerative function of NMN directly. Intriguingly, since SARM1 binds NAD for its degradation, one could speculate that SARM1 might bind NMN as well, thus acting as a sensor for the variations in NAD/NMN ratio that might be responsible for its activation. This possibility will be addressed in future studies.

In conclusion, current knowledge suggests that the Wallerian degeneration pathway can be blocked upstream (targeting NMN accumulation) or downstream of SARM1 activation (counteracting the catastrophic SARM1-dependent NAD depletion). After injury, blocking steps upstream of SARM1 is likely to be the most effective strategy to delay degeneration. However, boosting NAD might still have a significant protective effect in pathological conditions different from injury, where the activation of the pathway is likely to be less dramatic.

6.1.1 Role for this pathway in soma survival?

Studies to date have focused predominantly on the axonal role of this pathway. There are no studies directly addressing the hypothesis that this pathway can also control the survival of the neuronal soma. However, there are some recent data that support this hypothesis. First, expression of the artificial active form of SARM1 does promote death of neuronal cell bodies as well as axons and also induces death of non-neuronal cells (Gerdts et al., 2015; Gerdts et al., 2013). Next, deletion of SARM1 also protects neuronal cell bodies against rotenone and CCCP toxicity (Summers et al., 2014).

The possibility that a similar pathway could be activated in the soma is considered unlikely since the presence of NMNAT1 in the nucleus is thought to compensate for an eventual loss of NMNAT2 in the cell body. In fact, NMN is believed to move freely between the cytosol and the nucleus (Canto et al., 2015), where the presence of NMNAT1 could limit its accumulation. Consistent with this, DRG neurons lacking both NMNAT2 and SARM1 show a marked increase of NMN in axons, but no increase in cell bodies (Gilley et al., 2015). Nevertheless, a recent study reported a decline of NMNAT1, 2 and 3 levels in the liver of old mice (Camacho-Pereira et al., 2016). It would be interesting to test, in future experiments, whether a combined reduction of the three NMNAT isoforms can promote the activation of the Wallerian degeneration pathway in the soma.

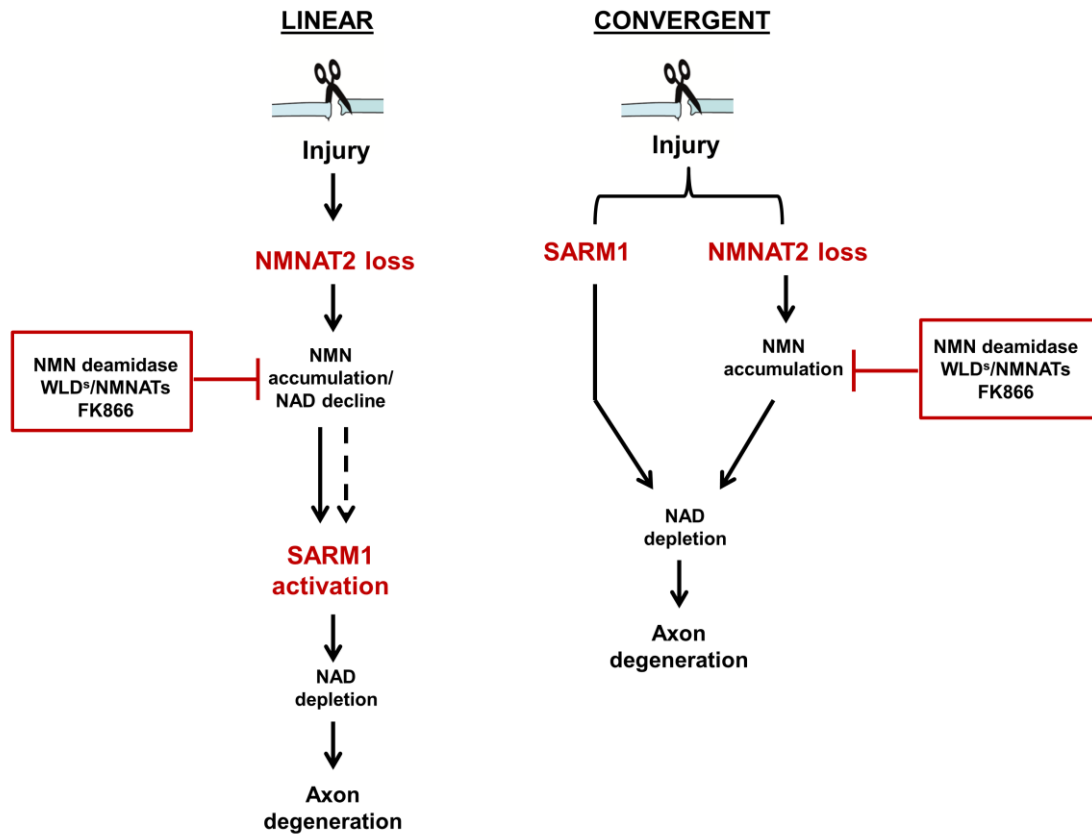


Figure 6.1 Proposed alternative pathways leading to axon degeneration after injury.

6.2 How can we correctly identify neurodegenerative disorders characterized by Wallerian-like degeneration?

The protection conferred by WLD^S/NMNATs in several disease models is the first indication that the Wallerian degeneration pathway might be involved in the development of neurodegenerative disorders. A list of diseases sensitive to WLD^S/NMNATs can be found in the introduction of this thesis and a recent review (Conforti *et al.*, 2014). Theoretically, any condition causing a failure in NMNAT2 delivery, reduced synthesis/increased degradation of NMNAT2 and impairment of NMNAT2 enzymatic activity is expected to trigger Wallerian-like degeneration. For instance, several chemotherapy drugs are associated with impairment of axonal transport and cause peripheral neuropathy (LaPointe *et al.*, 2013; Milde *et al.*, 2013b). Axonal transport is impaired also in several neurodegenerative disorders (De Vos *et al.*, 2008). WLD^S delays vincristine-

induced degeneration *in vitro* and paclitaxel-induced neuropathy *in vivo* (Conforti *et al.*, 2014). Therefore, these conditions represent an area of primary interest for clinical translation. However, WLD^S/NMNATs are effective in a wide spectrum of *in vitro* and *in vivo* disease models, triggered by very different kind of insults. Identifying a direct link with Wallerian degeneration is not always straightforward and the possibility that WLD^S/NMNATs are conferring protection with mechanisms unrelated to their function in axon degeneration has to be taken into account. For Instance, NMNATs chaperone activity has been recently related to protection in models of neurodegeneration (Ali *et al.*, 2012; Ljungberg *et al.*, 2012; Rallis *et al.*, 2013; Ali *et al.*, 2016; Lavado-Roldan & Fernandez-Chacon, 2016). Furthermore, the axon-protective action of WLD^S does not exclude a role in the nucleus (Gillingwater *et al.*, 2004; Verghese *et al.*, 2011; Tokunaga & Araki, 2012; Zhu *et al.*, 2013), especially given its high expression within this cellular compartment. NAD levels regulate the activity of several proteins (further discussed in the next paragraph) and this could underline the protection achieved with WLD^S/NMNATs in some conditions. A striking example of an extra-axonal protective function of WLD^S is its capacity to preserve β -cells functionality in experimental models of diabetes (Wu *et al.*, 2011; Zhu *et al.*, 2011).

The discussion above highlights, once again, the importance of fully characterizing the pathway leading to axon degeneration after injury. Diseases characterized by Wallerian-like degeneration should be sensitive to more than one (possibly all) regulator of Wallerian degeneration. In this thesis, I have followed this approach testing the involvement of several regulators of Wallerian degeneration in the toxicity caused by mitochondrial toxins. Importantly, recent studies reported that SARM1 deletion is protective in a model of traumatic brain injury (Henninger *et al.*, 2016) and against vincristine toxicity (Geisler *et al.*, 2016). In addition, SARM1 gene has been associated with ALS risk in a genome-wide association study (van Rheenen *et al.*, 2016). Future studies will also aim at identifying frequent variants of NMNAT2 in the human population which are associated with changes in NMNAT2 levels and/or activity. Data in this thesis suggest that reduced levels of NMNAT2 make axons more vulnerable and this might be particularly relevant in the context of

neurodegeneration caused by mitochondrial dysfunction. Importantly, a yet unpublished study identified a homozygous mutation in the NMNAT2 gene which reduces NMNAT2 activity in members of a family that developed an erythromelalgia-like phenotype (Huppke, Gilley, Orsomando et al., unpublished).

Finally, previous reports showed that the absence of SARM1 fully rescues axonal defects and perinatal lethality in NMNAT2 deficient mice (Gilley *et al.*, 2015). Further studies show that the strength of the rescue is extraordinary, with mice lacking both NMNAT2 and SARM1 showing a normal lifespan (>2 years) without any obvious abnormality (Gilley et al. unpublished). Therefore, if there are pathologies directly caused by defects in NMNAT2, the therapeutic benefits of targeting the Wallerian degeneration pathway could be extremely significant.

6.3 NMN supplementation as an anti-aging therapy

In recent years, supplementation of NAD precursors (mainly NMN and NR) has attracted a considerable amount of interest as an anti-aging therapy. The scientific base behind this approach is attempting to counteract the decline of NAD occurring with aging by stimulating its synthesis (Bonkowski & Sinclair, 2016). What causes NAD depletion in the first place is not fully understood (Schultz & Sinclair, 2016). A combination between a reduction in NAMPT levels (Yoshino *et al.*, 2011) and an increase in the expression of the NAD-consuming enzyme CD38 with aging (in mice) might be accountable for NAD decline (Camacho-Pereira *et al.*, 2016). Recent data also suggest that NMNATs might play a role in NAD decline (Gomes *et al.*, 2013; Prolla & Denu, 2014), with the levels of the three isoforms reduced in liver of older mice (Camacho-Pereira *et al.*, 2016).

Why are NAD levels associated with aging? NAD regulates the activity of several proteins, including a class of NAD-dependent deacylases called sirtuins (7 isoforms in humans, SIRT1-7) (Imai & Guarente, 2014). Sirtuins are linked to longevity in several different species (Kaeberlein *et al.*, 1999; Tissenbaum & Guarente, 2001; Rogina & Helfand, 2004; Satoh *et al.*, 2013; Imai & Guarente, 2014) and several studies report that sirtuins activity declines with aging as well

(Ramsey *et al.*, 2008; Chang & Guarente, 2013; Gomes *et al.*, 2013). This decline is thought to be strictly related to the decay in NAD levels (Imai & Guarente, 2014). Reduced sirtuins activity, in particular SIRT1, has been shown to have a deleterious effect on mitochondria (Gomes *et al.*, 2013). Dysfunctional mitochondria are a hallmark of aging and sirtuins are believed to slow aging by keeping mitochondria healthy (Imai & Guarente, 2014). Boosting NAD with supplementation of its precursors could potentially activate all 7 isoforms of sirtuins and has been associated with increased lifespan, improved mitochondrial function and a surprisingly broad protective effect in a number of pathological conditions, ranging from diabetes to heart disease and glaucoma (all reviewed in (Bonkowski & Sinclair, 2016)).

Whilst the results obtained with supplementation of NAD precursors are remarkable, our data envisage the possibility that long-term administration of NMN might be toxic for neurons under certain circumstances. For instance, NMN administration is likely going to be safe in people expressing normal levels of NMNAT2. However, the toxic effect on NMN could become apparent in people expressing lower levels of NMNAT2. NMN and NR are already available on the market classified as dietary supplements. Clinical trials are now ongoing to confirm the safety profiles of these molecules. Studies in mice showed that long-term supplementation of NMN did not cause any obvious toxicity (Mills *et al.*, 2016). Nevertheless, additional studies are needed and testing the safety of long-term supplementation of NMN in mice expressing reduced levels of NMNAT2 is of critical importance in this context.

6.4 NMNAT2 axonal transport and local synthesis in axons

To conclude this thesis, I am going to briefly discuss one of the most important open questions arising from our model of axon degeneration. NMNAT2 transport and presence in the axon is critical to preserve axonal integrity. However, NMNAT2 is an extremely labile protein, with a half-life of approximately 45 minutes (Milde *et al.*, 2013b). Thus, how can sufficient amounts of NMNAT2 reach the distal end of very long axons in time to avoid spontaneous degeneration? Some axons in the human body can be up to a

meter long; taking into account the maximum velocity of fast axonal transport, NMNAT2 would require more than two days to reach the end of these axons. At present, we do not have an adequate reply to this question. Several hypothesis have been made, including a difference in the half-life of NMNAT2 *in vitro* and *in vivo*, accelerated turnover after injury and post-translational modifications impacting on NMNAT2 stability (Milde *et al.*, 2013b). Another possibility is that NMNAT2 is locally translated in the axon. As mentioned in the introduction of this thesis, blocking translation exclusively in the axonal compartment does not promote axonal degeneration, at least within 24 hours (Gilley & Coleman, 2010). However, NMNAT2 mRNA has recently been found *in vivo* in axons (Dr. Jose Sotelo-Silveira, personal communication). This suggests that local synthesis of NMNAT2 might occur. This possibility deserves to be explored in depth, since local translation could explain how long axons get sufficient amounts of NMNAT2 and might also represent a therapeutic target to regulate axonal levels of NMNAT2.

List of abbreviations

AAD	acute axonal degeneration
ALS	amyotrophic lateral sclerosis
ANT	adenine nucleotide translocator
APP	amyloid precursor protein
ARM	armadillo
ATP	adenosine triphosphate
BAK	BCL-2 homologous antagonist killer
BAX	BCL-2-associated X
BCL-2	B-cell lymphoma 2
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CD38	cluster of differentiation 38
CICR	Ca ²⁺ -induced Ca ²⁺ release
CMT	Charcot-Marie-Tooth
CNS	central nervous system
CypD	cyclophilin D
CysA	cyclosporin A
DLK	dual leucine zipper-bearing kinase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
dNMNAT	homolog of NMNAT in <i>Drosophila melanogaster</i>
DRG	dorsal root ganglion
dSARM	homolog of SARM1 in <i>Drosophila melanogaster</i>
EAE	experimental autoimmune encephalitis
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
JNK	c-Jun N-terminal kinase
LRRK2	leucine-rich-repeat kinase 2
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase

MAPKKK	mitogen-activated protein kinase kinase kinase
MARTs	mono-ADP-ribosyl transferases
MCU	mitochondrial Ca^{2+} uniporter
mPT	mitochondrial permeability transition
mPTP	mitochondrial permeability transition pore
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NaAD	nicotinic acid adenine dinucleotide
NAD	nicotinamide adenine dinucleotide
NAM	nicotinamide
NaMN	nicotinic acid mononucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NaPRT	nicotinic acid phosphoribosyltransferase
NCX	$\text{Na}^+/\text{Ca}^{2+}$ exchangers
NGF	nerve growth factor
NMN	nicotinamide mononucleotide
NR	nicotinamide riboside
6OHDA	6-hydroxydopamine
PARPs	poly-ADP-ribose polymerases
PBS	phosphate buffered saline
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
<i>pmn</i>	progressive motor neuropathy
PNS	peripheral nervous system
PTU	1-phenyl 2-thiourea
RFP	red fluorescent protein
RGC	retinal ganglion cell
ROS	reactive oxygen species
SAM	sterile α motif
SARM1	sterile α -motif-, armadillo-motif-, and Toll-interleukin receptor homology domain-containing protein
SCA1	spinocerebellar ataxia 1
SCG	superior cervical ganglion
SCG10	superior cervical ganglion 10

SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIRT6	sirtuins
SMA	spinal muscular atrophy
SOD1	superoxide dismutase 1
TBST	TBS pH 8.3 with 0.05 % Tween 20
TCA	tricarboxylic acid cycle
TIR	Toll-interleukin receptor homology domain
TMRM	tetramethylrhodamine methyl ester
UBE4B	ubiquitin ligase E4B
UPS	ubiquitin proteasome system
UTR	5' untranslated region
VCP	valosin-containing protein
VDAC	voltage-dependent anion channel
VDCC	voltage-dependent Ca ²⁺ channels
WLD^s	Wallerian degeneration slow
<i>Wnd</i>	wallenda

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