Title
Mitochondrial Transport and Function in Axon Degeneration

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Publication Date
2013

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Mitochondrial Transport and Function in Axon Degeneration

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Neuroscience

by

Kelley O'Donnell

2013

ABSTRACT OF THE DISSERTATION

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Kelley O'Donnell

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2013

Professor Alvaro Sagasti, Chair

Axon degeneration plays a critical but ill-defined role in Parkinson disease (PD), and is actively regulated by pathways that are not well understood. Mitochondria orchestrate many of the processes that underlie axonal homeostasis, and mitochondrial dysfunction is implicated in PD pathogenesis. A better understanding of axonal mitochondria may therefore identify the cell biological processes that mediate degeneration and protection of the axon. Recent studies have suggested that mitochondrial transport is critical to axon protection after injury. I studied mitochondrial morphology, transport, and function in living zebrafish embryos. Using time-lapse confocal imaging of peripheral sensory neurons, I investigated the effect of injury on mitochondrial transport. I used two-photon laser axotomy to induce Wallerian degeneration (WD) of the distal axon. I found that acute transport arrest occurs in the distal axon, and motility does not recover before fragmentation. Although transport arrest was preserved in the proximal axon, which does not degenerate, increased mitochondrial trafficking after injury did not always correlate with axon protection. To determine whether mitochondrial ROS production is relevant to WD, I expressed the redox-sensitive biosensor roGFP2 in the mitochondrial matrix. After

injury, roGFP2 was rapidly and persistently oxidized in the distal, but not the proximal, axon. The axon-protective protein WldS, which had only a mild and temporary effect on transport arrest after axotomy, robustly inhibited roGFP2 oxidation and degeneration. To further investigate the importance of mitochondrial ROS production after injury, I expressed the transcriptional co-activator PGC-1α, which has roles in mitochondrial biogenesis and ROS detoxification. I saw that overexpression of this protein delays roGFP2 oxidation after injury, and delays WD. Mitochondrial ROS production is therefore a better predictor of axonal vulnerability than mitochondrial transport, and ROS detoxification may be a relevant therapeutic target to prevent axon degeneration.

I then studied degeneration induced by α-synuclein (aSyn), a protein that is associated with PD. I expressed human wild-type aSyn in peripheral sensory neurons and saw that axon pathology precedes cell death in these cells. Early changes in mitochondrial morphology were consistent with increased fragmentation, and axonal varicosities were filled with swollen mitochondria. I also saw reduced mitochondrial trafficking in aSyn-expressing cells. Motile mitochondria favored retrograde transport towards the cell body. I hypothesized that protection of mitochondria might prevent degeneration in this model, as it had after axotomy. I therefore expressed PGC-1α in cells expressing aSyn, and saw robust protection against axon degeneration and cell death. These results suggest that axon degeneration pathways converge on mitochondrial dysfunction. Protection of mitochondria may therefore be a promising therapeutic target in the prevention and treatment of neurodegenerative disease.

The dissertation of Kelley O'Donnell is approved.

Jeff Bronstein

Marie-Francoise Chesselet

Ming Guo

Alvaro Sagasti, Committee Chair

University of California, Los Angeles

2013

DEDICATION

For Trent

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ACKNOWLEDGMENTS

I am profoundly grateful to my advisor, Dr. Alvaro Sagasti. I have grown immeasurably as a scientist through his mentorship and his example. I am grateful to him for giving me both guidance and independence, and for handling that duality with grace and equanimity. I have not always made it easy. He is deeply inquisitive but intellectually generous, and from that foundation he has created a lab culture that is both stimulating and supportive.

I thank Alvaro and all the members of my committee for helping me to always think more deeply about my work. I have seldom been more excited about science than after our annual meetings. I also thank the members of my committee for contributing to my experience in ways that extend far beyond the walls of those meetings. Without Dr. Jeff Bronstein, Chapter 3 would not exist. I thank him for giving me so many opportunities to collaborate scientifically with his lab, and for the intellectual and personal support he has provided to me at critical moments in my graduate career. I thank Dr. Ming Guo for her invaluable expertise in mitochondrial neurodegeneration research, and for easing my fears regarding the two years I now face. Last, I thank Dr. Marie-Francoise Chesselet. Through her leadership of the Training Program in Neural Repair and the UCLA Center for the Study of Parkinson's Disease, she has provided generous support for my work in the lab. I thank her for this. Even more important to me, however, is the personal effort she put forth when she sensed I was experiencing a dark night of the scientific soul. I am profoundly grateful to her.

I thank Dr. Stephen Smale and Dr. Kelsey Martin for believing in me, and for seeing in my untraditional background the foundation of good science.

I am deeply indebted to Dr. David Krantz for the galvanizing effect he had on my spirit at a crucial time.

I thank Dr. Mauricio Vargas in the Sagasti lab for pioneering the studies of mitochondrial transport, and for his intellectual contribution to the work on Wallerian degeneration described in Chapter 2. I thank recent graduate Nickolas Wheat for his assistance with experiments in Chapter 3. I thank Dr. Mark Stahl for editing a draft of Chapter 1. I also thank him and Aaron Lulla for performing the immunohistochemistry in Chapter 3, and for their general efforts to keep me sane.

I thank all other members of the Sagasti lab, past and present, for making my time as enjoyable as possible through a combination of intellectual stimulation, general camaraderie, and wine.

I thank my family and my friends for their invaluable support. Most of all, I thank Trent, my beloved partner of ten years. His boundless love and generosity have sustained me throughout this endeavor. There are no words.

Chapter 2 is a version of a manuscript currently under review. I conducted and analyzed all experiments, and wrote the manuscript. Mauricio Vargas generated the UAS-mitoDsRed line of transgenic fish and developed early protocols for imaging mitochondrial transport. Both he and Alvaro made invaluable intellectual contributions to the work, and to the manuscript itself.

Chapter 3 is a manuscript in the final stages of preparation. All immunohistochemistry was performed by Mark Stahl and Aaron Lulla. I designed, performed, and analyzed all other

experiments, and wrote the manuscript. Nickolas Wheat injected embryos for experiments and contributed to data collection. Jeff Bronstein provided the original aSyn transgene, and both he and Alvaro contributed intellectually to the project.

Chapter 4 represents unpublished work that may contribute to future studies in the lab. I performed and analyzed all experiments.

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PUBLICATIONS

O'Donnell KC, Lulla A, Stahl MC, Wheat N, Bronstein JM, Sagasti A. Axon degeneration and PGC-1α-mediated protection in a vertebrate model of α-synuclein toxicity. In preparation.

O'Donnell KC, Vargas ME, Sagasti A. WldS and PGC-1α regulate mitochondrial transport and oxidation state after axonal injury. Submitted.
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Chapter 1. Introduction

Why and how should Parkinson disease be studied?

Despite decades of intensive study, disease-modifying treatments for neurodegenerative disorders such as Parkinson disease (PD) remain elusive. These diseases present a tremendous social and economic burden (Johnson et al., 2013; Kowal et al., 2013). In industrialized countries, the prevalence of Parkinson disease alone is estimated at 1% for individuals over 60 (de Lau and Breteler, 2006); recent estimates suggest it will double in the United States by 2040 (Kowal et al., 2013). These numbers are likely to be much lower than the true prevalence, as patients are often not diagnosed until late stages of the disease process. The identification of biomarkers of disease would allow earlier detection and intervention; to this end, a better understanding of pathogenesis and pathophysiology is critical (Adler, 2011). Considerable evidence implicates mitochondrial dysfunction in PD pathogenesis, and recent studies suggest that axon degeneration is an early, and potentially independent, aspect of pathophysiology. In this dissertation, I will explore the relationship between mitochondrial defects and axon pathology, first in the simplified model of Wallerian degeneration, then in cells overexpressing the PD-associated protein α-synuclein (aSyn).

The paradox of defining a neurodegenerative disease

The way in which a disease is defined affects not only diagnosis and treatment, but also how pathophysiology is studied, how putative biomarkers are identified, and how the disease is modeled in other systems. Traditionally, the pathological end-point of anatomically selective cell death has been used to classify neurodegenerative diseases.

More recently, molecular and genetic insights have suggested that these classification systems are at best crude, with a number of ostensibly unrelated genes giving rise to the same constellation of symptoms. With the discovery of disease-associated genes has come the development of new animal models for studying the basic cell biological mechanisms that underlie neuronal integrity and degeneration. That these models do not always—or even often—fully and faithfully recapitulate the anatomically selective and cell type-specific degeneration does not preclude their utility. The genetic hetereogeneity provides a starting point for a more complete study of pathogenesis that may ultimately refine or dramatically alter nosology and treatment. A better understanding of these genes and the subcellular processes they regulate may provide insight into both the cascade of events underlying neurodegeneration, and the most promising targets for therapeutic intervention.

Defining and studying Parkinson disease

Clinical symptoms and selective cell death

A clinical diagnosis of parkinsonism is based on the cardinal findings of low-amplitude rest tremor, bradykinesia, rigidity, and loss of postural reflexes (de Rijk et al., 1997; Litvan et al., 2003). These findings result from dopamine depletion caused by death of dopaminergic neurons in the substantia nigra pars compacta (SNc). Parkinsonism can be caused by insults that deplete dopaminergic transmission or damage the substantia nigra (e.g. stroke). Symptoms may therefore have a rapid onset that is distinct from the slow progression of PD. The discovery that ingestion of the street drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) gives rise to rapid-onset parkinsonism in human subjects (Davis et al., 1979; Langston et al., 1983; Wright et al., 1984) provided the basis for an early model for PD, and the selective dopaminergic cell death observed

after MPTP administration suggested that the nigral degeneration observed in the PD brain was the direct cause of parkinsonism (Przedborski and Jackson-Lewis, 1998). Animal models of the disease have traditionally been judged by their ability to reproduce this phenotype of selective dopaminergic death combined with motor symptoms (Waldmeier et al., 2006), though the value of that requirement has recently come into question (Jellinger, 2012; Lee et al., 2012; McDowell and Chesselet, 2012; Smith et al., 2012).

Characteristic pathology

In addition to the aforementioned cell death, a definitive diagnosis of PD, as opposed to parkinsonism, requires the presence of Lewy body pathology, which is confirmed at autopsy in 80-90% of cases (Hughes et al., 1992; Litvan et al., 2003). Lewy bodies are intracellular inclusions composed primarily of ubiquitin and aSyn (Spillantini et al., 1997; Spillantini et al., 1998). Their presence and regional distribution form the basis for the current PD staging system (Braak et al., 1999; Braak et al., 2003; Jellinger, 2004). The clinical relevance of this staging system, however, and of Lewy bodies altogether, is the subject of considerable debate (Burke et al., 2008; Parkkinen et al., 2008; Milber et al., 2012; Schulz-Schaeffer, 2012). Increased copy number or mutations in *SNCA*, the gene that encodes alpha-synuclein (aSyn), cause rare, autosomal-dominant PD (Polymeropoulos et al., 1997), and various polymorphisms in this gene are also associated with increased risk of sporadic PD (Mizuta et al., 2006; Ross et al., 2007), suggesting that aSyn aggregates have an active role in pathogenesis. Identification of the native conformation of aSyn has produced conflicting results, with various studies providing evidence that it is predominantly monomeric (Fauvet et al., 2012), tetrameric (Bartels et al., 2011), or oligomeric (Dettmer et al., 2013). Lewy bodies, however, are neither necessary nor sufficient for the development of Parkinson disease. For example,

they are not found in patients with autosomal-dominant PD caused by LRRK2 mutations (Funayama et al., 2005; Gaig et al., 2009), but they are found in patients with dementia with Lewy bodies (DLB), who do not have the motor symptoms characteristic of PD (Spillantini et al., 1998). More recent evidence suggests that aSyn oligomers, rather than the larger Lewy body aggregates, are toxic (Kramer and Schulz-Schaeffer, 2007; Brown, 2010; Jellinger, 2012; Wan and Chung, 2012). The precise mechanism of that toxicity is equally elusive.

Preclinical features

In PD, the overt striatal degeneration is a late feature of what is increasingly recognized as a multi-organ disease with both motor and non-motor deficits (Jellinger, 2012). Non-motor symptoms may be associated with aSyn accumulation elsewhere, including non-dopaminergic spinal cord axons (Bloch et al., 2006; Del Tredici and Braak, 2012), autonomic neurons including cardiac and enteric nerves (Wakabayashi and Takahashi, 1997; Courbon et al., 2003), and peripheral motor nerves (Mu et al., 2013). These symptoms often precede the motor manifestations of PD, and they are not improved with L-DOPA treatment. Dopamine depletion is therefore not the only mechanism relevant to disease, and dopamine replacement does not address the underlying neurodegenerative process. By evaluating genetic predisposition in combination with these non-motor symptoms, a preclinical form of PD is increasingly recognized, and may ultimately lead to the identification of biomarkers that facilitate diagnosis even in the pre-motor phase of the disease (Stern and Siderowf, 2010; Akhtar and Stern, 2012; Siderowf et al., 2012). Moreover, a better understanding of the relevant gene products may identify the molecular basis of degeneration, and the subcellular processes that underlie vulnerability and protection. The earlier diagnosis could then be matched by early, disease-modifying interventions that have not yet been developed.

Mitochondrial involvement in Parkinson disease

Although best known as the primary source of energy production in neurons, mitochondria take part in a broad range of other cellular processes, including cell death, calcium handling, and management of oxidative stress (McBride et al., 2006). Many lines of evidence implicate mitochondrial dysfunction in the pathophysiology of PD. Respiratory chain dysfunction can be observed in fibroblast (Piccoli et al., 2008) or platelet (Parker et al., 1989) samples from PD patients, and a similar mitochondrial phenotype has been confirmed in post-mortem analyses of neurons from PD patients (Mizuno et al., 1989; Schapira et al., 1989; Schapira et al., 1990; Mann et al., 1994). A higher frequency of mitochondrial DNA mutations have been observed in the substantia nigra of PD brains (Bender et al., 2006), and many of the genes associated with familial PD have roles in mitochondrial function and transport (Sai et al., 2012). Below I will discuss certain aspects of mitochondrial function. I will then discuss regulation of mitochondrial activity through morphology and transport, and evidence for mitochondrial disruption in PD.

Mitochondria as instigators of cell death

As neurodegenerative diseases are characterized by cell death, it is perhaps no surprise that mitochondria are implicated in PD. The specific mechanism of cell death in PD, however, is still a matter of debate (Bredesen et al., 2006; Jenner and Olanow, 2006). In neurons, the intrinsic apoptotic pathway, which is mitochondria- and caspase-dependent, is the primary form of programmed cell death (Bredesen et al., 2006). In a healthy cell, apoptosis is actively prevented by the presence of IAPs (inhibitor of apoptosis proteins), and by the fact that cytosolic caspases exists in as inactive

zymogens. Within the mitochondria are proapoptotic molecules such as cytochrome c and caspase-activating SMAC proteins. Their sequestration, which is critical to maintaining cellular integrity, relies on the impermeability of the mitochondrial membrane; pore formation and dramatic loss of the mitochondrial membrane potential (discussed briefly below in the section on oxidative phosphorylation) allows the escape of these proapoptotic factors into the cytosol, ultimately leading to caspase activation and cytoskeletal degradation (Saraste and Pulkki, 2000; Mattson and Chan, 2003; Danial and Korsmeyer, 2004; Riedl and Salvesen, 2007).

Loss-of-function mutations in the *PARK2* gene, which encodes the ubiquitin ligase Parkin, lead to dopaminergic cell death and autosomal recessive PD (Kitada et al., 1998). Parkin overexpression is antiapoptotic (Yang et al., 2005; Johnson et al., 2012), and one of the mechanisms of that protection may be the ubiquitination of proapoptotic proteins such as Bax (Johnson et al., 2012), which forms the pore through which cytochrome c escapes into the cytosol early in the apoptotic cascade. This does not necessarily mean, however, that PD-associated cell death is apoptotic (i.e., caspase-mediated). After pharmacologic ATP depletion or caspase inhibition—both of which prevent apoptosis—cytochrome c release from mitochondria is still toxic, eventually leading to cell death through a non-apoptotic pathway (Ikegami and Koike, 2003; Waldmeier et al., 2006; Vekrellis et al., 2009). Such a pathway may share some features of the excitotoxic cell death observed in glutamatergic neurons after chronic NMDA receptor activation, as there is evidence to suggest that calcium-dependent excitotoxicity is involved in cell death in PD (Mann et al., 1994; Jenner and Olanow, 2006; Koutsilieri and Riederer, 2007; Schulz, 2007; Caudle and Zhang, 2009; Zündorf and Reiser, 2011). Classically, excitotoxicity occurs when persistent activation of NMDA receptors leads to a condition of calcium overload, in which mitochondrial calcium buffering capacity is

exhausted, leading to mitochondrial dysfunction, rupture, and cell death. The subsequent calcium-mediated injury may begin with formation of the mitochondrial permeability transition pore (mPTP) across the inner mitochondrial membrane, though this model is controversial (Choi, 1985; Schinder et al., 1996; Panov et al., 2007; Bolshakov et al., 2008). Mitochondrial PTP opening results in mitochondrial depolarization, matrix swelling, outer membrane rupture, and expulsion of calcium, cytochrome c and other mitochondrial contents into the cytosol, leading to protease activation and cell death (Schinder et al., 1996; Brustovetsky et al., 2002; Brustovetsky et al., 2003). Dopaminergic neurons appear to be particularly susceptible to mPTP formation (Brustovetsky et al., 2003). Mutations in PTEN-induced kinase 1 (PINK1), which cause an autosomal recessive form of PD, increase neuronal vulnerability to calcium-induced cell death (Gandhi et al., 2009; Gandhi et al., 2012), possibly through reduced calcium buffering that results in a lower threshold for mPTP opening (Akundi et al., 2011). A better understanding of that vulnerability may provide insight into cell death in PD (Jenner and Olanow, 2006).

Although cell death is a conspicuous feature of Parkinson disease, considerable evidence suggests that more mild mitochondrial impairment begins much earlier in the disease process (Cardoso, 2011). Mitochondrial dysfunction is upstream of degeneration in toxin-induced models of PD, such as MPTP (Przedborski and Jackson-Lewis, 1998) and rotenone exposure (Betarbet et al., 2000; Sherer et al., 2002). In addition to the aforementioned roles in cell death pathways, mitochondria produce energy and reactive oxygen species (ROS) through oxidative phosphorylation, and regulate intracellular calcium levels; aberrations in any of these functions may be associated with PD, as discussed below. Dynamic changes in mitochondrial morphology and trafficking, which subserve these functions, have also been implicated in PD pathogenesis, and will also

be discussed.

Mitochondrial bioenergetics: ATP synthesis and ROS production

Mitochondrial bioenergetics refers to the process by which the energy generated through a series of oxidative-reduction (redox) reactions is used to produce ATP (Madeira, 2012). Oxidative phosphorylation (OXPHOS) couples activity of the electron transport chain (ETC) to ATP synthesis (Mitchell, 1961). Disruptions in this process are associated with a range of neurodegenerative diseases (Morán et al., 2012), and can result in an ATP deficit that may be relevant to degeneration of neurons, which have particularly high energy demands (Harris et al., 2012). Aberrant electron transfer or leakage through the ETC can also cause the production of ROS, which are oxygen-derived molecules capable of causing DNA damage and lipid peroxidation (Balaban et al., 2005). Although many mechanisms exist to scavenge these reactive molecules, they accumulate under conditions of overproduction or impaired detoxification. This imbalance between ROS production and detoxification is known as oxidative stress, and the resulting cellular damage is associated with both normal aging and age-related neurodegenerative diseases (Balaban et al., 2005; Jones, 2006). In the sections below, I will briefly discuss the OXPHOS machinery, its activity, and the evidence for its dysfunction in PD.

Oxidative phosphorylation machinery and mitochondrial membrane potential

The ETC consists of four transmembrane enzyme complexes that mediate electron transfer from one molecule of a redox pair to another: the molecule that loses an electron is oxidized, and the molecule that gains an electron is reduced. This process is thermodynamically spontaneous, and produces an electrochemical proton gradient, known as the mitochondrial membrane potential ($\Delta\Psi_m$) (Mitchell and Moyle, 1969),

between the mitochondrial matrix and the intermembrane space (IMS). As a result of this $\Delta\Psi_m$, protons in the IMS experience a driving force back across the inner membrane, down their electrochemical gradient. This proton flow causes a conformational change in the transmembrane protein ATP synthase, the final enzyme in the oxidative phosphorylation pathway, which generates ATP in the mitochondrial matrix (Yoshida et al., 2001).

ATP is required for a wide range of intracellular activities, and robust oxidative phosphorylation ensures that intracellular energy demands are met by aerobic respiration. Impaired ATP synthesis can result from dysfunction of any one of the enzymes involved in oxidative phosphorylation, either through failure to produce a driving force for proton re-entry into the matrix (i.e., dysfunction of complexes I-IV), or through direct inhibition of ATP synthase (e.g. by oligomycin). Highly metabolically active cells such as neurons may be particularly sensitive to impairments in this process, and energy depletion may therefore have a role in neurodegeneration. One recently identified role for the ubiquitin ligase Parkin is in regulating the physiologic turnover of ETC subunits (Vincow et al., 2013), which may be required for stable ETC activity and energy production. ATP depletion is seldom seen in isolation, however, and other aspects of respiratory chain dysfunction may have an earlier role in disease processes, as discussed below.

Disruptions in oxidative phosphorylation can produce reactive oxygen species

The final enzyme in the ETC is cytochrome c oxidase, which removes two pairs of electrons from cytochrome c and transfers them to molecular oxygen to produce water. When single electrons instead leak across the membrane, they can react with oxygen to generate the superoxide anion, a ROS that is the precursor to hydrogen peroxide

(H_2O_2). Under normal conditions, only a small percentage (<0.5%) of oxygen consumed in the ETC is reduced to superoxide (Liu et al., 2002), and there is evidence to suggest that at low levels, mitochondrial ROS act as cell signaling molecules (Sena and Chandel, 2012). Increases in $\Delta\Psi_m$, ATP synthesis, or matrix calcium concentration, however, can lead to increased production of mitochondrial ROS (Korshunov et al., 1997; Abramov et al., 2007; Komary et al., 2008; Tretter and Adam-Vizi, 2012), which can be mitigated by overexpression of uncoupling proteins (Nègre-Salvayre et al., 1997). It is notable that these relationships are not entirely well understood, and may depend on the precise value of the $\Delta\Psi_m$ and the particular ROS being measured (Sipos et al., 2003; Starkov and Fiskum, 2003; Tretter et al., 2007; Komary et al., 2008, 2010). On the other hand, ETC pathology or toxicity—and therefore low ATP synthesis—also dramatically increases ROS production (Liu et al., 2002; Castello et al., 2007). A disruption of OXPHOS in either direction can therefore cause ROS accumulation that may overwhelm mitochondrial defenses and lead to oxidative stress. In turn, an oxidizing environment promotes aggregation of disease-associated proteins such as aSyn (Giasson et al., 2000; Souza et al., 2000; Przedborski et al., 2001; Sherer et al., 2002).

The primary sites of mitochondrial superoxide production are complex I and complex III of the ETC. At complex I, ROS production is determined by the ratio of NADH/NAD^+ (Murphy, 2009). Buildup of NADH, which occurs in conditions of low ATP synthesis (either from low ATP demand in the presence of ample substrates, or from respiratory chain dysfunction), increases ROS production at this site (Murphy, 2009). Many of the toxins associated with parkinsonism and increased PD risk, such as MPP^+ (the toxic metabolite of MPTP), rotenone, and paraquat, impair complex I, which leads to increased ROS generation, reduced ATP synthesis, and cell death (Cassarino et al.,

1997; Betarbet et al., 2000; Sherer et al., 2002; Cochemé and Murphy, 2008). Likewise, in PINK1 or Parkin knock-out/mutant flies, which show increased vulnerability to oxidative stress (Pesah et al., 2004; Clark et al., 2006; Mai et al., 2010), enzymatic activity of both complex I and complex IV is impaired (Morais et al., 2009; Liu et al., 2011), leading to decreased $\Delta\Psi_m$. The impaired activity of complexes I and IV may similarly underlie the decreased ATP synthesis and increased ROS production observed in fibroblasts from patients with PINK1-deficient PD (Piccoli et al., 2008; Abramov et al., 2011).

Loss-of-function mutations in *PARK7*, the gene encoding DJ-1, lead to an early-onset, autosomal recessive form of PD (Bonifati et al., 2003). Although its function has not been fully elucidated, DJ-1 is redox-sensitive, with roles in superoxide-activated signaling cascades (Hao et al., 2010) and neuroprotection (Ved et al., 2005). It is possible that DJ-1 directly increases complex I assembly, as DJ-1 knock-out cells are less sensitive to paraquat-induced superoxide generation and apoptosis (Kwon et al., 2011). However, these knock-out cells also exhibit increased ROS production and mPTP formation that appears to be independent of an effect on the respiratory chain (Giaime et al., 2012), suggesting that DJ-1 may regulate mitochondrial function at multiple levels.

The relationship between respiratory chain dysfunction and aSyn toxicity has not been fully parsed and appears to vary according to the cell type being investigated, the form of aSyn being expressed, and the promoter used to drive expression. Oxidative stress induced by MPTP or pesticides may activate or potentiate aSyn oligomerization and aggregation (Souza et al., 2000; Vila et al., 2000; Uversky et al., 2001; Sherer et al., 2002), leading to toxicity relevant to both hereditary and idiopathic forms of PD. Alpha-synuclein may also have a direct effect on cellular vulnerability to oxidative stress. In

vitro, overexpression of aSyn in ectoderm-derived PC12 cells (Butler et al., 2012; Ma et al., 2013) or dopaminergic cells (Ma et al., 2011) does not affect baseline oxidative stress, but certain mutations increase sensitivity to OXPHOS uncoupling or pesticide exposure. Another in vitro study, however, suggested that overexpression of aSyn is actually protective against oxidative stress induced by H_2O_2 (Hashimoto et al., 2002). A more thorough understanding of aSyn toxicity in vivo may reconcile these conflicting reports and provide insight into cellular vulnerability and dysfunction in PD.

ROS detoxification

In healthy mitochondria, the production and clearance of ROS are thought to exist in equilibrium. When ROS production increases or antioxidant systems fail, these reactive molecules accumulate, causing oxidative damage to DNA and proteins. Superoxide is the proximal mitochondrial ROS, and mitochondrial superoxide dismutase reduces superoxide to H_2O_2. Although H_2O_2 is membrane-permeable and can diffuse into the cytoplasm, where it can be reduced to water by the enzyme catalase, neuronal mitochondria are also equipped with their own enzymatic systems for H_2O_2 detoxification: glutathione/glutathione peroxidase and thioredoxin/peroxiredoxin 3 and 5 (Drechsel and Patel, 2010). Through the relevant peroxidase (glutathione peroxidase or periredoxin) in each of these systems, H_2O_2 is reduced to water, while the reducing substrate (glutathione or thioredoxin) is oxidized. For continued ROS detoxification, that oxidized substrate is then reduced by glutathione reductase or thioredoxin reductase, respectively, in a redox reaction that requires a stable pool of reducing equivalents such as NADPH (Drechsel and Patel, 2008, 2010). Post-mortem samples have repeatedly demonstrated a lower level of the antioxidant glutathione in the PD brain (Riederer et al., 1989; Sian et al., 1994), and higher levels of glutathione peroxidase (Bellinger et al., 2011). This finding could simply be secondary to defective oxidative phosphorylation, as

seen with complex I inhibition; however, glutathione depletion can also precede complex I deficiency in PD (Jenner, 1993) suggesting that impaired ROS detoxification, and not merely increased ROS synthesis, may be relevant to pathogenesis. In a fly model, a Parkin loss-of-function mutation causes spontaneous neurodegeneration that can be prevented by overexpressing glutathione-S-transferase (Whitworth et al., 2005). This suggests that even if respiratory chain dysfunction and ROS accumulation is the proximal pathogenic insult, upregulation of downstream antioxidant activity may have therapeutic relevance.

Calcium buffering

A local increase in cytoplasmic calcium concentration can activate signaling pathways and gene expression, regulate metabolism and synaptic transmission, and, if excessive, initiate degeneration. Cytoplasmic calcium concentration is therefore tightly regulated by the endoplasmic reticulum and mitochondria, as well as calcium-binding proteins in the cytosol. At rest, free cytosolic calcium is maintained at approximately 100 nM, largely through ER sequestration (Meldolesi and Pozzan, 1998). Mitochondrial calcium buffering is characterized as low-affinity, high-capacity; matrix [Ca^{++}] in resting neurons is estimated to be 100 nM and it can rise to1 μM with stimulation (David et al., 2003). The calcium-sensitive dehydrogenases in the citric acid cycle are thought to be activated at this level, permitting energy production in the mitochondrial matrix to meet the newly increased demand (Glancy and Balaban, 2012; Tarasov et al., 2012). In addition to regulating bioenergetics, mitochondrial calcium buffering is required for normal synaptic transmission; as such, disruptions in either mitochondrial function or localization can impair neuronal signaling (Tang and Zucker, 1997; Billups and Forsythe, 2002; Medler and Gleason, 2002; David and Barrett, 2003).

Mitochondrial calcium buffering machinery

Calcium import from the cytosol to the mitochondrial matrix is mediated by a voltage-dependent anion channel (VDAC) in the outer membrane and a uniporter in the inner membrane (Vasington and Murphy, 1962; Gunter and Pfeiffer, 1990; Jonas et al., 1999; Kirichok et al., 2004; Baughman et al., 2011; De Stefani et al., 2011). As described above in the section on oxidative phosphorylation, the ETC generates a steep electrochemical gradient across the inner mitochondrial membrane, with a negative charge in the matrix; this gradient provides the driving force for calcium entry through the uniporter. As a result, mitochondrial depolarization inhibits calcium buffering (Werth and Thayer, 1994; Tang and Zucker, 1997; David and Barrett, 2003; Talbot et al., 2003). Inhibition of ATP synthesis, by contrast, has no effect on calcium buffering so long as $\Delta\Psi_m$ is intact (Talbot et al., 2003). The high-affinity calcium buffering prevents activation of cytosolic proteases that would otherwise effect degeneration. As cytoplasmic calcium levels fall, efflux from the mitochondrial matrix is mediated primarily by the Na^+/Ca^{++} exchanger (Gunter and Pfeiffer, 1990; Carafoli, 2012), though there is some evidence that a low-conductance state of the mPTP is also involved (Gunter et al., 1998; Ichas and Mazat, 1998; Murchison and Griffith, 2000). Under conditions of calcium overload (> 25μM) (Allen et al., 1993; Gunter et al., 1994; Barsukova et al., 2011) in the matrix, a high-conductance state of the mPTP permits efflux of high levels of calcium into the cytosol; if unchecked, this leads to cell death (Choi, 1985; Schinder et al., 1996).

Miro is a calcium sensor

Local calcium buffering by mitochondria occurs through the coordination of calcium sensing and microtubule-based transport. Upon sensing calcium, mitochondrial transport

along microtubules terminates, permitting rapid, local modulation of calcium uptake and ATP synthesis at sites of high demand. The mitochondrial GTPase Miro mediates both the sensing of calcium and the calcium-mediated transport arrest (Saotome et al., 2008; Macaskill et al., 2009; Wang and Schwarz, 2009). Miro is required for mitochondrial transport; it binds to kinesin motors through the adaptor protein Milton (Guo et al., 2005; Rice and Gelfand, 2006; Macaskill et al., 2009; Brickley and Stephenson, 2011). Calcium binding to the EF-hand domains of Miro induces a conformational change that causes the mitochondrial transport complex to dissociate from the microtubule (Saotome et al., 2008; Wang and Schwarz, 2009). Calcium buffering and ATP production can then proceed as necessary.

Calcium dysregulation and degeneration

As discussed earlier, calcium overload is associated with excitotoxicity (Choi, 1985, 1987), and prolonged elevation of cytoplasmic calcium levels can activate calcium-dependent cysteine proteases (calpains), calcineurin, and other effectors of degeneration (Zündorf and Reiser, 2011). Mitochondria provide local, high-affinity calcium buffering, and impairments in that process are implicated in a number of neurodegenerative disease models (Verkhratsky and Fernyhough, 2008; Fernyhough and Calcutt, 2010; Pandey et al., 2010; Crish and Calkins, 2011; Misko et al., 2012). High levels of calcium cause ROS release (Votyakova and Reynolds, 2005), which is associated with degeneration as described earlier. ROS accumulation in the mitochondrial matrix can also facilitate mPTP opening (Vercesi et al., 1997; Panov et al., 2007), suggesting that moderate levels of matrix ROS and calcium can combine to cause irreversible damage. Because ATP depletion inhibits calcium efflux through the Na^{+}/Ca^{++} exchanger, impaired bioenergetics can also facilitate calcium overloading in the matrix, which in turn leads to mPTP formation. Mitochondrial calcium handling, ROS

production, and ATP synthesis are thus interconnected, with $\Delta\Psi_m$ affecting and being affected by all three (Brookes et al., 2004).

Mitochondrial dynamics and quality control

Mitochondrial morphology is regulated by fission and fusion

Mitochondrial morphology varies widely both across cell types and within a single cell (Collins et al., 2002; Benard and Rossignol, 2008). In healthy neurons, tubular networks of mitochondria exist in a dynamic equilibrium of fission and fusion; within a single neuron, the morphology of individual mitochondria ranges from a cylindrical to a more spheroidal shape (Collins et al., 2002). The overall shape of both the network and an individual mitochondrion both affect and are affected by changes in mitochondrial membrane potential, bioenergetics (including both ATP synthesis and ROS production), calcium content, and transport (Rintoul et al., 2003; Benard and Rossignol, 2008; Plecitá-Hlavatá et al., 2008; Saotome et al., 2008; Dagda et al., 2009; Gautier et al., 2012) The morphology of single mitochondria, however, is thought to be primarily regulated by fusion (mitofusins on the outer membrane, and OPA1 on the inner membrane) and fission (the dynamin-related GTPase Drp1) proteins (McBride et al., 2006). Through fusion with healthy mitochondria, the effects of mild depolarization and DNA damage are essentially diluted in the network. Impairments in fusion proteins prevent this protective effect, and are directly implicated in certain forms of neurodegeneration (Baloh et al., 2007; Chen et al., 2007; Lutz et al., 2009; Palau et al., 2009; Pham et al., 2012). Aberrant fusion of highly depolarized mitochondria is detrimental, however, as described below in the section on mitochondrial quality control.

Bioenergetics, dynamics, and dysfunction

The relationship between mitochondrial dynamics and function is not linear. Reduced fusion or increased fission can result in an energy deficit, as seen with knock-down of various mitofusins (Chen et al., 2005) or cells overexpressing aSyn, which exhibit mitochondrial fragmentation prior to a loss of $\Delta\Psi_m$ (Nakamura et al., 2011). However, the converse is also true, as PINK1 knock-down results in complex I deficiency and loss of $\Delta\Psi_m$ that can precede a change in mitochondrial morphology (Clark et al., 2006; Morais et al., 2009). Other PD-associated mutations are also associated with increased fission and mitochondrial dysfunction: loss-of-function mutations in the redox sensor DJ-1, as mentioned above, exhibit increased fission and ROS production; both phenotypes are rescued by knocking down the pro-fission protein Drp1, suggesting that excessive fission either mediates or potentiates ROS accumulation and oxidative damage (Wang et al., 2012).

Reduced fusion and increased fission is not always destructive: PINK1 knock-out flies exhibit reduced complex I and complex IV activity, resulting in impaired OXPHOS that leads to ATP depletion and ROS production; these deficits are attenuated by overexpressing Drp1 (Dagda et al., 2009; Liu et al., 2011). The pro-fission activity of PINK1 is therefore, in some cases, a protective mechanism. Indeed, Drp1 knock-down inhibits fission and leads to the accumulation of mitochondrial DNA damage, impaired OXPHOS, and ROS accumulation (Parone et al., 2008). Precise regulation of mitochondrial dynamics, rather than simple inhibition of fission, is therefore essential to mitochondrial function, as discussed in the following section.

Mitochondrial quality control: the role of PINK1 and Parkin

Despite the protective effect of fusion, mitochondria ultimately accumulate enough

damage to warrant their clearance: brain mitochondria have an average half-life of 25 days (Menzies and Gold, 1971). Damaged mitochondria, as identified by a depolarized $\Delta\Psi_m$, are selectively cleared by an autophagic process known as mitophagy (Narendra et al., 2008; Chu, 2010). This selectivity is mediated by the coordinated activity of the mitochondrial kinase PINK1 and the E3 ubiquitin ligase Parkin (Narendra et al., 2008; Ziviani et al., 2010). Although an interaction between these proteins was identified several years ago (Clark et al., 2006; Yang et al., 2006), it has only been in the last three years that the mechanism and consequences of that interaction have begun to be parsed (Poole et al., 2008; Yang et al., 2008; Park et al., 2009; Yu et al., 2011). As discussed in earlier sections, mutations in these proteins alter mitochondrial bioenergetics and calcium buffering; these phenotypes may result, at least in part, from impaired mitophagy.

PINK1 is continually imported into mitochondria (Matsuda et al., 2010), where it is normally degraded by voltage-dependent proteolysis in the inner mitochondrial membrane (Whitworth et al., 2008; Jin et al., 2010; Narendra et al., 2010; Meissner et al., 2011; Lazarou et al., 2012). When the $\Delta\Psi_m$ dissipates, PINK1 accumulates on the outer mitochondrial membrane, where it recruits Parkin from the cytoplasm (Matsuda et al., 2010; Narendra et al., 2010; Cai et al., 2012; Lazarou et al., 2012; Okatsu et al., 2012). PINK1 phosphorylates a number of targets, including Miro (Glater et al., 2006), the mitochondrial transport adaptor protein described above in the discussion of calcium-mediated transport arrest. Miro phosphorylation by PINK1 results in its Parkin-mediated ubiquitination and degradation (Weihofen et al., 2009; Wang et al., 2011; Liu et al., 2012). This transport arrest sequesters damaged mitochondria, preventing their interaction with the healthy network.

Once sequestered, further phosphorylation by PINK1 and ubiquitination by Parkin flags depolarized mitochondria for destruction by mitophagy (Matsuda et al., 2010; Wang et al., 2011). This clearance is essential to neuronal function, as evidenced by animal models in which impaired mitophagy is accompanied by neurodegeneration. PINK1 depletion in flies, for example, causes mitochondrial fragmentation and increased sensitivity to oxidative stress, as well as neurodegeneration (Clark et al., 2006; Yang et al., 2006). PINK1 has a number of effects on mitochondrial function, disruption of which would be expected to lead to mitochondrial pathology; however, the neurodegenerative phenotype can be rescued by Parkin overexpression, suggesting it results from impaired sequestration and degradation of damaged mitochondria (Clark et al., 2006; Yang et al., 2006). In some cases, Parkin-mediated mitophagy can occur even in the absence of PINK1 (Dagda et al., 2009; Chu, 2010), which indicates that this pathway is regulated by multiple mechanisms. These processes are still being delineated, and the majority of studies on mitochondrial dynamics and mitophagy have been carried out in vitro, often in non-neuronal cells. There are therefore some inconsistencies across studies that will require more attention in living systems, if we are to parse these pathways in the context of neuronal physiology and dysfunction (Dagda et al., 2009; Lutz et al., 2009; Sandebring et al., 2009; Van Laar et al., 2011).

The effect of aSyn on mitochondrial dynamics and quality control may result from a direct association between this protein and mitochondria (Banerjee et al., 2010; Kamp et al., 2010; Nakamura et al., 2011). This association can inhibit fusion (Kamp et al., 2010; Xie and Chung, 2012) and cause Drp1-independent mitochondrial fragmentation (Nakamura et al., 2011) that can be prevented by overexpressing PINK1, Parkin, or DJ-1 (Kamp et al., 2010). Asyn-induced mitochondrial fragmentation can be observed prior to

both a decrease in $\Delta\Psi_m$ (Banerjee et al., 2010; Nakamura et al., 2011) and an increase in sensitivity to oxidant stress (Butler et al., 2012), suggesting that its regulation of mitochondrial dynamics may initiate a cascade of events that cause further dysfunction. In addition, in vitro studies suggest that aSyn aggregates decrease Parkin solubility (Kawahara et al., 2008), which may limit the degradation of damaged mitochondria.

Mitochondrial transport and axon degeneration

Mitochondrial distribution in the neuron depends on bidirectional transport

As discussed above, mitochondria have a number of critical functions in the cell, with roles in bioenergetics, calcium buffering, ROS production and detoxification, and initiation of programmed cell death. Mitochondria thus form an integrated system that is both vulnerable and responsive to cellular changes on many levels (Jenner, 2003; Schon and Przedborski, 2011; Tradewell et al., 2011). Dysfunction at any one of these levels is likely to affect the others, causing a broad range of effects that could culminate in degeneration.

The maintenance of mitochondrial homeostasis in the nervous system is particularly complicated because neurons are highly polarized, with elaborate dendrites and axons that can extend far beyond the cell body. Neuronal mitochondria are not evenly distributed; they are concentrated at sites of high demand, such as the presynaptic terminal, and transported along microtubules as needed (Berthold et al., 1993; Fabricius et al., 1993; Hollenbeck and Saxton, 2005; Mironov, 2007; Edgar et al., 2008; Wang and Schwarz, 2009). Depletion of axonal and synaptic mitochondria correlates with impaired synaptic transmission and axon degeneration, even in the absence of cell death (Guo et

al., 2005; Li et al., 2009b; Russo et al., 2009; Misko et al., 2010). Moreover, although mitochondrial turnover can occur within the axon (Amiri and Hollenbeck, 2008; Vincent et al., 2010), the primary site of both biogenesis and mitophagy is thought to be the cell body (Davis and Clayton, 1996; Cai et al., 2012). The regulation of bidirectional mitochondrial transport is therefore essential to ensure that healthy mitochondria are locally available to support neuronal function, and that damaged mitochondria are permanently cleared.

Mitochondrial transport machinery

Neuronal mitochondria are transported along microtubules by means of motor complexes that include kinesin or dynein, associated with multiple adapter proteins (Kondo et al., 1994; Hurd and Saxton, 1996; Glater et al., 2006; Pilling et al., 2006; Karle et al., 2012). As described briefly in the sections above, the mitochondrial GTPase Miro on the outer mitochondrial membrane is indispensible for mitochondrial transport along the axon. Miro interacts with the adapter protein TRAK1/Milton (Stowers et al., 2002; Górska-Andrzejak et al., 2003; Saotome et al., 2008; Cai and Sheng, 2009; Pathak et al., 2010), and proper localization of mitochondria requires both proteins (Russo et al., 2009; Misko et al., 2010). The mechanisms by which transport direction (anterograde or retrograde along the axon) is regulated are not clear, but Milton, which binds both kinesin and dynein-dynactin (van Spronsen et al., 2013), may have an active role in this process.

Regulation of mitochondrial transport and docking

Cytoplasmic calcium and ADP

Mitochondrial trafficking and distribution are affected by a number of factors, though

many of the mechanisms, as well as their relative importance, are still being investigated. Transport of healthy mitochondria is reduced at sites of high ADP (Mironov, 2007) and, as discussed earlier, at sites of high cytoplasmic calcium (Saotome et al., 2008; Wang and Schwarz, 2009; Chang et al., 2011), to ensure that local metabolic and calcium-buffering demands are met. Once microtubule-based transport has terminated, mitochondrial retention at these sites is mediated by adaptor proteins such as syntaphilin (Kang et al., 2008; Chen et al., 2009) and syntabulin (Cai et al., 2005), and there is some evidence that myosin-based transport may also contribute to this process (Chada and Hollenbeck, 2004; Pathak et al., 2010).

Mitochondrial damage

As discussed earlier, a dramatic loss of $\Delta\Psi_m$ causes PINK1/Parkin-mediated transport arrest through Miro degradation (Liu et al., 2011; Wang et al., 2011). Beyond this mechanism, the relationship between the $\Delta\Psi_m$ and mitochondrial transport has not been fully elucidated. Some studies have shown a correlation between the direction of transport and $\Delta\Psi_m$, with polarized mitochondria moving primarily in the anterograde direction, and depolarized mitochondria preferentially moving back towards the cell body (Miller and Sheetz, 2004), presumably to undergo mitophagy. This correlation is not universal, however, and may depend on the degree of depolarization (Verburg and Hollenbeck, 2008).

Mitochondrial transport defects in neurodegenerative disease models

Mitochondrial transport deficits have been observed in a number of neurodegeneration models (Morfini et al., 2009), though it is not always the case that rescuing such defects is protective (Zhu and Sheng, 2011; Marinkovic et al., 2012; Millecamps and Julien,

2013). If the observed “defect” is secondary to PINK1/Parkin-mediated degradation of Miro, for example, then to rescue transport without repairing mitochondrial function could be deleterious. In some cases, however, a trafficking defect may be primary, and its rescue is essential to both synaptic transmission and neuronal integrity. For example, mutations in mitofusin proteins are associated with the axonal neuropathy Charcot-Marie-Tooth disease type 2A2, and cause defective fusion and trafficking through separate mechanisms (Baloh et al., 2007; Misko et al., 2010; Misko et al., 2012). The transport defect results in depletion of mitochondria at the presynaptic terminal, which may underlie the phenotype of axon degeneration observed in this disease (Palau et al., 2009; Misko et al., 2010; Misko et al., 2012). The axonal compartment is particularly sensitive to transport impairments, and even subtle dysregulation of mitochondrial distribution in the axon can initiate local hypoxia and focal degeneration (Misko et al., 2012; Shahpasand et al., 2012). Preservation of mitochondrial trafficking may therefore be critical to preventing axon degeneration, particularly if considered in the context of mitochondrial function (Hollenbeck and Saxton, 2005; Iijima-Ando et al., 2009; Li et al., 2009b; Sterky et al., 2011).

Parkinson disease axonopathy

In recent years, attention has begun to shift from the cell body to the axon in the question of PD pathogenesis (Cheng et al., 2010; O'Malley, 2010; Burke and O'Malley, 2012). Mutations in LRRK2 and aSyn are associated with both hereditary and idiopathic PD, and roles in the presynaptic terminal have been described for both of these proteins (Burré et al., 2010; Matta et al., 2012; Boassa et al., 2013). In some neuronal subtypes and disease models, aSyn accumulates in the axon prior to the cell body (Marui et al., 2002; Orimo et al., 2008; Schulz-Schaeffer, 2010; Volpicelli-Daley et al., 2011; Boassa

et al., 2013), a phenotype also observed in some post-mortem studies of PD pathology (Braak et al., 1999; Galvin et al., 1999). Moreover, synaptic dysfunction and axonal degeneration precedes cell death in both toxin-induced (Herkenham et al., 1991; Li et al., 2009a; Cartelli et al., 2010; Arnold et al., 2011; Kim-Han et al., 2011; Mijatovic et al., 2011) and genetic (Li et al., 2009c; Decressac et al., 2012) models of PD. Because axon degeneration impairs neurotransmission, it causes functional deficits even if the cell body remains intact. Attempts to preserve neuronal circuitry and prevent neurodegeneration will therefore require a better understanding of the mechanisms by which axons degenerate (Coleman and Perry, 2002; Cheng et al., 2010; Pienaar et al., 2012).

As discussed in the sections above, mitochondrial dysfunction is directly implicated in PD pathogenesis, and exposure to various environmental toxins that impair mitochondrial bioenergetics is a strong risk factor for the disease. Because the axonal compartment is especially sensitive to disruptions in mitochondrial function and transport, a better understanding of the relationship between these disruptions and axonal integrity may identify new therapeutic targets that act on pathways either upstream of cell death or parallel to it.

Wallerian degeneration as a model to study axon degeneration

Molecular regulation of axonal integrity

The most tractable model of axon degeneration is Wallerian degeneration, the process by which a distal axon degenerates after physical transection. Once thought to entail a passive “starvation” caused by axonal separation from the cell body, Wallerian

degeneration (WD) is now known to be an active process of self-destruction, analogous to, but distinct from, apoptosis (Saxena and Caroni, 2007). This insight into the regulated nature of WD had its origin in the discovery of the *Wallerian degeneration slow* (WldS) mouse, a spontaneous mutant with no obvious phenotype beyond a dramatic delay in axon degeneration following transection (Perry et al., 1991). That a genetic mutation could prevent degeneration suggested that WD was not the passive result of a general lack of nutrients, but instead a process of self-destruction that could be regulated at the molecular level. The mutation that caused this phenotype was later identified as a genomic rearrangement that fused two genes: full-length nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), an enzyme that catalyzes the synthesis of NAD from nicotinamide ribonucleotide, and the N-terminus of Ube4b, an E4-type ubiquitin ligase (Coleman et al., 1998; Mack et al., 2001). Structure-function analyses in a number of model organisms have implicated Nmnat1 enzymatic activity in WldS-dependent protection, and the N-terminal 16 amino acids of WldS in the relocalization of the protective protein from the nucleus to the axon (Laser et al., 2006; Avery et al., 2009; Conforti et al., 2009). The mouse WldS protein retains its protective effects in both flies and zebrafish, suggesting that the mechanisms by which it protects axons are conserved across species (Coleman, 2005).

Compartment-specific neurodegeneration

In a number of cellular models characterized by both cell death and axon degeneration, WldS selectively protects the axons, while the cell bodies degenerate normally (Adalbert et al., 2006; Beirowski et al., 2008). Moreover, caspase-3, one of the primary effectors of apoptosis, does not appear to be activated in the axon after transection (Finn et al., 2000), and deletion of the pro-apoptotic proteins Bax and Bak does not delay WD

(Whitmore et al., 2003). Wallerian degeneration does not, therefore, appear to activate classical pathways of cell death. The study of WldS in other degeneration models has provided further evidence for the existence of molecular pathways that selectively regulate degeneration of the axon. After nerve growth factor withdrawal or direct caspase activation, which cause cell death and axon degeneration, WldS delays axon degeneration, but has no effect on cell death (Deckwerth and Johnson, 1994), which is prevented by Bax and Bak deletion (Whitmore et al., 2003). These and other studies suggest that neurodegeneration is compartment-specific, with different processes underlying degeneration of the soma and the axon after injury. This finding may be relevant to the treatment of neurodegenerative disease. In PD, for example, approximately 30% of dopaminergic neurons in the substantia nigra have died by the time a diagnosis is typically made on the basis of mild motor symptoms, but efforts to prevent death of the remaining cells have failed to delay symptom progression (Waldmeier et al., 2006; Investigators, 2007; Hart et al., 2009). There are a number of reasons why this might be the case (Waldmeier et al., 2006), but the model of compartment-specific neurodegeneration suggests that more effective treatment might require a dual approach in which survival of the cell body and the axon are targeted separately (Coleman and Perry, 2002). Indeed, increased neurotrophic signaling delayed nigral cell death, but not striatal axon degeneration, in the MPTP model of PD (Mijatovic et al., 2011).

Convergent mechanisms of axon degeneration

WldS protects axons in neurodegenerative disease models

Expression of WldS or overexpression of various Nmnat isoforms protects axons not only after transection, but also in a number of disease models (Coleman, 2005; Saxena

and Caroni, 2007), including several that are relevant to PD, such as MPTP (Hasbani and O'Malley, 2006; Antenor-Dorsey and O'Malley, 2012), rotenone (Press and Milbrandt, 2008), and 6-hydroxydopamine toxicity (Sajadi et al., 2004; Cheng and Burke, 2010). That WldS is protective against such varied insults suggests that axon degeneration pathways are highly convergent (Coleman, 2005). This notion is the primary rationale for the widespread use of axotomy as a model for the study of axon degeneration and protection. Indeed, protection by WldS has become the single criterion by which axon degeneration is classified as "Wallerian-like," regardless of the initial insult. Even in the absence of physical transection, various disorders associated with impaired axonal transport may result in a functional separation from the cell body that initiates Wallerian-like degeneration (Coleman and Perry, 2002). Axon degeneration in a number of models and diseases, including the gracile axonal dystrophy (GAD) mouse(Mi et al., 2005), the neurodegeneration of glaucoma (Beirowski et al., 2008), and the axonopathy of motor neuron disease (Ferri et al., 2003), has been classified as Wallerian-like, suggesting that the identification of the downstream target(s) of WldS may give insight into axon degeneration in a number of disorders. Still, WldS is not universally protective (Vande Velde et al., 2004), and understanding the nature of its selectivity may help to distinguish different axon degeneration pathways, and to identify sites at which those pathways converge.

Mitochondria are implicated in Wallerian degeneration and WldS protection

Multiple lines of evidence suggest that mitochondrial function and transport are impaired during Wallerian degeneration, and that WldS protects axons by preventing those impairments. During WD, ATP depletion and mitochondrial swelling occur (Ikegami and Koike, 2003; Adalbert et al., 2005; Wang et al., 2005), accompanied by a loss of $\Delta\Psi_m$

(Ikegami and Koike, 2003; Sievers et al., 2003) that can be prevented by WldS (Zheng et al., 2010). In addition to its effects on WD, WldS protects against a number of mitochondrial toxins, including the complex I inhibitors MPTP and rotenone (Hasbani and O'Malley, 2006; Press and Milbrandt, 2008; Antenor-Dorsey and O'Malley, 2012), though it does not appear to do so by increasing expressing of respiratory chain components (Yahata et al., 2009). Its protective effect declines with age (Perry et al., 1992; Ferri et al., 2003), which may reflect a role in the prevention or regulation of ROS generation (Press and Milbrandt, 2008), though this hypothesis has not yet been confirmed in vivo.

WldS may improve mitochondrial calcium buffering

Application of vincristine, a neurotoxic chemotherapeutic agent that destabilizes microtubules, causes apoptotic cell death and non-apoptotic axonal degeneration characterized by mitochondrial depolarization and ATP depletion (Ikegami and Koike, 2003). WldS expression prevents vincristine axonopathy (though not the caspase-dependent death of the cell body), and preserves axonal ATP and $\Delta\Psi_m$, indicating that its protective target is upstream of these changes. Consistent with this hypothesis, in the presence of sufficiently high calcium, both wild-type and WldS-expressing axons undergo proteolytic cytoskeletal degeneration, indicating that calcium-activated proteases (calpains) are present and functional in the cytosol; however, WldS-expressing axons can tolerate a higher calcium load before that occurs (Glass et al., 1994). Likewise, mitochondria isolated from wild-type and WldS-expressing mouse brains can tolerate a higher calcium load before mPTP formation, suggesting that improved calcium buffering may be a protective mechanism in these cells (Avery et al., 2012). Indeed, calpain activation has long been associated with cytoskeletal breakdown in WD (Schlaepfer and Bunge, 1973; Yawo and Kuno, 1985; Xie and Barrett, 1991;

Glass et al., 1994; George et al., 1995; Ziv and Spira, 1995), and mitochondrial calcium overload and consequent mPTP activation may represent an irreversible step in axon degeneration (Wang et al., 2000; Barrientos et al., 2011; Adalbert et al., 2012). Moreover, treatment with the mitochondrial uncoupler CCCP (carbonyl cyanide m-chloro phenyl hydrazine) causes axon degeneration that WldS cannot prevent (Ikegami and Koike, 2003), suggesting that mitochondrial function is required for WldS-mediated protection. Indeed, because mitochondrial uncoupling prevents calcium efflux from the mitochondrial matrix, it facilitates formation of the mPTP, which could abrogate any protective effect of WldS on mitochondrial calcium handling.

Consistent with a model in which WldS acts directly on mitochondria, the mitochondrially localized isoform of mouse Nmnat, Nmnat3, confers potent axonal protection after injury in both mice and flies (Avery et al., 2009; Yahata et al., 2009). Moreover, depletion of axonal mitochondria may prevent WldS-mediated protection (Avery et al., 2012), though this finding is controversial (Kitay et al., 2013). A mitochondrial, and specifically calcium-mediated, effect on axon degeneration and protection is further supported by the fact that knock-out of the sole *Drosophila* Nmnat isoform, *dNmnat*, leads to spontaneous axon degeneration, suggesting that this protein regulates axonal protection (Zhai et al., 2006). The spontaneous axon degeneration observed in dNmnat knock-out flies can be prevented by inhibiting neuronal activity (Zhai et al., 2006). Since activity-dependent degeneration is often associated with calcium overload (as in the case of excitotoxicity), it is possible that axonal Nmnat actively and constitutively regulates axonal integrity through effects on calcium buffering. Indeed, there is evidence in vitro that Nmnat acts as an endogenous axonal survival factor that is rapidly depleted after axotomy, and that WldS substitutes for it (Gilley and Coleman, 2010). Regulation of both cytosolic and mitochondrial calcium levels would be a plausible mechanism for such a survival

pathway, particularly as mPTP activation has recently been identified as a critical event in axon degeneration (Barrientos et al., 2011). As discussed above, this regulation would not have to be direct, since a primary protective effect on mitochondrial bioenergetics would also affect calcium handling.

WldS may protect through increased mitochondrial transport

In addition to a possible role in mitochondrial function, increased mitochondrial trafficking has also been suggested as a mechanism by which WldS protects axons. In WldS-expressing flies, baseline mitochondrial transport is higher than in wild-type, and inhibition of that transport prevents WldS-mediated protection (Avery et al., 2012). However, reports of the relationship between WldS and mitochondria are inconsistent, with some suggesting that their transport is not required for protection (Kitay et al., 2013). Moreover, in the SOD1 model of amyotrophic lateral sclerosis, a mitochondrial transport deficit is often observed, but does not correlate with axon degeneration (Marinkovic et al., 2012), and increased mitochondrial trafficking is not protective (Zhu and Sheng, 2011). This suggests that a correlation between WldS or Nmnat protection, as seen in flies, might not reflect causation. However, WldS is not actually protective in the SOD1 model of axon degeneration (Vande Velde et al., 2004), so the relationship between mitochondrial transport and WldS protection may be more meaningful in Wallerian-like degeneration pathways. This uncertainty could be addressed by an approach in which the effects of mitochondrial transport and function on axon degeneration and WldS-mediated protection can be monitored and manipulated in vivo.

Larval zebrafish peripheral sensory neurons as a model for studying axon degeneration

The larval zebrafish provides an excellent model for studies of the subcellular events underlying axon degeneration. By exploiting the optical transparency and genetic tractability of this system, dynamic processes of growth, degeneration, and regeneration can be observed in vivo (Sagasti et al., 2005; O'Brien et al., 2009b; Martin et al., 2010; Rieger and Sagasti, 2011). The trigeminal and Rohon-Beard peripheral sensory neurons are ideal for such studies. The cell bodies of these neurons are located behind the eye and along the length of the spinal cord, respectively, but their axons arborize between the two layers in the skin (O'Brien et al., 2012). This superficial orientation is ideal for in vivo imaging, and also permits precise transection of an individual branch using a two-photon laser (O'Brien et al., 2009a; Martin et al., 2010). Wallerian degeneration of the injured axon can then be observed in vivo through time-lapse confocal imaging (O'Brien et al., 2009a).

Three phases of WD have been characterized in this model (Martin et al., 2010).First, after the initial injury, there is a lag phase in which the axon is separated from the cell body but remains intact. This is followed by abrupt, synchronous fragmentation along the length of the transected axon. Finally, there is phagocytic clearance of the fragments. These three phases are complete within approximately two hours after injury (Martin et al., 2010). This rapid time frame permits continuous imaging of the entire process. In *Drosophila*, by contrast, axons do not appear dystrophic until at least 8 hours after axotomy, both in larvae and adult animals (MacDonald et al., 2006; Fang et al., 2012), and the process takes at least 24 hours in various peripheral nerves in mice (Lubińska, 1977; Lunn et al., 1989; Brown et al., 1991; Beirowski et al., 2005). As such, time-lapse

imaging throughout the lag phase in these systems is not practical, and transient changes in degenerative processes may go unnoticed.

The optical transparency and genetic tractability of the larval zebrafish model can also be harnessed to visualize dynamic intracellular processes that may be relevant to degeneration, including axonal transport of various organelles (Plucinska et al., 2012; Drerup and Nechiporuk, 2013). A recent study of zebrafish peripheral sensory neurons revealed impairments in axonal transport of mitochondria in a tauopathy model (Plucinska et al., 2012), confirming its utility in the study of cell biological processes implicated in neurodegenerative disease. It is therefore ideally situated to study the relevance of mitochondrial transport and function to axon degeneration and protection.

Goals of the dissertation

To summarize, axon degeneration is relevant to neurodegenerative diseases such as Parkinson disease, and may be compartment-specific. Because the WldS protein protects axons in a wide range of disease models, the pathways that regulate axonal integrity and degeneration are likely to be highly conserved. Wallerian degeneration therefore provides a simplified experimental model in which the cell biological processes underlying axon degeneration and protection can be studied.

This thesis explores the relevance of mitochondrial transport and function to axon degeneration. In Chapter 2, I evaluate mitochondrial transport in peripheral sensory neurons both during development and after injury. After two-photon laser transection, I observed abrupt mitochondrial transport arrest, as has previously been reported in flies (Avery et al., 2012). By studying the effect of WldS expression or PINK1 knock-down on

injury-induced transport arrest, however, I found that transport and function did not consistently correlate. By expressing the redox-sensitive biosensor roGFP2 (Hanson et al., 2004) in the mitochondrial matrix, I observed dynamic redox changes after injury that were mitigated by WldS, suggesting that mitochondrial ROS production might mediate axonal vulnerability after injury, and could ultimately lead to degeneration. Activation of the transcriptional co-activator PGC-1α, a critical regulator of mitochondrial biogenesis (Wu et al., 1999), has recently been associated with ROS detoxification and neuroprotection in vitro (St-Pierre et al., 2006). I therefore overexpressed this protein to determine whether it would alter the redox changes after axotomy. I observed that PGC-1α did temporarily attenuate the injury-induced oxidation of roGFP2, and delayed Wallerian degeneration. These results suggest mitochondrial redox state, likely a reflection of mitochondrial ROS production, is more relevant to axonal integrity than mitochondrial transport deficits alone.

In Chapter 3, I used many of the same tools to describe degeneration induced by overexpression of wild-type human aSyn in zebrafish peripheral sensory neurons. In this model, I observed cell death in approximately 20% of neurons between 2 and 3 days post-fertilization. I observed significant axon degeneration in aSyn-expressing cells, with a higher percentage of dystrophic axons than cell bodies. This model provides a new system in which to study the cell biological processes underlying aSyn toxicity in vivo, particularly within the axonal compartment. Mitochondrial imaging in aSyn-expressing axons revealed early morphological changes consistent with increased fragmentation, as well as early transport deficits, which may reflect a protective response to fragmentation and dysfunction. In highly dystrophic axons, varicosities were occupied by swollen and/or aggregated mitochondria. Consistent with a primary effect of mitochondrial

dysfunction, PGC-1α expression robustly prevented both the early axonopathy and the later cell death in this model.

In Chapter 4, I briefly discuss other models of degeneration, one through genetically induced apoptosis, and the other through mitochondrial ROS accumulation. These models can be used in the future to further explore compartment-specific degeneration and protection.

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Chapter 2. WldS and PGC-1α regulate mitochondrial transport and oxidation state after axonal injury

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ABSTRACT

Mitochondria carry out many of the processes implicated in maintaining axon health or causing axon degeneration, including ATP and reactive oxygen species (ROS) generation, as well as calcium buffering and protease activation. Defects in mitochondrial function and transport are common in axon degeneration, but how changes in specific mitochondrial properties relate to degeneration is not well understood. Using cutaneous sensory neurons of living larval zebrafish as a model, we examined the role of mitochondria in axon degeneration in vivo. We monitored mitochondrial morphology, transport, and redox state before and after laser axotomy. Mitochondrial transport terminated locally after injury in wild-type axons, an effect that was moderately attenuated by expressing the axon-protective fusion protein Wallerian degeneration slow (WldS). However, mitochondrial transport eventually ceased in WldS-protected axons, and increasing mitochondrial transport after axotomy by knocking down PTEN-induced putative kinase 1 (PINK1) accelerated degeneration, indicating that mitochondrial transport is neither necessary nor sufficient for axon protection. By contrast, the redox-sensitive biosensor roGFP2 was rapidly oxidized in the mitochondrial matrix after injury, and WldS expression prevented this effect, suggesting that

stabilization of ROS production may mediate axon protection. Overexpression of PGC-1α, a transcriptional co-activator with roles in both mitochondrial biogenesis and ROS detoxification, dramatically increased mitochondrial density, attenuated roGFP2 oxidation, and delayed WD. Collectively, these results indicate that mitochondrial oxidation state is a more reliable indicator of axon vulnerability to degeneration than mitochondrial motility.

INTRODUCTION

Axons are vulnerable to changes in energy production, organelle transport, and oxidative stress, all of which accompany axon degeneration (Coleman, 2005). The relative importance of each of these processes in degeneration has been difficult to parse, given their interdependence and the challenge of monitoring them in vivo. Mitochondria perform many of the processes implicated in degeneration, including ATP and reactive oxygen species (ROS) generation, as well as calcium buffering and protease activation. Therefore, it is not surprising that changes in mitochondrial function and transport are frequently observed during axon degeneration (Court and Coleman, 2012); however, the specific role that mitochondria play in these processes remains poorly understood.

Mitochondria have been linked to Wallerian degeneration (WD), a stereotyped program by which injured axons degenerate that shares features with axon pathology in neurodegenerative diseases (Coleman, 2005). Recent studies suggest that mitochondria may directly initiate WD, potentially through activation of the mitochondrial permeability transition pore (mPTP) (Barrientos et al., 2011). WD can be dramatically delayed by the Wallerian degeneration slow protein *(*WldS), a mutant mouse protein created by a spontaneous genomic rearrangement (Perry et al., 1991; Coleman et al., 1998; Mack et

al., 2001). Recent evidence suggests that the protective effects of WldS are mediated by mitochondria (Avery et al., 2012; Fang et al., 2012). For example, mitochondria isolated from WldS-expressing axons exhibit increased ATP production (Yahata et al., 2009) and calcium buffering (Avery et al., 2012), and a recent study found a correlation between mitochondrial transport and axon protection by WldS (Avery et al., 2012). However, whether mitochondrial transport is necessary or sufficient for axon protection after injury is not clear. Moreover, conflicting data indicate that mitochondria can either promote or inhibit axon degeneration in different contexts (Fang et al., 2012; Kitay et al., 2013).

To examine the role of mitochondria in axon degeneration we have imaged them in zebrafish peripheral sensory neurons undergoing WD. This model can be used to monitor the entire process of WD in vivo (O'Brien et al., 2009b; Martin et al., 2010) and to characterize dynamic subcellular events (Plucinska et al., 2012). By imaging mitochondria after axotomy in these neurons, we have characterized mitochondrial density, morphology, transport, and redox state during WD. Axonal injury led to mitochondrial transport arrest and oxidation of the redox-sensitive biosensor roGFP2 (Hanson et al., 2004) in the mitochondrial matrix, suggesting that mitochondrial ROS production may be relevant to axon degeneration. Increased mitochondrial transport was neither necessary nor sufficient for axon protection. By contrast, expression of WldS or the transcriptional co-activator PGC-1α prevented roGFP2 oxidation and delayed axon degeneration. Collectively, our results suggest that stabilization of mitochondrial redox state may regulate axon protection after injury.

MATERIALS AND METHODS

Fish

Fish were raised on a 14 h/10 h light/dark cycle at 28.5°C. Embryos were maintained in a 28.5°C incubator. All experiments were approved by the Chancellor's Animal Research Care Committee at the University of California, Los Angeles.

Injections

For transient, mosaic gene expression in sensory neurons, approximately 10 pg of each transgene was injected at the one-cell stage into wild-type AB or nacre (Lister et al., 1999) embryos or embryos from the transgenic lines described below. Embryos were screened for fluorescent transgene expression in Rohon-Beard (RB) neurons at 24-48 hours post fertilization (hpf), using a Zeiss Discovery.V12 SteREO fluorescence dissecting microscope.

Transgenes

The stable transgenic lines used to visualize sensory neurons have been described elsewhere (Sagasti et al., 2005; Palanca et al., 2012). Briefly, the *isl1(ss*) enhancer from the islet-1 gene (Higashijima et al., 2000) was used to drive expression of Gal4VP16, which activated expression of EGFP or DsRed under control of the Gal4 upstream activation sequence (14 copies; 14XUAS) (Köster and Fraser, 2001). For studies of mitochondrial transport after injury, fish from a previously described stable transgenic line expressing GFP under the control of the *Fru.trkA* enhancer (Palanca et al., 2012) were also used, because their reporter expression was sparse, allowing the identification of embryos expressing GFP in a single RB neuron. For developmental experiments in wild-type embryos, sensory neurons were visualized by transient injection of the *isl1(ss)*:Gal4VP16–14XUAS–reporter plasmids used to generate the stable lines described above. The Tol2/Gateway zebrafish kit (Kwan et al., 2007) was used for

construction of all remaining plasmids. In all cases transgene expression was driven by the *CREST3* enhancer (gift of H. Okamoto; (Uemura et al., 2005). Like *isl1(ss)*, the *CREST3* enhancer is derived from the *islet-1* gene and drives expression in peripheral sensory neurons.

WldS: For co-expressing GFP and WldS in sensory neurons, we used a previously described transgene (*CREST3*:Gal4VP16–14xUAS–GFP–14xUAS–WldS) (Martin et al., 2010). To achieve equimolar expression of DsRed and WldS, we used the viral T2A sequence, which causes ribosomal skipping (Donnelly et al., 2001; Tang et al., 2009). This sequence was inserted into the Gateway system (Invitrogen) behind a multiple cloning site. WldS was amplified using primers to add XhoI and HindIII restriction sites to the 5' and the 3' ends of the sequence, respectively, and cloned into the system to generate *CREST3*:Gal4VP16–14XUAS–WldS-2A-DsRed.

PGC-1α: A plasmid encoding mouse PGC-1α, which was previously expressed in zebrafish (Hanai et al., 2007), was a gift from Dr. Shintaro Imamura. The PGC-1α coding sequence was amplified using primers to add XhoI and BamHI to the 5' and 3' ends of the sequence, respectively. The sequence and the p3E–MCS-2A-DsRed and p3E–MCS-2A-GFP Gateway donor vectors were cloned with XhoI and BamHI sites to generate p3E–PGC-1α-2A-DsRed and p3E–PGC-1α-2A-GFP. These donor vectors were then recombined to generate *CREST3*:Gal4VP16–14XUAS–PGC-1α-2A-GFP and *CREST3*:Gal4VP16–14XUAS–PGC-1α-2A-DsRed.

MitoDsRed: To visualize mitochondria, a cox8 mitochondrial targeting sequence was added to DsRed (mitoDsRed, gift of Meghan E. Johnson and Carla Koehler). The

reporter UAS–mitoDsRed–polyA was used to generate a *MitoDsRed* transgenic line using the Tol2 system (Kawakami, 2004). The plasmid, which contained Tol2 sites flanking UAS–mitoDsRed–polyA, was injected with transposase mRNA into zebrafish eggs at the one cell stage. These fish were raised and crossed to *isl1(ss)*:Gal4VP16:14XUAS-GFP fish. Progeny from this cross were then screened for the expression of GFP and mitoDsRed in sensory neurons. These double-transgenic fish were then crossed to WT AB fish to obtain a line of single-transgenic UAS-mitoDsRed fish.

Mito-roGFP2: To monitor changes in the mitochondrial redox potential, roGFP2 (Hanson et al., 2004) was localized to the mitochondrial matrix with a cox8 targeting sequence (mito-roGFP2, gift from Meghan E. Johnson and Carla Koehler). Mito-roGFP2 was cloned into the Gateway system to generate UAS–mito-roGFP2–polyA. This transgene was coinjected with one of the *CREST3*:DsRed reporters described above, for expression of DsRed (+/- WldS or PGC-1α) in the axon and mito-roGFP2 in the mitochondrial matrix.

Synaptophysin-GFP: The transgene encoding the zebrafish Synaptophysin-EGFP fusion protein (Syp-GFP) has been described (Meyer and Smith, 2006), and was a gift from Martin Meyer.

Morpholinos

Morpholino oligonucleotides targeting the zebrafish *pink1* gene (NM_001008628) (Anichtchik et al., 2008) were acquired from GeneTools, LLC (Philomath, OR). Sequences were as previously described (ATG targeting, 5′-GCT GAG AAC ATG CTT TAC TGA CAT T-3′; 5′-untranslated region (UTR) targeting, 5′-ATA TTG ACT ATG AGA

GGA AAT CTG A-3′) and 7 ng of each morpholino were coinjected into one-cell stage embryos (Anichtchik et al., 2008). Control embryos were injected with 14 ng of the standard GeneTools control morpholino (5’-CCT CTT ACC TCA GTT ACA ATT TAT A-3’).

Imaging axons and mitochondria

Embryos were dechorionated, anesthetized in 0.01-0.02% tricaine, mounted in 1.2% low-melt agarose (Promega, V2111) in sealed chambers (O'Brien et al., 2009a), and imaged on a heated stage kept at 28.5°C. For time-lapse analysis of mitochondrial movement and WD, GFP- and mitoDsRed-expressing embryos were imaged at various intervals for up to 3 hours on a confocal microscope (Zeiss LSM 510). Images were compiled into projections and movies with QuickTime software.

Mitochondrial imaging: To monitor mitochondrial morphology and density, GFP reporter transgenes were injected into UAS–mitoDsRed-expressing embryos. Embryos were imaged at 2-4 dpf using a confocal microscope with a 40X oil objective and 3X digital zoom. Mitochondrial and vesicular transport imaging was limited to a single optical section. Before each time-lapse movie, an image was taken using both 488 nm and 543 nm lasers, so that the axon could be visualized for later analysis. Mitochondria or vesicles were imaged every second for 6 minutes. In the case of axotomy, a time-lapse movie was made before axon transection for comparison. Only axons in which at least one mitochondrion moved prior to injury were then cut and imaged for post-injury analysis. For evaluation of relative redox potential, mito-roGFP2-expressing embryos were imaged with a 20x air objective and 4X digital zoom. Images were taken before and at various intervals after injury, scanning at 543 nm to visualize red axons, and at 405 nm and 488 nm to monitor roGFP2 oxidation.

Axon transection

Axons were cut using a Zeiss microscope (LSM 710) equipped with a multiphoton laser (O'Brien et al., 2009a). Embryos were imaged with a 20X water objective and 488/543 nm laser scanning to identify the axonal region of interest, then 1-5 scans of the multiphoton laser (tuned to 910 nm for green axons, 850 nm for red axons) were used to transect an axonal region of interest at 100X digital zoom.

Data Analysis

Mitochondrial morphology: Morphology was quantified from 2D projections of confocal stacks generated from images taken at 40X, 3X digital zoom. In the green channel, all axons within an image were traced using ImageJ software. Line length was calibrated to convert pixels to distance, and the Measure plugin for ImageJ was used to determine the length and diameter of each mitochondrion in an image. All mitochondria within an image were quantified, and the ratio of length/width was taken as a measure of mitochondrial morphology. Mean morphology was compared with unpaired t-tests. To further analyze morphology, GraphPad Prism software was used to divide mitochondria into bins according to their length/width ratio (bin width: 1.0; i.e., all mitochondria with a length/width ratio between 1-2 were in Bin 1), and the relative frequency of each bin was determined as a percent of the total, and represented in a histogram. Note that all discussions of mitochondrial “length” refer to this ratio, to control for any differences in expression levels that might increase or decrease mitoDsRed fluorescence intensity and thereby affect measurement.

Mitochondrial transport: The percent of total mitochondria that moved in a 50-μm axon segment during a 6-minute time-lapse movie was quantified using the Kymograph macro

for ImageJ. Kymograph color was inverted so that mitochondria appeared black. A mitochondrion was considered to be moving only if it traveled at least 2 μm, at a speed of at least 0.1 μm/s, to be consistent with published work (Misgeld et al., 2007). Speed was calculated as the slope of distance (x) over time (y) on the kymograph. Vesicular transport was quantified using the same parameters. For measures of transport after axotomy, data from all axons were binned according to time after injury, and statistical analysis was performed.

Mitochondrial density: Mitochondrial density in axons of WT and WldS-expressing embryos, and in control and PINK1 morphants, was determined by counting all mitochondria (stationary and motile) in a kymograph. In PGC-1-expressing neurons, mitochondrial distribution was irregular. For this reason, mitochondrial density was determined from still images in multiple regions of the axon, and values therefore primarily represented the density of stationary mitochondria. Mitochondrial density in still images from WT axons was evaluated as a control.

Relative redox potential: The excitation maxima of roGFP2 are ~400 and ~475-490 nm; under reducing conditions, the 475-490 nm excitation peak dominates, whereas oxidation results in relatively higher excitation at 400 nm (Hanson et al., 2004). Using confocal images taken before and at various time points after injury, projections were generated to calculate the 405/488 fluorescence ratio of mitochondria in the Z stack. Measures were taken from all mitochondria within 100 μm of the eventual injury site, and values were pooled to generate a mean fluorescence ratio for each axon before injury. All post-injury values for a given axon were calculated as a percentage of the initial. Data

from all axons were then binned according to time after injury, and statistical analysis was performed.

Statistical Analysis: GraphPad Prism software was used for statistical analysis. Planned, unpaired Student's t-tests were used to compare independent groups. A paired t-test was used to compare mito-roGFP2 oxidation in individual axons early and late in the lag period before degeneration. One-way ANOVA was used to compare three or more groups, with Dunnet's post-tests used for comparison to the control. Two-way ANOVA was used to evaluate the effect of time and genotype/location on changes in transport and roGFP2 oxidation after injury, followed by Bonferroni's post-tests to evaluate significance at each time point.

RESULTS

Monitoring mitochondria in living zebrafish sensory neurons

To study morphology and motility of mitochondria in a live, vertebrate system, we created reporters to label them in zebrafish somatosensory neurons (Figure 2.1A). DsRed fused to the cox8 mitochondrial matrix targeting sequence (mitoDsRed) was placed under the control of UAS elements and used to generate a stable transgenic line (UAS:mitoDsRed) (Figure 2.1B). The *CREST3* enhancer element, which drives expression in peripheral sensory neurons (Uemura et al., 2005; Palanca et al., 2012), was used to drive Gal4VP16, and this transgene was injected into mitoDsRed embryos to achieve mosaic expression in small numbers of sensory neurons (Figure 2.1C). Mitochondrial density and transport were evaluated at 2 and 4 days post-fertilization

(dpf) in 50-μm segments of individual peripheral axons. Cells were monitored for six minutes at one frame per second (Movie 1, Figure 2.1D), and transport was quantified using kymograph analysis (Figure 2.1E). Mitochondrial density was slightly but significantly lower at 4 dpf (2 dpf: 2.80 ± 0.24 mitos/mm; 4 dpf: 2.08 ± 0.19 mitos/mm; n ≥ 16 cells, $p<0.05$) but mitochondrial motility, defined as the percentage of mitochondria that moved at any point within a 6-minute time-lapse movie, did not differ significantly between 2 and 4 dpf (2 dpf: 23.2 ± 4.0%; 4 dpf: 20.1 ± 5.1%; n ≥ 16 cells, $p=0.59$). Among motile mitochondria, the speed of uninterrupted runs in the retrograde direction did not change between 2 and 4 dpf (2 dpf: 0.69 ± 0.06 μm/s; 4 dpf: 0.58 ± 0.06 μm/s; n ≥ 16 mitos in n ≥ 11 cells, $p=0.383$); however, anterograde velocity was higher at 4 dpf (2 dpf: 0.50 ± 0.03 μm/s; 4 dpf: 0.65 ± 0.04 μm/s; n ≥ 51 mitos in n ≥ 11 cells, $p = 0.003$). The mitoDsRed system can thus be used to detect quantitative changes in axonal mitochondria during development, similar to the recently described *MitoFish* system (Plucińska et al., 2012).

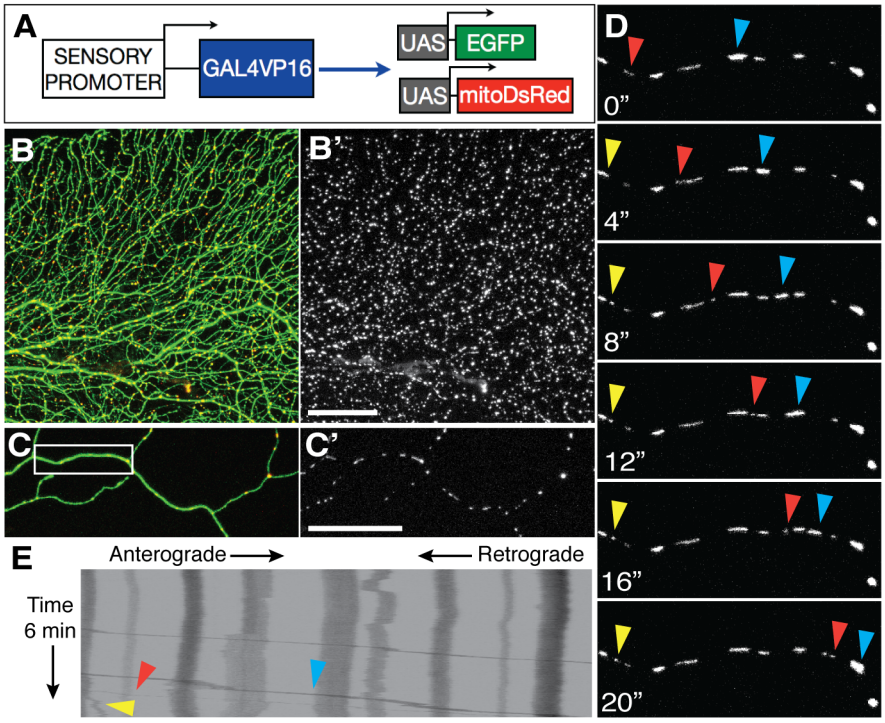

Figure 2.1 In vivo imaging of mitochondrial transport in zebrafish somatosensory neurons.

A) Transgenes to visualize GFP in neurons and DsRed in the mitochondrial matrix. B) When a stable UAS–mitoDsRed transgenic fish line was crossed to a previously described line in which the *isl1(ss)* sensory promoter drives Gal4 and GFP expression in somatosensory neurons, red fluorescent mitochondria (B') were visible in green fluorescent axons. C-C') To permit quantification of mitochondrial transport in a single cell, the *CREST3*:Gal4-UAS–GFP reporter transgene was injected into UAS–mitoDsRed embryos at the one-cell stage for transient mosaic gene expression. D) A single optical section was repeatedly imaged at a frequency of 1 Hz (see Movie 1) to quantify mitochondrial motility (the percentage of mitochondria that moved within a 6-minute movie), and to characterize transport speed and direction. Images in (D) represent still frames every 4" near the end of Movie 1. E) Kymograph representing transport in Movie 1. Arrows point to moving mitochondria in (D). Scale bars: 50 μm. White box in (C) represents axonal segment in (D) and corresponds to Movie 1.

Mitochondrial transport arrest after injury is local and specific

Recent studies in invertebrate and in vitro systems have suggested that mitochondrial number and transport decrease upon injury (Avery et al., 2012; Fang et al., 2012). To determine whether mitochondrial transport was also affected in a vertebrate system in vivo, we transected peripheral axons of sensory neurons with a laser at 3 dpf. At this stage Wallerian degeneration (WD) in peripheral sensory neurons is highly stereotyped, and fragmentation occurs approximately 90 minutes after axotomy (Martin et al., 2010). MitoDsRed-expressing axons were imaged before (Figure 2.2A) and after (Figure 2.2B) laser transection. Mitochondrial transport was monitored along 50-μm axon segments either immediately adjacent (0-50 μm) or 100-200 μm distal to the site of injury. Within two minutes post axotomy (mpa), mitochondrial transport completely terminated in the axonal segment immediately distal to the site of injury and never recovered (Figure 2.2C-D,I). By contrast, transport in axonal segments further from the cut site was not immediately affected (Figure 2.2E-F,I). However, motile mitochondria in this region spent significantly less time moving in the anterograde direction (away from the cut site), and a greater amount of time paused between runs (Figure 2.2J), and the velocity of uninterrupted runs in the retrograde direction was decreased (Figure 2.2K). Thus, although degeneration is synchronous throughout the distal fragment (Figure 2.2G-H) (Martin et al., 2010), acute transport arrest was limited to the region nearest the site of injury; however, all mitochondrial transport ultimately stops in the distal axon prior to fragmentation.

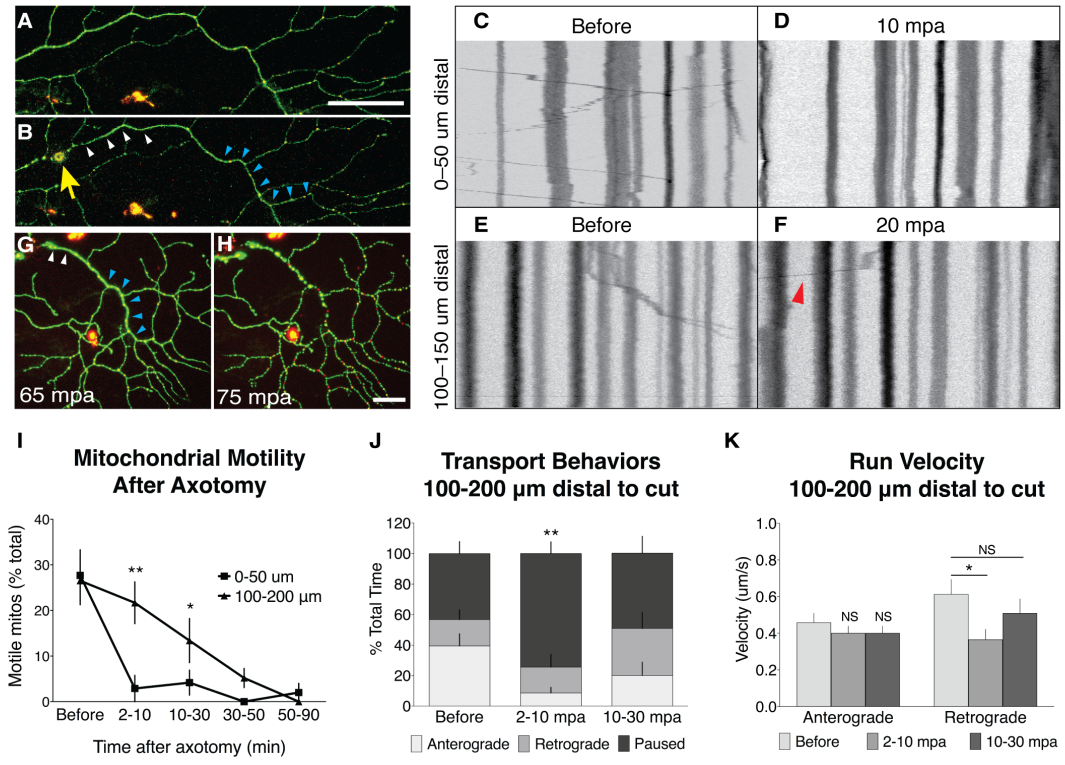

Figure 2.2 Axonal injury causes mitochondrial transport arrest in a separated axon fragment.

A-B) Mitochondrial transport was imaged in a 50-µm axon segment before (A) and after (B) GFP- and mitoDsRed-expressing axons were severed with a laser (yellow arrow points to injury site). Transport was monitored either immediately distal to the injury (white arrowheads) or 100-200 µm distal (blue arrowheads). C-F) Representative kymographs from 50-µm axonal segments before and after axotomy at the two locations. Red arrowhead in (F) points to motile mitochondrion in more distal region 20 minutes post-axotomy (mpa). G-H) The distal axon ultimately fragments synchronously: the entire detached axon is intact at 65 minutes post-axotomy (G), and has fragmented by 75 mpa (H). I) Quantification of mitochondrial motility before and at various time-points after axotomy. Adjacent to the injury, motility stopped abruptly (% motile before: 27.69 ± 5.63%; 2-10 mpa: 2.89 ± 2.89%; n = 8 axons, p=0.002) and did not recover (10-90 mpa: 1.96 ± 1.10%; p<0.0001). Further from the injury (100-200 µm), mitochondrial transport did not immediately terminate (% motile before: 26.51 ± 5.25% motile; 2-10 mpa: 21.68 ± 4.57, n ≥ 5 axons; p=0.5386); however, the characteristics of transport were acutely affected (J-K). J) At the 100-200 µm distal site, motile mitochondria spent a higher percentage of time paused immediately after axotomy (before: 43.28 ± 7.91%; 2-10 mpa: 74.48 ± 7.73%), and proportionally less time moving in the anterograde direction, away from the cut site (before: 39.45 ± 7.96%; 2-10 mpa: 8.55 ± 3.71%, n ≥ 21 mitochondria from ≥ axons, p<0.001). By 10-30 mpa, however, this difference was no longer significant (Bonferroni's post-test, p>0.05). K) Quantification of the speed of uninterrupted runs (not counting time paused) revealed that retrograde transport was reduced acutely in mitochondria after injury (retrograde speed before: 0.67 ± 0.09 µm/s; 2-10 mpa: 0.36 ± 0.06 µm/s; n ≥ 8 mitochondria from ≥ 5 axons, p=0.0286), but anterograde speed was not affected (before: 0.46 ± 0.05 µm/s; 2-10 mpa: 0.40 ± 0.04 µm/s; n ≥ 8 mitochondria from ≥ 5 axons). Later in the lag phase, retrograde transport velocity recovered (10-30 mpa: 0.51 ± 0.08 µm/s; p=0.238). Despite this transient recovery, transport eventually stopped throughout the distal axon (I) prior to fragmentation. Scale bars: 50 µm.

The eventual loss of all distal mitochondrial movement after injury could be caused by a global impairment of axonal transport, or the effect could be specific to mitochondria. To distinguish between these possibilities, we imaged vesicular transport by expressing a Synaptophysin-GFP fusion protein (Meyer and Smith, 2006) in DsRed-labeled axons (Figure 2.3A-C). In contrast to the acute mitochondrial transport arrest, vesicular transport was unaffected immediately after axotomy, and persisted at baseline rates until shortly before axon fragmentation (Figure 2.3D-F). Mitochondrial transport arrest after axotomy is therefore not likely caused by generalized microtubule breakdown or dysfunction of microtubule motors, but represents a specific and local cessation of mitochondrial transport.

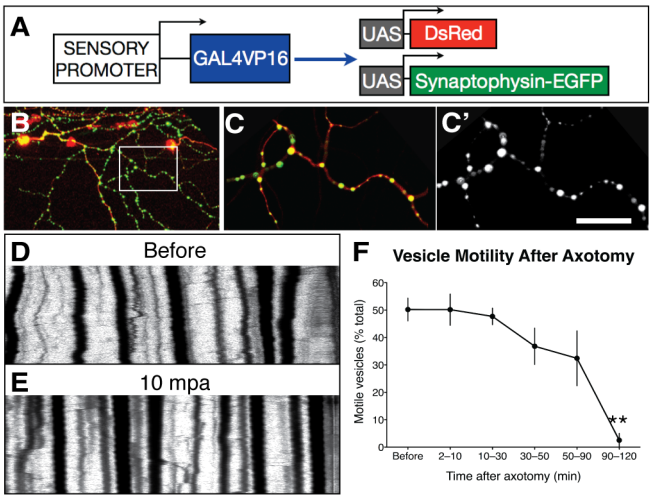

Figure 2.3 Vesicular transport is not affected by axonal injury.

A-C) DsRed and a Synaptophysin-GFP fusion protein were expressed in axons to visualize vesicle transport before and after axotomy in 2 dpf embryos. Image in (C-C') is a single optical section taken from boxed region in (B). D-E) Representative kymographs illustrating vesicle motility before and 10 minutes post-axotomy (mpa). F) In contrast to the acute mitochondrial transport arrest, vesicular transport was not acutely affected by injury (% motile before: 50.22 ± 4.14%; 2-10 mpa: 50.17 ± 5.70%; n = 8 axons, p=0.99), and persisted at baseline rates until shortly before axon fragmentation (90-120 mpa: 24.54 ± 6.81%, p=0.0014). Scale bar: 50 μm.

To examine whether mitochondrial transport arrest correlates with axon degeneration, we monitored mitochondrial motility in the proximal portion of the transected axon (0-50 μm proximal to the injury, Figure 2.4A), which remains connected to the cell body and does not degenerate. Overall mitochondrial motility in the proximal axon was not altered at any point after axotomy (Figure 2.4B-E). Motile mitochondria did, however, spend a higher percentage of time paused for the first hour after axotomy (Figure 2.4F), and the duration and speed of retrograde movement were also reduced (Figure 2.4G). However, the net direction of transport was not significantly different (Figure 2.4H). Together, these changes in the proximal axon stump favored mitochondrial retention near the injury. One hour later the differences in mitochondrial transport were no longer statistically significant (Figure 2.4E). Despite the mild and transient changes in the characteristics of transport, overall mitochondrial motility in the proximal axon was thus preserved after axotomy, supporting the possibility that mitochondrial transport arrest in the distal axon might underlie degeneration.

WldS mildly affects baseline mitochondrial morphology and transport

Expressing WldS in zebrafish somatosensory axons (Figure 2.5) dramatically delays degeneration after transection (Figure 2.6A-B) (Martin et al., 2010). To determine whether WldS alters mitochondrial motility in zebrafish sensory axons we co-expressed WldS and GFP in mitoDsRed-expressing embryos (Figure 2.5A-C). Overall mitochondrial density in uninjured WldS-expressing axons did not differ from wild-type axons (Figure 2.5B-D; WT: 2.20 ± 0.16 mitos/μm; WldS: 2.33 ± 0.25 mitos/μm; $n \geq 18$ cells, $p=0.64$), but the average mitochondrial morphology (ratio of length/width) was slightly elongated (Figure 2.5B-D). Overall mitochondrial motility in uninjured WldS-expressing axons was not significantly different from WT (Figure 2.5E). The amount of

time motile mitochondria spent either paused or moving in either direction was also not different from WT (Figure 2.5F). The speed of anterograde runs was higher in WldS mitochondria than WT, but retrograde speed was unchanged (Figure 2.5G). Thus, WldS expression in zebrafish somatosensory neurons appears to affect mitochondria only mildly.

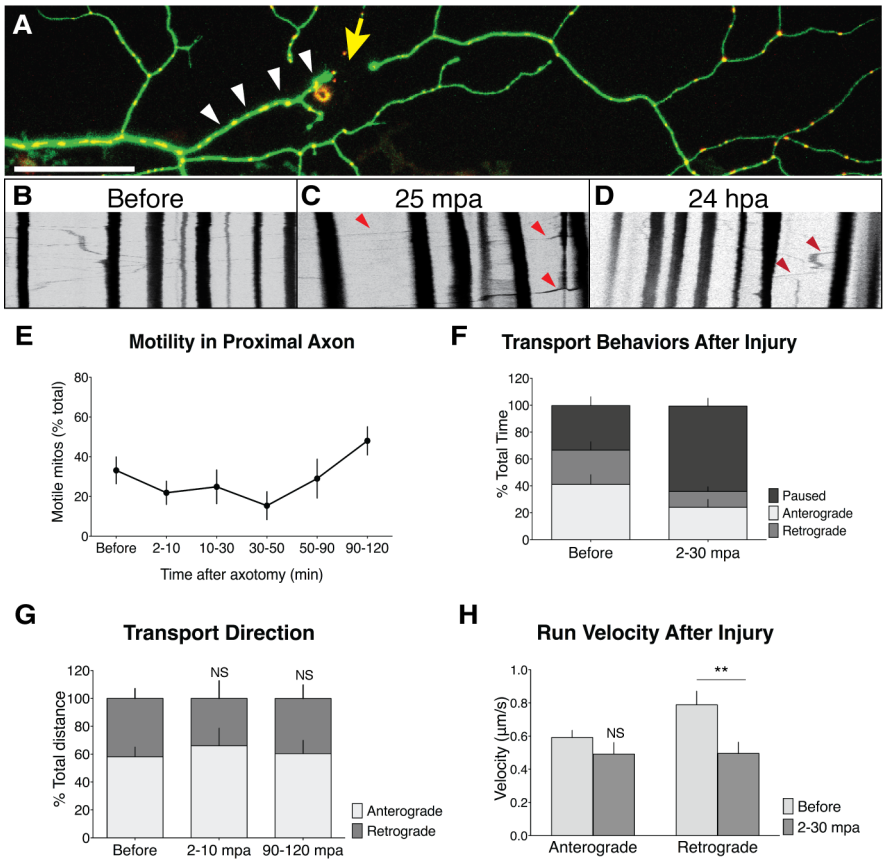

Figure 2.4 Mitochondrial transport persists in the proximal axon after injury.

A) Mitochondrial motility was monitored in the 50-μm axonal segment immediately proximal to the site of injury (white arrowheads) at various time points after axotomy (yellow arrow points to injury site). B-D) Representative kymographs before and after axotomy. E) Overall mitochondrial motility in the proximal axon was not significantly different from baseline at any point after axotomy ($p=0.2158$). F) Motile mitochondria spent a higher percentage of time paused for the first 30 minutes after axotomy (before: 33.10 ± 6.55%; 2-30 mpa: 63.38 ± 5.86%; $n \geq 29$ mitos from ≥ 6 axons, $p=0.0014$). G) The net direction of transport in the proximal axon was unchanged before and after axotomy ($p=0.7959$). H) The speed of uninterrupted runs in the retrograde direction was reduced during the first 30 minutes after axotomy (before: 0.60 ± 0.07 μm/s; 2-60 mpa: 0.41 ± 0.06 μm/s; $n \geq 12$ mitos from ≥ 6 axons, $p=0.0118$), but the anterograde velocity was unchanged ($p=0.2138$). NS: not significant. Scale bar: 50 μm.

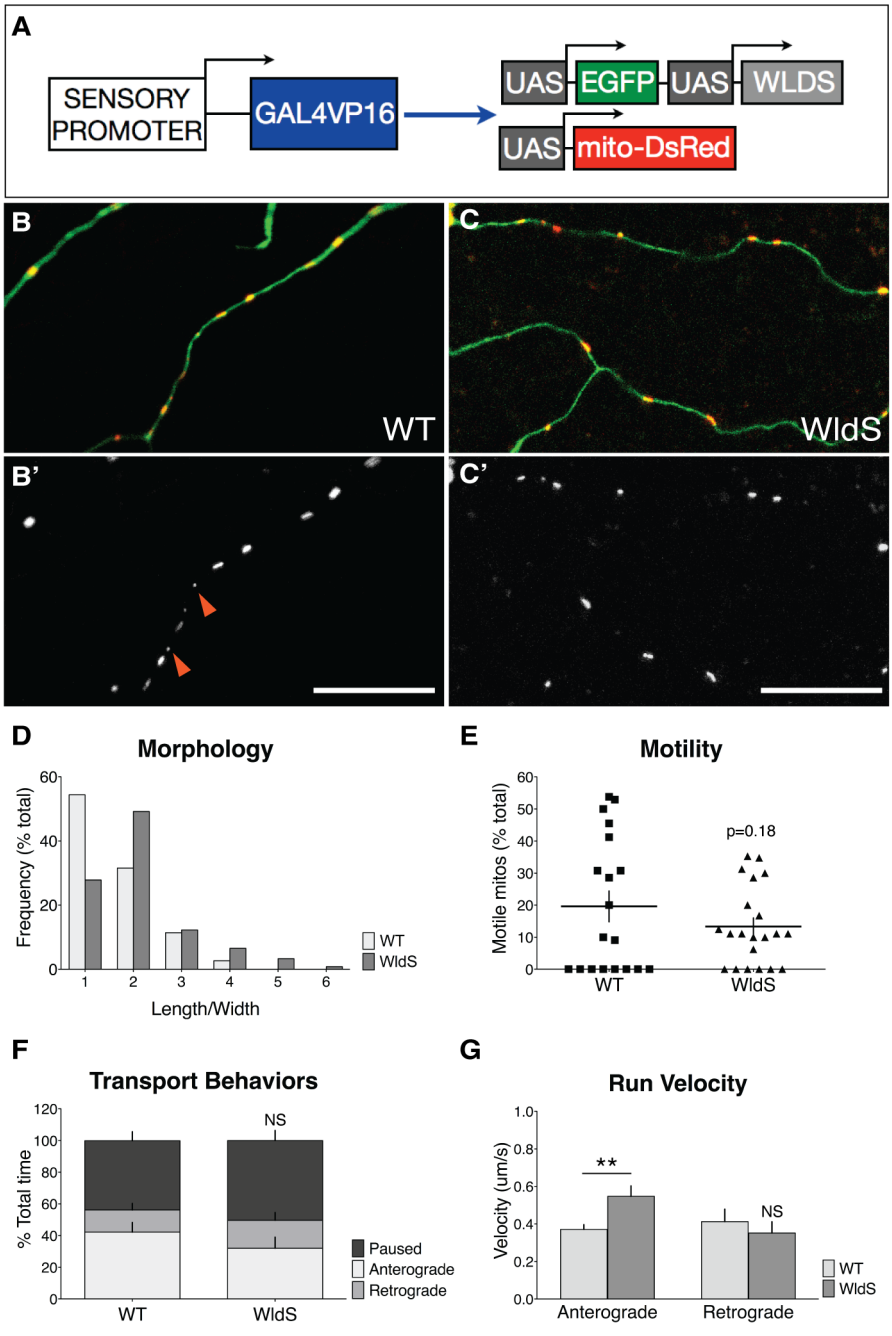

Figure 2.5 WldS mildly affects mitochondrial morphology and transport in uninjured axons.

A) Transgenes used to visualize mitochondria in WldS-expressing axons. A sensory promoter drove expression of either GFP (WT, B-B') or WldS and GFP (C-C') in UAS-mitoDsRed embryos. B-D) Mitochondria in WldS-expressing axons were, on average, elongated compared to WT mitochondria (WT

average length/width ratio: 1.70 ± 0.07; WldS: 2.12 ± 0.09, p=0.0002). This difference was primarily attributable to the presence of fewer small, spherical mitochondria (length/width ratio of 1; WT example indicated by arrowheads in B'), as indicated by the histogram in D. E) Overall mitochondrial motility in uninjured WldS-expressing axons was not significantly different from WT (WT % motile: 20.71 ± 4.96%; WldS 13.32 ± 2.66%; n ≥ 18 axons, p=0.18). F) The amount of time motile mitochondria spent paused or moving in either direction was not different from WT (p>0.05). G) Mitochondrial transport in the anterograde direction was significantly faster in WldS-expressing axons (WT anterograde speed: 0.37 ± 0.03 μm/s; WldS: 0.55 ± 0.06 μm/s; n ≥ 23 mitochondria from ≥ 11 axons), but retrograde speed was not significantly different (WT: 0.41 ± 0.07 μm/s; WldS: 0.35 ± 0.06 μm/s; n ≥ 18 mitochondria from ≥ 11 axons; p=0.5282). Scale bar: 20 μm.

WldS modestly and temporarily mitigates mitochondrial transport arrest after axotomy

Although there was no significant difference in baseline mitochondrial motility in WldS-expressing axons, it is possible that preservation of motility after injury underlies its protective effect (Avery et al., 2012). To test this hypothesis, we evaluated mitochondrial transport before and after axotomy. As in wild-type axons, mitochondria in the portion of the axon immediately distal to the injury stopped moving acutely after transection in WldS-expressing axons, but shortly thereafter mitochondrial transport recovered to baseline values (Figure 2.6C-D; WT values in Figure 2.6D are replotted from Figure 2.2). At that time, motile mitochondria moved more in the retrograde direction than before injury, as measured both by time (Figure 2.6E), and by distance (Figure 2.6F). The velocity of uninterrupted runs in either direction was not different (Figure 2.6G). Mitochondrial transport during this phase thus favors retrograde movement towards the cut site, similar to the effect seen in the WT proximal axon.

The early transport recovery observed in transected WldS-expressing axons was temporary. At all later time points, mitochondrial motility was reduced relative to baseline (Figure 2.6D). This observation suggests that long-term protection is not mediated by

long-term transport preservation. To further examine this idea, we monitored axonal mitochondria in WldS-expressing neurons 24 hours post-axotomy (hpa) and compared mitochondrial motility to uncut axons from age-matched controls. Although distal axons from these WldS-expressing cells were still intact (Figure 2.6B), all mitochondria were essentially stationary (4 dpf uncut: 13.71 ± 3.16% motile mitochondria; 4 dpf, 24 hpa: 0.45 ± 0.45% motile; 14/15 axons had no motile mitos, p=0.0004). This finding demonstrates that mitochondrial motility is not required for the maintenance of axonal integrity. It also suggests that WldS may not protect axons by regulating mitochondrial transport.

Some studies suggest that uneven mitochondrial distribution induces local hypoxia that initiates axon degeneration (Misko et al., 2012). It is therefore possible that the early attenuation of mitochondrial transport arrest by WldS allows mitochondria to redistribute to a more uniform pattern. To examine this possibility, we measured mitochondrial distribution in WldS-expressing axons before axotomy and 50-90 mpa, after the transport recovery had occurred. There was no difference in average intermitochondrial distance or the variation from the mean (before: 4.36 ± 0.35 μm, 50-90 mpa: 4.73 ± 0.36 μm, n ≥ 97 mitos in 7 axons), suggesting that WldS likely does not protect axons by altering mitochondrial distribution after injury.

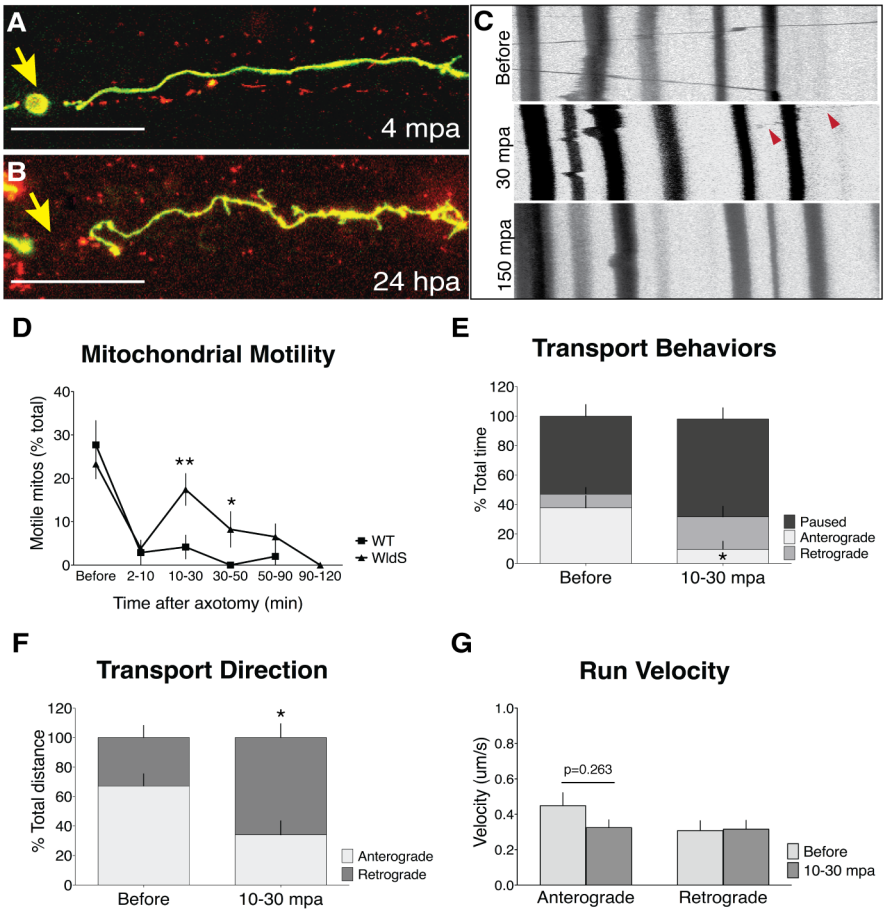

Figure 2.6 WldS moderately and temporarily mitigates mitochondrial transport arrest after injury.

A-B) WldS-expressing axons were transected and imaged at various time points after injury. B) WldS-expressing axons remained intact 24 hours post-axotomy (hpa), despite persistent detachment from the cell body. C) Representative kymographs from movies of mitochondrial transport at various time points before and after axotomy. All mitochondrial transport was evaluated in 50-mm axonal segments immediately distal to the injury. D) As in WT axons, acute mitochondrial transport stopped in the distal axon immediately after transection in WldS-expressing axons (% motile before: 23.29 ± 3.40%; 2-10 mpa: 3.85 ± 1.73%, n = 13 cells, $p<0.0001$), but shortly thereafter transport temporarily recovered to baseline values (10-30 mpa: 17.43 ± 3.67%, n = 12 cells). Eventually, mitochondrial transport terminated altogether in WldS-expressing axons (% motile 50-90 mpa: 5.376 ± 2.511 n = 17 axons; $p=0.0002$). E) After recovering from acute transport arrest, motile mitochondria spent a lower percentage of time moving in the anterograde direction than before injury (before: 37.72 ± 8.32%; 10-30 mpa: 9.58 ± 5.48%; n ≥ 18 mitos from 12 cells; $p=0.0143$). F) The net direction of mitochondrial movement also changed, with mitochondria traveling a higher percentage of their total distance in the retrograde direction towards the cut site (retrograde before: 32.85 ± 8.33%; 10-30 mpa: 65.97 ± 9.5%; n ≥ 18 mitos from ≥ cells, $p=0.013$). G) Run velocity also did not change in either direction (anterograde before: 0.45 ± 0.07 μm/s; 10-30 mpa: 0.33 ± 0.04 μm/s; $p=0.2633$; retrograde before: 0.31 ± 0.06 μm/s; 10-30 mpa: 0.32 ± 0.05 μm/s; $p=0.9170$). Scale bar in A: 50 μm.

PINK1 knock-down increases mitochondrial transport after axotomy without delaying degeneration

Observing mitochondria in WldS-expressing axons demonstrated that ongoing motility is not required for protection. To test whether early motility might be sufficient to protect axons from degeneration we sought to prevent their arrest after axotomy with an independent manipulation. Damaged mitochondria accumulate PTEN induced putative kinase 1 (PINK1) on their surface (Matsuda et al., 2010), which ultimately leads to their dissociation from microtubules (Liu et al., 2012). We hypothesized that if transport arrest after axotomy is mediated by PINK1 stabilization on depolarized mitochondria, PINK1 knock-down might promote recovery of transport after transection. We used a previously characterized morpholino (Anichtchik et al., 2008) to knock down PINK1 expression and conducted experiments at 2 dpf, to ensure a robust morphant phenotype. We observed the same gross morphological changes previously reported in these morphants, such as cerebral edema (Figure 2.7A-B). Axonal mitochondria were elongated in PINK1 morphants (Figure 2.7C-D), consistent with the fact that PINK1 promotes mitochondrial fission (Deng et al., 2008; Poole et al., 2008). There was no difference in baseline mitochondrial motility between PINK1 and control morphant embryos (Figure 2.7E). After axotomy, mitochondrial motility recovered modestly in PINK1 morphants (Figure 2.7F), but this increased motility was not accompanied by axon protection. On the contrary, axon degeneration was slightly accelerated, with a shorter lag phase prior to fragmentation in PINK1 morphants compared to controls (Figure 2.7G). PINK1 knock-down therefore phenocopies the post-injury mitochondrial transport recovery observed in WldS-expressing axons, but does not provide axon protection. Together our results demonstrate that axon motility is neither required nor sufficient for axon protection.

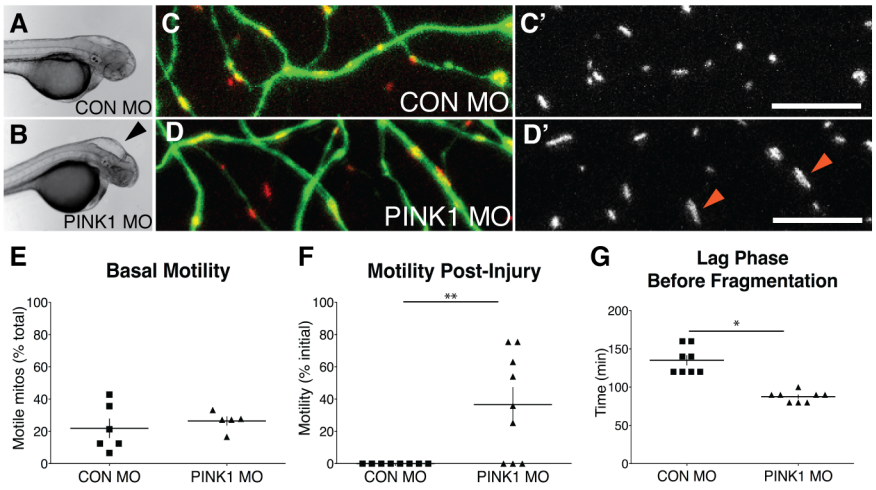

Figure 2.7 PINK1 knock-down increases mitochondrial motility after injury and accelerates axon degeneration.

A-B) UAS-mitoDsRed embryos were coinjected with control or PINK1 morpholinos and the sensory:Gal4:UAS-GFP transgene used in Figures 1-2. Phenotypes such as cerebral edema (arrowhead in B) have previously been reported with this PINK1 morpholino. C-D: Control (C-C') and PINK1 morphants (D-D') were imaged at 2 dpf. Mitochondria were on average longer in PINK1 morphants than controls (arrowheads in D). E) There was no difference in baseline mitochondrial motility between PINK1 and control morphant embryos (Control MO: 21.93 ± %; PINK1 MO: 26.48 ± 2.70%; n ≥ 5 axons, p=0.5285). F) After axotomy, mitochondrial transport stopped in control morphants, but PINK1 morphants experienced mild recovery (quantified as % original motility; Control MO: 0.00 ± 0.0 motile; PINK1 MO: 36.59 ± 10.66%; n ≥ 5 cells, p=0.0081). G) Axon degeneration after injury was accelerated in PINK1 morphants (quantified as the duration of the lag period between injury and axon fragmentation; Control MO: 135.0 ± 6.3 min; PINK1 MO: 87.5 ± 2.5 min; n = 8 axons per group; p<0.0001). Scale bar: 10 μm.

Axonal injury alters the redox state of mitochondria

Since mitochondrial motility per se does not appear to be instructive in WD, we investigated another feature of mitochondrial function. In addition to their roles in ATP synthesis and calcium buffering, mitochondria are the primary site of ROS production in the cell. Because oxidative stress is associated with aging and neurodegeneration (Balaban et al., 2005; Lin and Beal, 2006) and has been proposed to play a role in axon degeneration (Press and Milbrandt, 2008; Fischer and Glass, 2010; Calixto et al., 2012), we determined whether mitochondrial redox state was affected by axotomy, using the ratiometric, redox-sensitive roGFP2 (Hanson et al., 2004) localized to mitochondria

(Figure 2.8A). The emission intensity of roGFP2 at two excitation wavelengths (~400 nm and ~475-490 nm) is a function of the redox state of the subcellular compartment to which it is localized: an increase in the 405/488 fluorescence ratio of roGFP2 reflects oxidation (Hanson et al., 2004). We evaluated mito-roGFP2 fluorescence before and after transecting wild-type axons with a laser. Mito-roGFP2 was rapidly and significantly oxidized in the distal axon after injury (2-10 mpa: 161.9 ± 13.14% initial) and remained elevated but stable until shortly before fragmentation, when it was further oxidized (Figure 2.8B). In the proximal axon, by contrast, the early increase in oxidation was mild (2-10 mpa: 107.9 ± 10.49). Later, mito-roGFP2 oxidation in the proximal axon fell below baseline, and was significantly lower than that observed in the distal axon at all later time points (Figure 2.8C).

To determine whether WldS expression might regulate mitochondrial redox state, we examined mito-roGFP2 fluorescence in severed WldS-expressing axons. Unlike the change in WT axons, mito-roGFP2 was only mildly and temporarily oxidized after injury in WldS-expressing axons (2-10 mpa: 121 ± 14.62% initial), and this effect was significantly lower than that seen in WT (Figure 2.8B; $p<0.05$). At all later time points, mito-roGFP2 was less oxidized in WldS-expressing axons than in WT (Figure 2.8B-C). Together, these results demonstrate that injury changes the oxidation state of axonal mitochondria, and the ability to prevent that change correlates with axon protection.

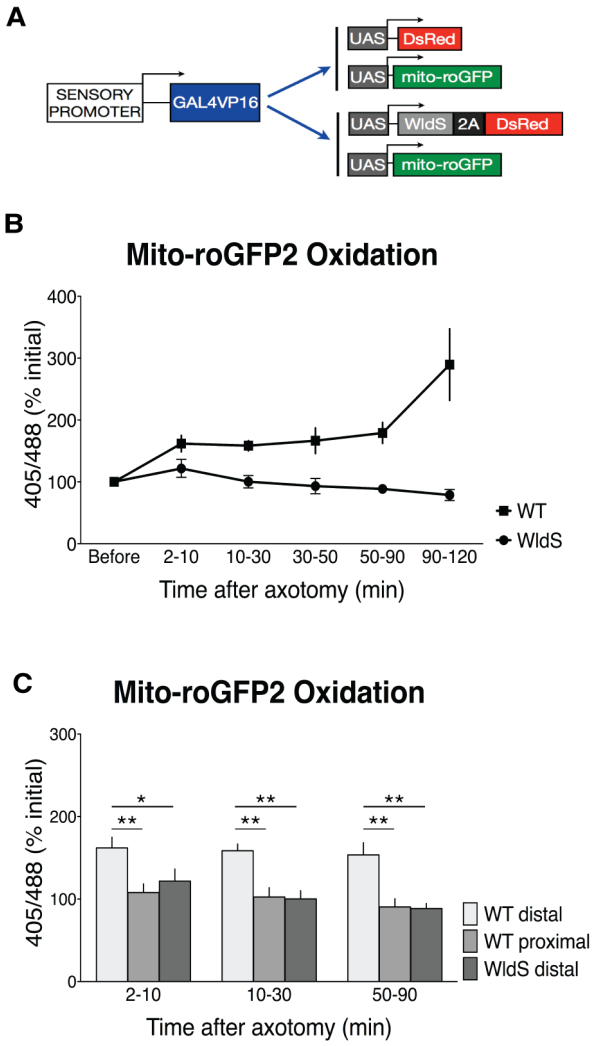

Figure 2.8 Mito-roGFP2 oxidation after injury correlates with axon degeneration.

A) Transgenes were generated to express DsRed +/- WldS in sensory neurons and the redox-sensitive biosensor roGFP2 in the mitochondrial matrix. B) Mito-roGFP2 was rapidly oxidized in the distal axon after injury (2-10 mpa: 167.21 ± 13.91%, n = 13 axons), and remained elevated but stable for most of the lag period before fragmentation. Shortly before fragmentation of the distal axon, however, mito-roGFP2 was further oxidized (90-120 mpa: 289.62 ± 57.79%; n = 5 axons). In the distal axon of WldS-expressing cells, mito-roGFP2 was slightly oxidized acutely after axotomy (2-10 mpa: 121.8 ± 14.62%); however, this effect was still significantly lower than mito-roGFP2 oxidation in WT axons ($p<0.05$, two-way ANOVA with Bonferroni post-tests), and WldS entirely suppressed the long-term oxidation ($p<0.001$). C) Mito-roGFP2 was not significantly oxidized in the proximal axon any point after axotomy (2-10 mpa: 107.9 ± 10.49%; 10-30 mpa: 102.6 ± 11.13%; 50-90 mpa: 91.93 ± 12.21%). Later in the lag phase, mito-roGFP2 oxidation returned to baseline levels in WldS-expressing axons, and was significantly lower than that observed in the distal axon at all time points ($p < 0.001$).

PGC-1α delays axon degeneration and roGFP2 oxidation after injury

The transcriptional co-activator PGC-1α regulates mitochondrial biogenesis (Wu et al., 1999; Lehman et al., 2000) and ROS detoxification (St-Pierre et al, 2006). Because mitochondrial redox state correlated with axon degeneration, we tested whether PGC-1α overexpression could affect WD. We generated reporters to express mouse PGC-1α and either mitoDsRed or mito-roGFP (Figure 2.9A). PGC-1α expression increased mitochondrial content (Figure 2.9B-F): PGC-1α-expressing axons had a greater density of mitochondria (Figure 2.9F), and mitochondria had a dramatically elongated morphology (Figure 2.9C,E,G), likely reflecting an increase in mitochondrial biogenesis and/or fusion. PGC-1α had no effect on baseline mitochondrial transport (WT % motile: 19.62 ± 4.82%; PGC-1α: 23.13 ± 5.81; n ≥ 13 axons; p=0.322).

To determine whether PGC-1α improved axon survival after injury, we axotomized PGC-1α-expressing sensory neurons, and saw markedly decreased mito-roGFP2 oxidation after injury compared to WT (Figure 2.9H). This result suggests that PGC-1α might help to regulate mitochondrial redox homeostasis. PGC-1α did not prevent acute mitochondrial transport arrest (% motile before: 43.92 ± 7.48; 2-10 mpa 16.19 ± 5.14%, p=0.010, n=5 axons). However, relative to WT, transport moderately and temporarily recovered later in the lag phase (Figure 2.9I). PGC-1α expression thus phenocopied the effects of WldS on mitochondrial transport and mito-roGFP2 oxidation after injury. To determine whether these effects correlated with axon protection, we monitored axonal integrity after injury. PGC-1α delayed the onset of fragmentation relative to WT (Figure 2.9J), suggesting that its effects on mitochondria are functionally relevant to axon protection.

The axon protection afforded by PGC-1α, while significant, was not as robust as WldS-induced protection. Because mitochondrial transport could be dissociated from axon protection, we sought to determine whether mito-roGFP2 oxidation was a more accurate predictor of axon vulnerability. We therefore compared mito-roGFP2 oxidation in individual axons at 10-30 mpa and shortly before fragmentation. As in wild-type axons, mito-roGFP2 was oxidized in PGC-1α-expressing axons shortly after axotomy (Figure 2.9K). This observation further strengthens the correlation between mitochondrial redox state and axonal integrity, suggesting that ROS production in the mitochondrial matrix may be critical to axonal vulnerability and degeneration.

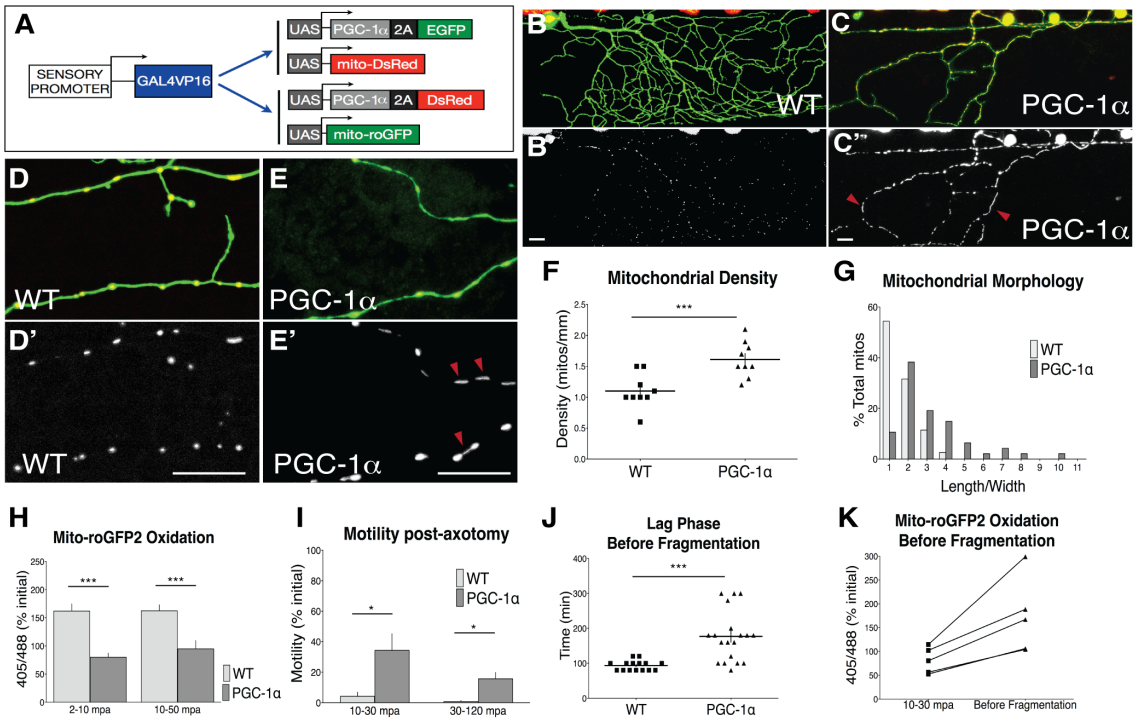

Figure 2.9 PGC-1α alters axonal mitochondrial properties and delays degeneration.

A) Transgenes used to image mitochondria in PGC1α–expressing neurons. B-G) Mouse PGC1α expression in zebrafish sensory neurons caused a significant increase in mitochondrial content, quantified as a higher density of stationary mitochondria (F; WT: 1.1 ± 0.09; PGC1α: 1.611 ± 0.1-; n ≥ 9 axons) and an elongated mitochondrial phenotype (C,E,G; arrowheads in C' and E' point to elongated mitochondria). H) In the first hour after axotomy, PGC1α-expressing axons did not exhibit mito-roGFP2 oxidation. I) Mitochondrial transport (quantified as % initial) recovered moderately after acute post-injury arrest in PGC1α–expressing axons (WT 10-30 mpa: 4.17 ± 2.73% initial; PGC1α: 34.29 ± 10.99%; n=6 axons in each group; p=0.0110; WT 30-120 mpa: 0.77 ± 0.77% initial; PGC1α: 15.67 ± 4.20%; p=0.0124). J) Changes in mitochondrial transport and function correlated with a delay in axon degeneration, with a longer lag period before fragmentation in injured PGC1α–expressing axons (WT: 94.6 ± 4.7 min; PGC1α: 176.8 ± 16.6 min; n ≥ 15 axons; p<0.0001). K) Despite the early protection in PGC-1a-expressing axons, mito-roGFP2 was oxidized shortly before axons fragment. Data are plotted from five representative axons; the 405/488 fluorescence ratio represents pooled mitochondria from each axon either 10-30 mpa or shortly before fragmentation. A paired t-test permitted evaluation of significance (p=0.0202) despite a relatively wide range in oxidation and degeneration times. Scale bar in B-C: 50 μm. Scale bar in D-E: 20 μm.

DISCUSSION

In vivo imaging of mitochondrial transport and oxidation state after injury

Mitochondrial defects are associated with multiple neurodegenerative disease processes (Sheng and Cai, 2012); however, the extent to which different mitochondrial functions actively contribute to degeneration is unclear. We have described a model that allows mitochondrial transport and physiology to be visualized noninvasively in zebrafish larvae, a living vertebrate system that readily lends itself to genetic and pharmacologic manipulation. Like the recently described *MitoFish* (Plucińska et al., 2012), in which zebrafish axonal mitochondria were labeled with CFP, mitoDsRed expression in zebrafish peripheral sensory neurons permits quantitative analysis of mitochondrial morphology and transport both during development and in conditions relevant to neurodegeneration. We also report the use of mito-roGFP2 (Hanson et al., 2004) to monitor dynamic changes in the mitochondrial redox state in a living, vertebrate system. The development and characterization of these and other genetically encoded reporters in living zebrafish neurons (Drerup and Nechiporuk, 2013) has the potential to provide insight into many cell biological processes underlying axon degeneration and protection.

Mitochondrial motility can be dissociated from axonal integrity after injury

Mitochondrial transport defects are associated with many forms of neurodegeneration (Coleman and Perry, 2002), including WD of injured *Drosophila* axons (Avery et al., 2012). The extent to which such defects are instructive in degeneration, however, is unclear. We observed that mitochondrial transport terminates in zebrafish peripheral sensory neurons after injury, but acute transport arrest was local, limited to the region of the axon immediately distal to the site of injury. Ultimately, mitochondrial transport terminated throughout the distal axon, while transport in the proximal axon persisted.

Because the proximal axon does not degenerate, this correlation raised the possibility that mitochondrial transport arrest might underlie axon degeneration. Further experiments, however, dissociated mitochondrial transport from axon degeneration. In contrast to recent findings in flies (Avery et al., 2012), we detected no difference in overall mitochondrial motility in uninjured WldS-expressing axons in our system. Transport arrest after injury was attenuated in WldS-expressing axons, but this effect was temporary: 24 hours after injury, mitochondria in WldS-expressing axons were immobile, though the detached axon remained intact. This result confirms that, as was recently observed in a neurodegenerative disease model system (Marinkovic et al., 2012), mitochondrial transport is not required for axon survival. Moreover, the observation revealed that long-term mitochondrial transport preservation does not mediate the protective effect of WldS.

Beyond the correlation between mitochondrial transport and axon protection by WldS, Avery and colleagues (2012) reported that *miro* mutations, which impair axonal transport of mitochondria (Russo et al., 2009), weakened the effect of WldS expression on mitochondrial transport and survival. This finding raised the intriguing possibility that increased mitochondrial transport, which correlated with axon protection, was also required for it. However, our findings suggest otherwise, as mitochondria in WldS-expressing axons ultimately stop moving after injury. The inconsistency may be due to differences between vertebrate and invertebrate axons, or to the fact that *miro* mutants often exhibit reduced density and altered distribution of axonal mitochondria (Guo et al., 2005; Russo et al., 2009; Kitay et al., 2013).

Two-phases of mitochondrial transport arrest

Analysis of mitochondrial transport in WT and WldS-expressing axons allowed us to

distinguish different phases of transport arrest after axotomy. The first phase occurs immediately after injury, and is not suppressed by WldS, PINK1 knock-down, or PGC-1α. This acute arrest is limited to the region immediately adjacent to the injury site, and may be mediated by rapid calcium influx, which occurs upon axotomy (Yawo and Kuno, 1985; Ziv and Spira, 1995; Adalbert et al., 2012; Avery et al., 2012). Calcium-induced mitochondrial transport arrest is an adaptive response that permits local calcium buffering and ATP synthesis at sites of high demand (Saotome et al., 2008; Macaskill et al., 2009; Wang and Schwarz, 2009). The retention of mitochondria at the injury site via this mechanism may minimize acute damage.

Observation of mitochondria later in the lag phase revealed a second phase of transport arrest that was mitigated by WldS and PGC-1α. In contrast, mitochondrial transport never recovered after injury in WT axons, and eventually terminated throughout the distal axon, suggesting that a different mechanism might underlie the long-term transport arrest. To test whether mitochondrial damage might be involved in this second phase, we knocked down the kinase PINK1. When the mitochondrial membrane potential dissipates in damaged mitochondria, PINK1 is stabilized on the outer mitochondrial membrane, where it recruits Parkin, an E3 ubiquitin ligase (Matsuda et al., 2010; Narendra et al., 2010; Okatsu et al., 2012). Stabilized PINK1 phosphorylates Miro, leading to its Parkin-dependent proteasomal degradation and mitochondrial detachment from microtubules (Wang et al., 2011). PINK1 knock-down did not affect the first phase of injury-induced transport arrest but moderately rescued transport during the second phase, suggesting that the persistent transport arrest in WT axons may reflect mitochondrial damage and may be mediated by PINK1. Interestingly, axon degeneration was accelerated by PINK1 knock-down. It is possible that the aberrant transport of

damaged mitochondria in PINK1-deficient axons may propagate damage throughout the mitochondrial network, actively promoting axon degeneration. Alternatively, mitochondria in PINK1 morphants may be more sensitive to injury, regardless of transport. Indeed, PINK1 knock-down has been associated with increased vulnerability to oxidative stress (Wang et al., 2006; Mai et al., 2010). Both of these explanations for the faster degeneration in PINK1 morphants are consistent with the recent observation that the mitochondrial permeability transition is a late-stage effector of axon degeneration (Barrientos et al., 2011).

Dynamic changes in mitochondrial redox state after injury

Mitochondria are the primary source of intracellular ROS production, and oxidative stress may play an active role in neurodegeneration (Press and Milbrandt, 2008; Calixto et al., 2012). To determine whether mitochondrial ROS production is relevant to axon degeneration and protection, we evaluated mitochondrial redox state before and after axotomy using the redox-sensitive biosensor mito-roGFP2 (Hanson et al., 2004). After axotomy, mito-roGFP2 was rapidly oxidized in the disconnected axon branch, consistent with increased ROS production in the mitochondrial matrix. This effect was not observed in the proximal axon (still attached to the cell body) or in WldS-expressing axons. Because mitochondrial ROS production can be induced by calcium (Yan et al., 2006), increased calcium buffering by WldS-expressing mitochondria (Avery et al., 2012) could contribute to this difference. It is also possible that WldS directly affects mitochondrial ROS production. Consistent with this possibility, overexpression of Nmnat (the critical catalytic component of the WldS fusion protein) prevents degeneration of cultured neurons exposed to hydrogen peroxide (Press and Milbrandt, 2008). Moreover, antioxidant treatment was recently shown to be protective against Wallerian-like degeneration in *C. elegans* and mice (Calixto et al., 2012).

Oxidation of roGFP2 remained elevated but stable in the WT distal axon for the majority of the lag phase. Shortly before axon fragmentation, however, mito-roGFP2 oxidation rose further. This increase, which was not observed in WldS-expressing axons, may reflect formation of the mPTP, which occurs under conditions of calcium overload (Haworth and Hunter, 1979; Hunter and Haworth, 1979b, a), particularly in the presence of ROS (Kowaltowski et al., 1996; Vercesi et al., 1997). The permeability transition results in further ROS production (Hansson et al., 2008), loss of membrane potential, and calcium efflux into the cytoplasm, where it activates calcium-dependent cysteine proteases that degrade the cytoskeleton (George et al., 1995).

PGC-1α expression delays mito-roGFP2 oxidation and axon degeneration

The transcriptional co-activator PGC-1α has many targets that together regulate mitochondrial biogenesis, bioenergetics, and ROS detoxification (Wu et al., 1999; Lehman et al., 2000; St-Pierre et al., 2006; Cantó and Auwerx, 2009). Impairments in PGC-1α-mediated transcription have recently been associated with Huntington disease and Parkinson disease, and PGC-1α has been proposed as a therapeutic target for treatment (Cui et al., 2006; Weydt et al., 2006; Zheng et al., 2010; Shin et al., 2011; Tsunemi and La Spada, 2012). PGC-1α expression delayed axon degeneration of zebrafish sensory neurons after transection. Because the distal axon is no longer connected to the cell body after axotomy, injury-induced transcriptional activation by PGC-1α cannot affect contribute to this protection. However, PGC-1α increased mitochondrial density, and a larger number of mitochondria may be able to buffer more calcium. Additionally, PGC-1α may upregulate ROS detoxification programs at baseline, making mitochondria more resistant to ROS buildup after injury. Indeed, PGC-1α

overexpression protects cultured neurons from ROS-mediated degeneration, an effect associated with increased mRNA expression of multiple enzymes involved in ROS detoxification (St-Pierre et al., 2006). Nevertheless, detachment from the cell body prevents the distal axon from enjoying any further effects of PGC-1α activation after injury. This limitation could explain why the delay of WD in PGC-1α-expressing axons, while significant, was not comparable to that provided by WldS expression. In many neurodegenerative disease models, axon degeneration proceeds via a WD-like mechanism without physical separation from the cell body; PGC-1α overexpression may provide improved protection in these models, as axons could still benefit from upregulation of protective gene expression after the initial insult.

ACKNOWLEDGMENTS

We thank Meghan E. Johnson and Carla Koehler for the mitoDsRed and mito-roGFP constructs, and Jeffrey Rasmussen for comments on the manuscript. KCO was supported by a training grant from the UCLA Training Program in Neural Repair (NINDS T32 NS07449:13). MEV was supported by the Jules Stein EyeSTAR Training Program. This work was supported by grants to AS from the National Institutes of Dental and Craniofacial Research (RO1 DE018496) and the American Parkinson Disease Association Pilot Fund (20082501).

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Chapter 3. Axon degeneration and PGC-1α-mediated protection in a vertebrate model of α-synuclein toxicity

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ABSTRACT

α-Synuclein (aSyn) is a small neuronal protein that is implicated in a number of neurodegenerative processes, including Parkinson disease (PD) and dementia with Lewy bodies (DLB). In animal models of these diseases, axon pathology often precedes cell death, raising the question of whether aSyn has compartment-specific toxic effects that may require early and/or independent therapeutic intervention. The relevance of axonal pathology to degeneration is therefore an important question in neurodegenerative disease research. However, it can only be addressed through longitudinal, in vivo monitoring of the different neuronal compartments. With current imaging methods, dopaminergic neurons do not readily lend themselves to such a task in any vertebrate system. We therefore expressed human, wild-type aSyn in zebrafish peripheral sensory neurons, which project elaborate and superficial axons that can be continuously imaged in vivo. Axonal outgrowth was normal, but at 2 dpf, many aSyn-expressing axons were dystrophic, with focal varicosities or diffuse beading. Approximately 20% of aSyn-expressing cells died by 3 dpf. Time-lapse imaging revealed that focal axonal swelling, but not overt fragmentation, usually preceded cell death. When aSyn was co-expressed with DsRed localized to the mitochondrial matrix (mitoDsRed), we observed early deficits in mitochondrial transport and morphology even in axons that appeared normal. Large, swollen mitochondria were found in dystrophic axons. The axon-protective protein *Wallerian*

degeneration slow (WldS) delayed axon degeneration but not cell death caused by aSyn. By contrast, the transcriptional co-activator PGC-1α, which has roles in mitochondrial biogenesis and reactive oxygen species detoxification, abrogated aSyn toxicity in both the axon and the cell body. The rapid onset of axonal toxicity in this system, and the relatively moderate degree of cell death, provide a new model for the study of aSyn toxicity and protection. Moreover, the accessibility of peripheral sensory axons will allow the effects of aSyn to be studied in different neuronal compartments, and may have utility in screening for novel disease-modifying compounds.

INTRODUCTION

Parkinson disease (PD) is a movement disorder characterized pathologically by the loss of dopaminergic cells in the midbrain, and by the appearance of Lewy bodies (Braak et al., 1999; Braak et al., 2003), intracellular protein aggregates composed primarily of ubiquitin and α-synuclein (aSyn) (Spillantini et al., 1997; Spillantini et al., 1998). *SNCA*, the gene that encodes aSyn, was the first gene to be associated with PD: duplications, triplications, and mutations in this gene are associated with rare hereditary forms of the disease (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Fuchs et al., 2007), and variants are also associated with the more common sporadic form of PD (Satake et al., 2009; Simón-Sánchez et al., 2009; Wu-Chou et al., 2012). Alpha-synuclein is a synaptic protein (Maroteaux et al., 1988; Boassa et al., 2013), and aggregate formation in the synapse and the axon precedes Lewy body formation and cell death in multiple cell types (Galvin et al., 1999; Orimo et al., 2008; Schulz-Schaeffer, 2010; Nakata et al., 2012). These recent findings have led to the hypothesis that PD degeneration is initiated in the axon (O'Malley, 2010; Burke and O'Malley, 2012). Whether axon degeneration leads to cell death or proceeds independently, however, is unknown.

A number of lines of evidence support the hypothesis that mitochondrial dysfunction contributes to PD pathogenesis. Mitochondrial dysfunction has been observed in post mortem samples from PD patients (Schapira et al., 1990; Penn et al., 1995; Navarro et al., 2009), and a number of genes associated with mitochondrial function are associated with hereditary forms of the disease (Martin, 2006; Dodson and Guo, 2007; Sai et al., 2012). Although aSyn itself is not a mitochondrial protein, it is capable of binding mitochondria directly (Nakamura et al., 2011) and can accumulate on the inner and outer mitochondrial membranes (Li et al., 2007; Zhu et al., 2012). Its overexpression or mutation alters mitochondrial morphology in a number of systems and cell types (Martin et al., 2006a; Li et al., 2007; Kamp et al., 2010; Nakamura et al., 2011;

Xie and Chung, 2012; Zhu et al., 2012), and is associated with respiratory chain defects, oxidative stress, and mitochondrial fragmentation (Parihar et al., 2008; Chinta et al., 2010; Zhu et al., 2012). A better understanding of the effect of aSyn on mitochondrial transport and function in vivo may provide insight into PD pathophysiology and potential therapeutic targets.

Each of the models used to study aSyn-induced degeneration has limitations. In vitro studies can shed light on the cell biology of aSyn oligomerization and aggregation, but their relevance to pathophysiology in living animals is unknown. By contrast, studies in mammalian systems recapitulate some of the disease phenotypes, but in vivo cell biological studies are difficult (Martin et al., 2006a; Chesselet, 2008). A better understanding of aSyn toxicity requires a model system in which neurons can be visualized and manipulated in vivo. Larval zebrafish are increasingly recognized as a genetically and pharmacologically tractable model system useful in high-throughput screens for PD-associated phenotypes (Bretaud et al., 2004; Flinn et al., 2008). Moreover, their optical transparency permits the visualization of cellular processes in living animals, including mitochondrial transport (Plucinska et al., 2012). Zebrafish are therefore an attractive model for studying the relationship between aSyn expression and neuronal degeneration at the cellular level.

We expressed human aSyn in zebrafish Rohon-Beard neurons, the peripheral sensory neurons in the developing spinal cord. Both the cell bodies and the elaborate peripheral arbors of these cells can be monitored in vivo, permitting visualization of axonal transport and degeneration (Plucińska et al., 2012). Human aSyn was cloned upstream of the viral T2A sequence (Donnelly et al., 2001) and GFP. We observed moderate toxicity to the cell body, but a high number of axons exhibited diffuse or focal swellings that have been associated with degeneration of this compartment (Nikić et al., 2011). Expression of the axon-protective protein WldS (Lunn et al., 1989; Coleman et al., 1998) delayed axon degeneration, but did not affect cell death. Early

defects in mitochondrial morphology and transport suggested that mitochondrial toxicity might be relevant to pathogenesis (Banerjee et al., 2010; Butler et al., 2012; Siddiqui et al., 2012; Zhu et al., 2012). Consistent with this hypothesis, expression of PGC-1α, a transcriptional co-activator with roles in mitochondrial biogenesis and reactive oxygen species (ROS) detoxification, prevented both axonopathy and cell death in this model.

RESULTS

Generation of transgenes

Zebrafish Rohon-Beard neurons in the spinal cord arborize in the skin, and are therefore readily accessible to in vivo imaging of dynamic intracellular processes. We generated transgenes to overexpress aSyn in these cells, using a sensory neuron promoter and the Gal4/UAS binary transcription system to drive robust gene expression (Figure 3.1A,C-D). We took advantage of the viral T2A system to co-express aSyn and GFP, which provides bright reporter expression earlier than the aSyn-2A-DsRed transgene that has been reported previously (Prabhudesai et al., 2012). The viral T2A system permits visualization of cells expressing the transgene, but circumvents the possibility of increased aggregation that could be observed with a fusion protein. Consistent with a previous report (Prabhudesai et al., 2012), immunostaining for human aSyn revealed protein expression and aggregate formation by 2 dpf in aSyn-injected cells, but not wild-type embryos (Figure 3.2).

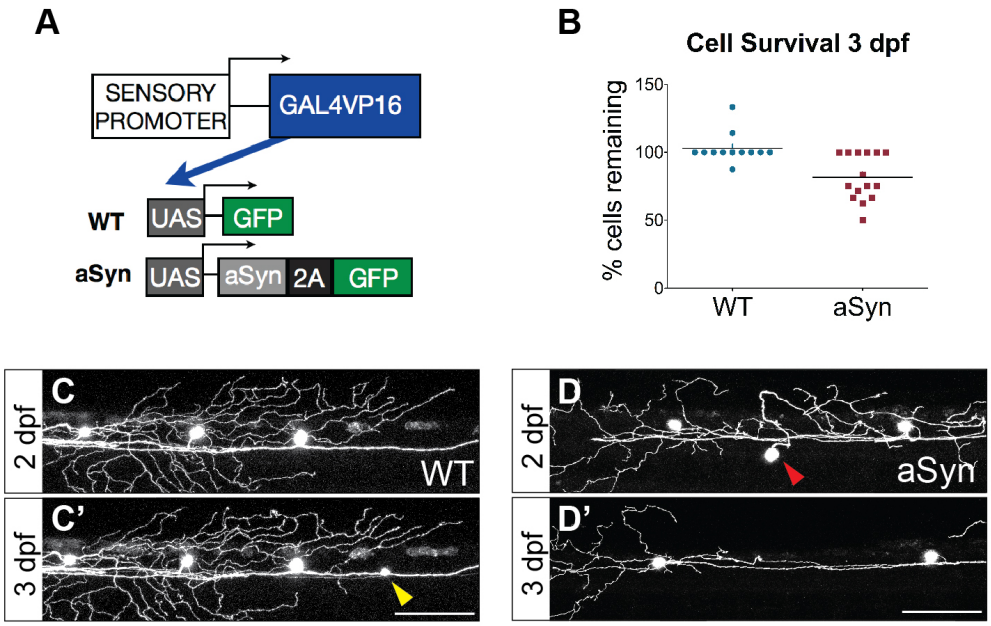


Figure 3.1 Alpha-synuclein is moderately toxic to cell bodies between 2 and 3 dpf.

A) Transgenes to express GFP (WT) or asyn-2A-GFP (aSyn) were injected into wild-type embryos at the one-cell stage. The *CREST3* enhancer drove expression in peripheral sensory neurons. The Gal4/UAS system was used to amplify gene expression, and a viral T2A sequence was cloned between aSyn and GFP to generate two proteins from a single transcript. B-D) Approximately 20% of aSyn-expressing neurons died between 2 and 3 dpf (WT 3 dpf survival: 102.9 ± 3.2%; aSyn: 81.7 ± 4.4%; n ≥ 12 embryos, p = 0.0010). Red arrowhead in C points to cell that newly expresses GFP at 3 dpf. White arrowhead in D points to a cell that died between 2 and 3 dpf.

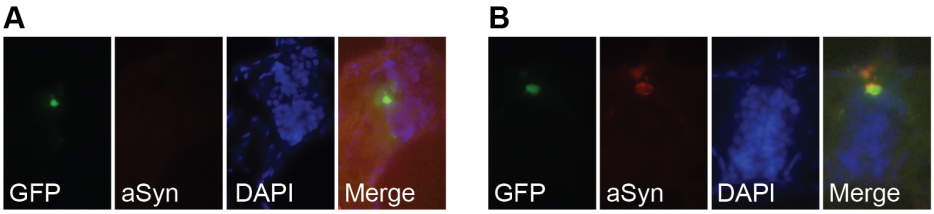


Figure 3.2 Alpha-synuclein aggregates in zebrafish peripheral sensory neurons

Embryos were injected at the 1-cell stage with transgenes indicated in Figure 3.1A. At 48 hours post-fertilization, embryos were fixed in 4% PFA, sectioned, and stained with anti-alpha-synuclein primary antibody, followed by Alexa 594-conjugated goat anti-mouse IgG secondary antibody. DAPI staining was used to visualize nuclei. Green fluorescence indicates cells expressing the transgene. A) No aSyn staining was observed in control-injected cells. B) All aSyn-injected cells exhibited red aSyn staining.

Normal gross morphology in most aSyn-expressing embryos

When the HuC promoter is used to drive aSyn expression in larval zebrafish neurons, embryos exhibit massive cell death and gross morphological abnormalities, and die within 2-3 dpf (Prabhudesai et al., 2012). When we drove expression using a sensory neuron promoter (Figure 3.1A), only a small number of embryos exhibited such defects; most were morphologically normal. Only the latter were retained for subsequent studies, and in these embryos lethality was not observed at levels higher than in wild-type (data not shown).

Alpha-synuclein does not impair early development or axonal outgrowth of sensory neurons

We first injected the aSyn-2A-GFP transgene into embryos from a stable line expressing DsRed in sensory neurons, and screened for reporter expression at 1 dpf. To determine whether aSyn was immediately toxic to sensory neurons, we imaged cells hourly between 32 and 44 hpf (Figure 3.3A-B). Because transient aSyn-2A-GFP expression was sparse, some neurons expressed only DsRed; these served as an internal control for development and cell death. Over the course of the imaging period, peripheral sensory axons extended normally in aSyn-expressing neurons (Figure 3.3B), cell survival between the first and last time point was not different between the two groups (Figure 3.3C). This result indicates that aSyn is not toxic at this early stage.

Alpha-synuclein causes moderate cell death in zebrafish sensory neurons

Having determined that aSyn expression does not impair development of peripheral sensory neurons by 44 hpf, we investigated whether it affected cell survival at later time points (Figure 3.1B-D). We monitored cohorts of embryos expressing GFP or aSyn-2A-GFP between 2 and 3 dpf, and counted Rohon-Beard neurons at each time point (Figure

3.1B-D). Approximately 20% of cells aSyn-expressing cells died between 2 and 3 dpf (Figure 3.1B; WT 3 dpf survival: 102.9 ± 3.2%; aSyn: 81.7 ± 4.4%; n ≥ 12 embryos, p = 0.0010).

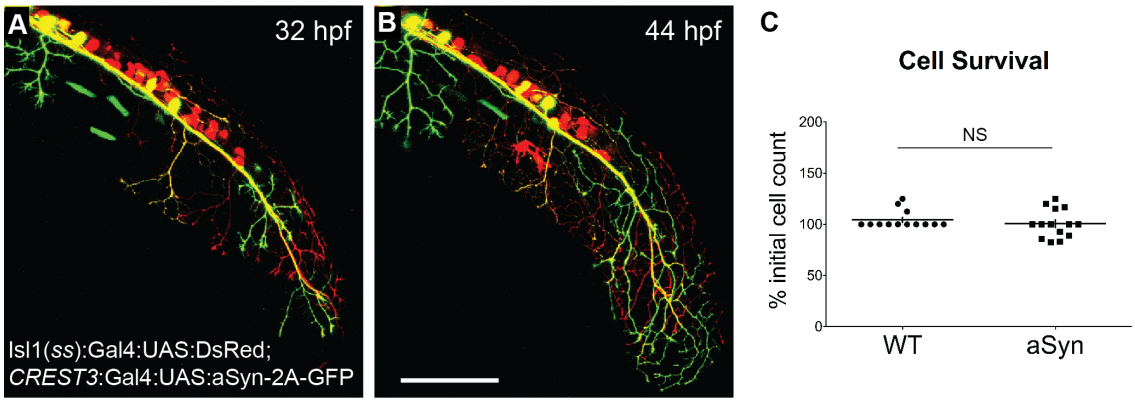


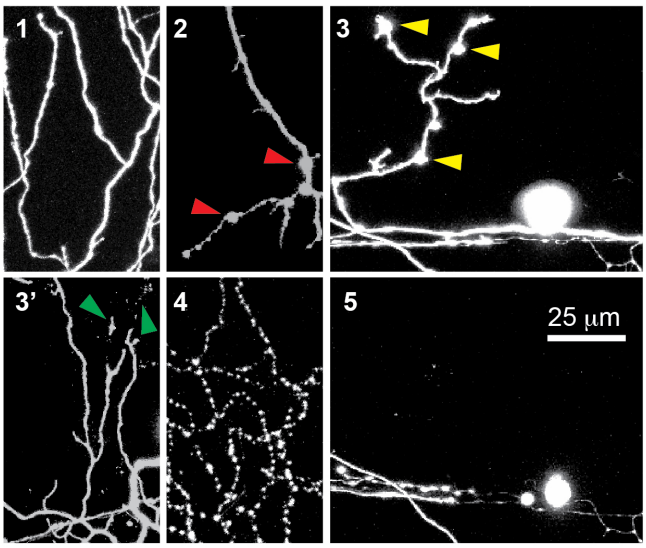
Figure 3.3 Alpha-synuclein expression does not impair early survival or axonal outgrowth of peripheral sensory neurons.

The *CREST3*:Gal4:UAS:aSyn-2A-GFP transgene was injected into embryos from a previously described stable line (Palanca et al., 2012) expressing DsRed in peripheral sensory neurons (*isl1(ss)*:Gal4-UAS-DsRed). Embryos were imaged every hour between 32 (A) and 44 (B) hours post-fertilization (hpf). Axons grew normally during this time. C) Cell bodies were counted, and cell survival at 44 hpf was quantified as a percentage of the earlier time point. There was no difference in survival between WT and aSyn-expressing cells by 44 hpf (WT: 104.4 ± 2.4% aSyn: 100.7 ± 3.7%; n ≥ 13 embryos; p = 0.4203).

Alpha-synuclein expression causes axonopathy

We developed a 5-point staging scheme for quantifying axonal dystrophy (Figure 3.4), and compared axons expressing DsRed and either GFP or aSyn-2A-GFP at 2 and 3 dpf (Figure 3.5A). At 2 dpf, before cell death had been observed, a majority (14/19) of aSyn-expressing axons exhibited a beaded morphology (Figure 3.5A, arrowheads), quantified as a degeneration stage of 2-3 (Figure 3.5B; WT: 1.08 ± 0.08; aSyn: 2.05 ± 0.14; n ≥ 12 axons, p<0.0001). When the same axons were imaged the following day, degeneration was even more advanced (Figure 3.5B; WT: 1.42 ± 0.33; aSyn: 3.05 ± 0.35; n ≥ 12 axons; p = 0.0033). In control axons, one cell died between 2 and 3 dpf (degeneration

stage 5), and one exhibited mild beading (stage 2); the remaining 10 axons were smooth and continuous (stage 1). Among aSyn-expressing axons, by contrast, 17/19 axons (89.5%) had a degeneration score of 2 or higher, with 6 having degenerated entirely (stage 5).



1	Smooth, continuous axons
2	Mild beading (diffuse or locally restricted)
3	Severe beading, +/- limited fragmentation
4	Diffuse fragmentation
5	Most/all fragments cleared

Figure 3.4 Axon degeneration index

A five-point scoring system was devised to quantify axon morphology between 2 and 3 dpf. A score of 1 was given to smooth, continuous axons. A score of 2 was given to axons with mild and diffuse or moderate but localized swellings. A score of 3 was given to axons that either had severe swelling or a small amount of fragmentation, (as seen at the tips in 3'). Axons that had more extensive fragmentation were given a score of 4. When fragments were mostly or entirely cleared, axons received a score of 5.

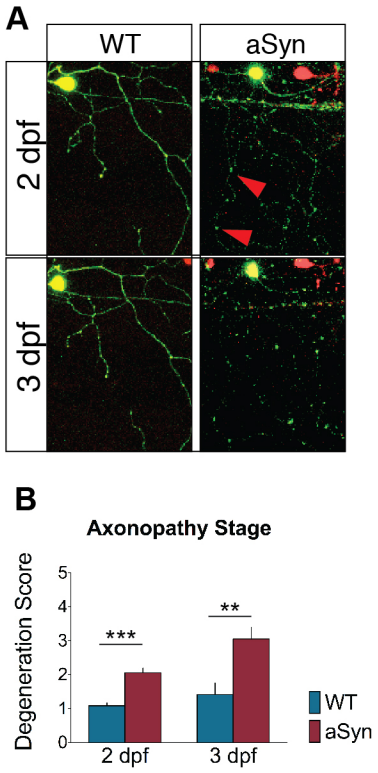


Figure 3.5 Early axonopathy in aSyn-expressing neurons

Axon pathology was scored at 2 and 3 days post-fertilization (dpf) in WT and aSyn-expressing axons, using the five-point staging system described above. A) At 2 dpf, WT axons were smooth and continuous, but a beaded morphology (degeneration score of 2-3) was observed in many aSyn-expressing axons (red arrowheads). By 3 dpf, WT axons were still continuous, but approximately 90% of aSyn-expressing axons were dystrophic. B) Quantification of average axonopathy stage. ASyn-expressing axons were more dystrophic at both 2 dpf (WT: 1.03 ± 0.08; aSyn: 2.05 ± 0.14; $n \geq 12$ axons; $p<0.0001$) and 3 dpf (WT: 1.42 ± 0.34; aSyn: 3.05 ± 0.35; $n \geq 12$ axons; $p=0.0033$).

Axonopathy, but not axonal fragmentation, precedes cell death

Recent reviews have raised the question of whether the axon degeneration observed in PD represents an early, and potentially independent, process in pathophysiology (O'Malley, 2010; Burke and O'Malley, 2012; Jellinger, 2012). The percentage of cells with dystrophic axons between 2 and 3 dpf was higher than the percentage of cells that died during that time. To determine whether severe axonopathy always preceded cell death, we conducted time-lapse imaging at 20-minute intervals between 56 and 68 hpf

(Figure 3.6A-B). In cells that died during the movies, we compared the onset of axonal dystrophy (beading or fragmentation) with the morphological changes in the soma that herald cell death. In all cases (n=9), focal or diffuse swellings (axonopathy stage 2-3) were seen in the axons several hours before cell death (Figure 3.6A-B). Axonal fragmentation, however, did not precede apoptotic changes in the cell body (Figure 3.6A-B, arrows). Overt axonal breakdown therefore does not proceed in a retrograde direction in this model. However, because axonal dystrophy preceded cell death, it is likely that the axonal compartment is more vulnerable to aSyn toxicity.

Axonal injury increases death rate in aSyn-expressing neurons

To further investigate the sensitivity of the axon and the cell body to aSyn toxicity, we examined the effect of aSyn expression on the rate of Wallerian degeneration after injury. In most neuronal subtypes, including zebrafish peripheral sensory neurons (Martin et al., 2010), Wallerian degeneration after axonal transection is compartment-specific. After a lag period, the distal axon undergoes fragmentation and clearance, while the proximal axon and the cell body survive. To determine whether aSyn expression altered these characteristics, we transected axons at 2 dpf and conducted time-lapse confocal imaging to visualize WD in vivo (Figure 3.6C-D). Alpha-synuclein did not change the duration of the lag phase before fragmentation (Figure 3.6E), or the clearance of axonal debris (Figure 3.6F). WD in aSyn-expressing axons therefore proceeds with the same rapid and stereotyped kinetics as in wild-type axons. In aSyn axons, as in wild-type, fragmentation of the distal axon was synchronous (Figure 3.6C-D), unlike the axon degeneration observed in uninjured cells (Figure 3.6A-B).

Consistent with the compartment-specificity of WD, in both WT and aSyn-expressing axons the cell body and proximal axon remained intact while the distal fragment

underwent degeneration (data not shown). However, when we imaged transected cells at 3 dpf, 24 hours after injury, 50% of aSyn-expressing cells (5/10) had died, whereas all axotomized WT cells (n = 11) were still intact. Because 20% of uninjured aSyn-expressing cells died between 2 and 3 dpf (Figure 3.1B), this higher percentage suggests that direct axonal injury exacerbates aSyn toxicity.

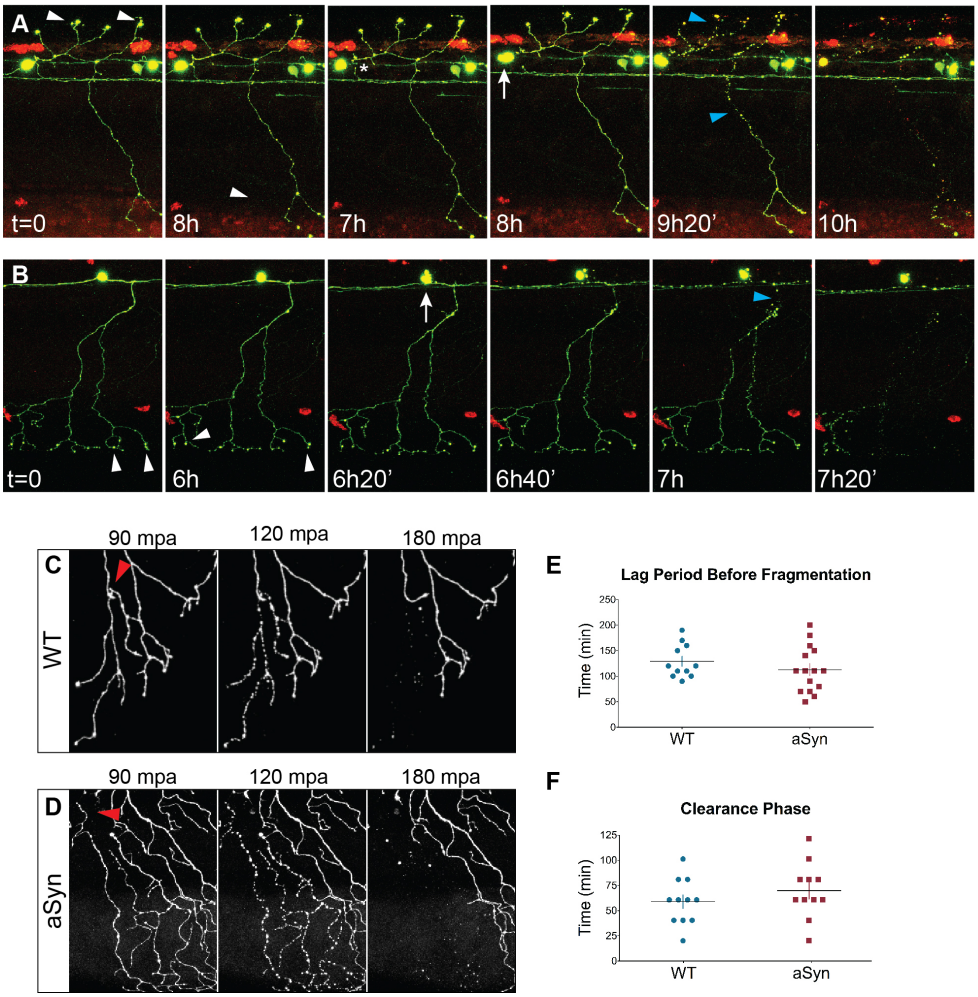


Figure 3.6 Axonopathy is not followed by "dying-back" or Wallerian-like degeneration in aSyn-expressing neurons

A-B) Time-lapse imaging of degeneration in two aSyn-expressing axons. Cells were imaged every 20 minutes beginning 32 hpf. Axonal varicosities were observed (white arrowheads) several hours before cell death (arrows point to morphological changes indicative of cell death). Axonal fragmentation was not stereotyped (observe that it does not occur in a retrograde direction or synchronously along the length of the axon), and usually did not occur before cell death. C-D) Wild-type (C) and aSyn-expressing axons (D) were transected with a two-photon laser at 2 dpf, and embryos were imaged every 30 minutes for up to 12 hours. In both groups, axons rapidly fragmented approximately two hours after axotomy, and were cleared approximately one hour later. Arrows point to site of transection. E) There was no difference in the duration of the lag period between transection and fragmentation (WT: 129.1 ± 10.0 min; aSyn: 112.7 ± 11.7 min; $n \geq 11$ axons, $p = 0.3173$). F) The time between fragmentation and clearance of all axonal debris was not significantly different between the two groups (WT: 58.2 ± 6.9 min; aSyn: 69.1 ± 8.3 min; $p = 0.3213$).

WldS delays axon degeneration caused by aSyn toxicity

To further characterize aSyn-induced degeneration, we sought to determine whether it could be prevented by the axon-protective protein WldS (Figure 3.7). This protein was first discovered to delay WD of transected axons (Coleman et al., 1998; Mack et al., 2001), and was subsequently found to be protective of axons in many animal models of neurodegenerative disease (Sajadi et al., 2004; Hasbani and O'Malley, 2006; Press and Milbrandt, 2008; Cheng and Burke, 2010). We co-expressed aSyn and WldS in peripheral sensory neurons (Figure 3.7A,C), and quantified cell survival and axon pathology between 2 and 3 dpf (Figure 3.7B,D). WldS did not prevent cell death induced by aSyn (Figure 3.7B; 3 dpf WT survival: 95.65 ± 4.35%; aSyn: 79.36 ± 6.62%; WldS: 86.86 ± 4.43%; n = 22 cells; p = 0.3515). Axon degeneration, however, was delayed in WldS-expressing cells (Figure 3.7D). Degeneration scores were lower at 2 dpf in WldS-expressing cells (2 dpf aSyn: 2.05 ± 0.14; 2 dpf WldS+aSyn: 1.57 ± 0.11; n ≥ 19; p = 0.0114), but by 3 dpf, this difference was no longer significant (3 dpf aSyn: 3.05 ± 0.35; WldS+aSyn: 2.54 ± 0.29; n ≥ 19 axons; p = 0.2766). When we analyzed the frequency distribution of axonopathy scores, however, we saw that a higher percentage of WldS-expressing axons of intact aSyn-expressing cells were still smooth and continuous (stage 1) at 3 dpf (Figure 3.7E-F). In cells that had died, WldS had no protective effect (stage 5; Figure 3.7C,F). WldS therefore provides moderate protection against aSyn toxicity in the axonal compartment, reducing the incidence of focal axonal swellings. However, WldS cannot delay either death of the cell body or the axonal fragmentation associated with cell death.

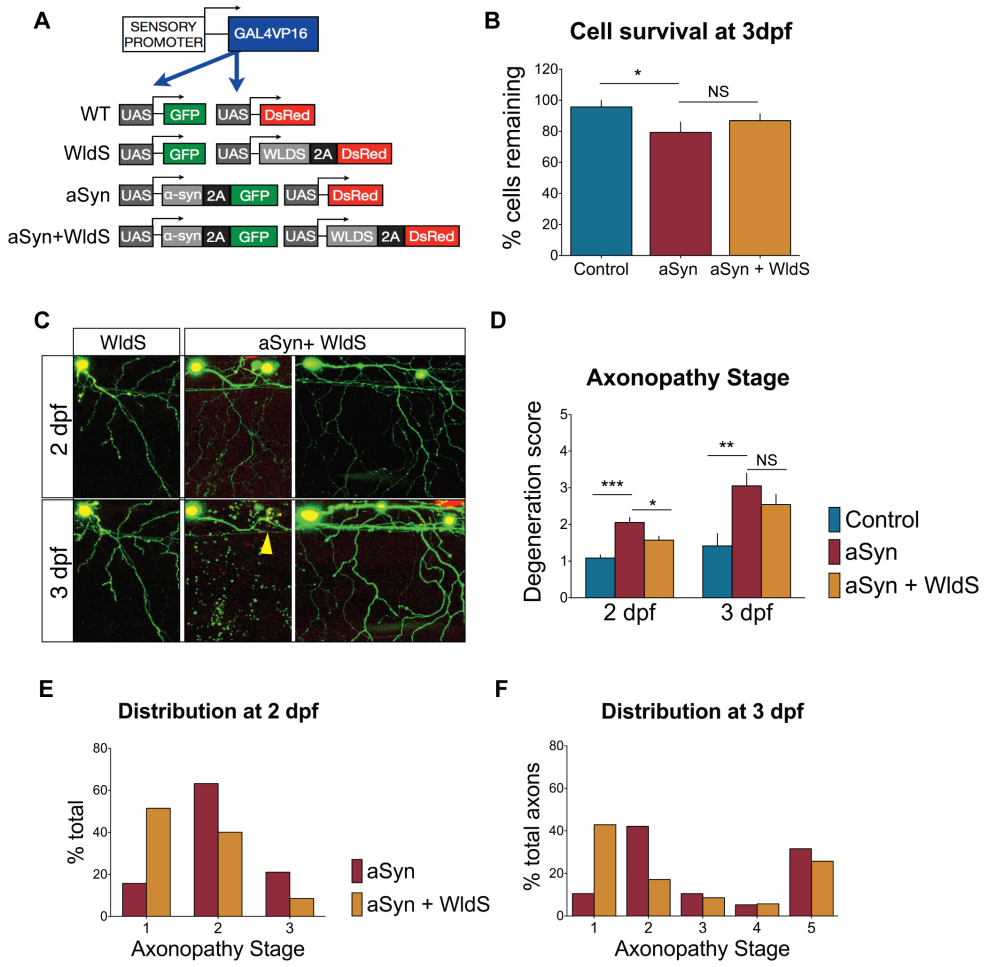


Figure 3.7 WldS delays axonopathy but does not prevent aSyn-induced cell death

A) Transgenes used to visualize the effect of aSyn and WldS expression on peripheral sensory neurons. B) WldS did not affect survival of aSyn-expressing cells between 2 and 3 dpf (p=0.3515). WldS alone did not affect cell survival (data not shown). C) WldS delays the beaded morphology observed in aSyn expressing axons. At 2 dpf, WldS-expressing axons are relatively continuous (compare to aSyn in Figure 3.5). At 3 dpf, WldS-expressing axons of cell bodies that died overnight are undergoing degeneration (yellow arrowhead points to remains of the dead cell body). However, axons that remain connected to cell bodies are relatively preserved (right panel). D) Quantification of average axonopathy stage at 2 and 3 dpf. WldS-expressing axons are significantly protected (2 dpf aSyn: 2.05 ± 0.14; 2 dpf WldS+aSyn: 1.57 ± 0.11; $n \geq 19$ axons, p=0.0114). By 3 dpf, this difference was no longer significant (3 dpf aSyn: 3.05 ± 0.35; 3 dpf WldS+aSyn: 2.54 ± 0.28; $n \geq 19$ axons, p=0.2766). E-F) Frequency distribution of axonopathy scores at 2 (F) and 3 dpf (G). Significantly more WldS-expressing axons have an axonopathy score of 1 at both time points. At 3 dpf, axons that underwent cell death have fully degenerated (axonopathy stage 5), regardless of whether or not WldS was expressed. Wild-type and control data in D are replotted from Figure 3.5.

Mitochondria are swollen in dystrophic axons of aSyn-expressing neurons

Multiple in vitro and histological studies suggest that wild-type or mutant aSyn interacts with mitochondria (Martin et al., 2006b; Parihar et al., 2008; Banerjee et al., 2010; Chinta et al., 2010; Devi and Anandatheerthavarada, 2010; Nakamura et al., 2011; Calì et al., 2012; Reeve et al., 2012; Zhu et al., 2012). To determine whether axonal mitochondria were affected by aSyn expression in our model, DsRed was expressed in the mitochondrial matrix of sensory neurons expressing either GFP or aSyn-2A-GFP (Figure 3.8A-C). Mitochondrial density was significantly higher in aSyn-expressing cells, even in the absence of overt axonopathy (Figure 3.8C-D). Mitochondria in aSyn-expressing axons were less elongated than in wild-type cells (Figure 3.8C,E; WT length/width: 2.01 ± 0.11; aSyn: 1.48 ± 0.05; n ≥ 54 mitos from ≥ 5 embryos; $p < 0.0001$), with a higher percentage of spherical mitochondria (ratio of 1), a phenotype associated with respiratory chain dysfunction (Benard and Rossignol, 2008). In dystrophic aSyn-expressing axons (Figure 3.8F), many mitochondria exhibited pathological swelling that is observed with the mitochondrial permeability transition (Haworth and Hunter, 1979; Kowaltowski et al., 1996; Brustovetsky et al., 2002).

Alpha-synuclein expression causes early mitochondrial transport impairment

Because mitochondrial transport arrest is associated with axon degeneration (Baloh et al., 2007; Kim-Han et al., 2011; Sterky et al., 2011; Avery et al., 2012), we investigated whether aSyn expression induced mitochondrial transport impairments at 2dpf, prior to axonal fragmentation and cell death. Mitochondrial transport was evaluated along 50-μm axonal segments for 6 minutes in WT or aSyn-expressing sensory neurons. Kymographs were generated to quantify overall motility, defined as the percentage of mitochondria that moved within a 6-minute time-lapse movie. Mitochondrial motility was significantly reduced in aSyn-expressing axons (Figure 3.8G), and motile mitochondria favored

transport back towards the cell body. A higher percentage of the total distance traveled by motile mitochondria was in the retrograde direction (Figure 3.8H). Motile mitochondria spent less time moving in the anterograde direction, and a greater percentage of time paused (Figure 3.8I). The speed of uninterrupted runs in either the anterograde or retrograde direction, however, was not significantly different between WT and aSyn-expressing cells (WT anterograde speed: 0.56 ± 0.04 μm/s; aSyn: 0.53 ± 0.06 μm/s; n ≥ 27 mitos, p = 0.7137; WT retrograde speed: 0.57 ± 0.04 μm/s; aSyn: 0.64 ± 0.07 μm/s; n ≥ 38 mitos).

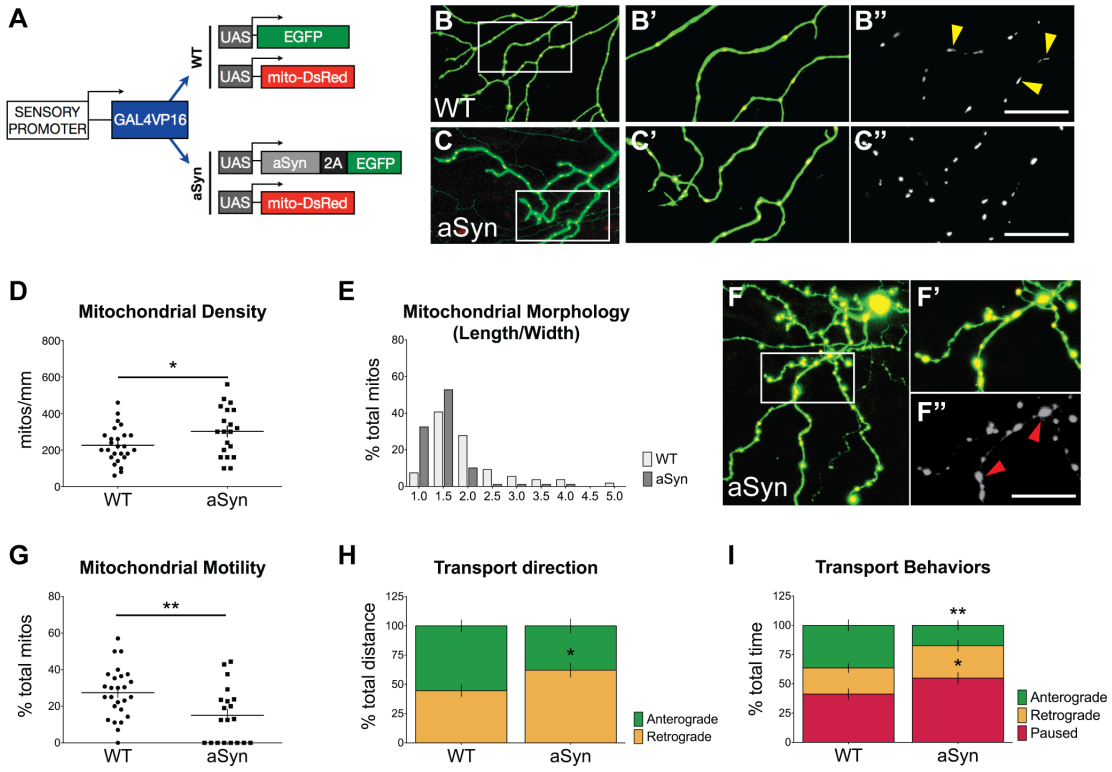


Figure 3.8 Early mitochondrial pathology and transport impairments in aSyn-expressing axons

A) Transgenes used to visualize mitochondria in GFP- or aSyn-2A-GFP-expressing peripheral sensory neurons. Transgenes were co-injected into wild-type embryos at the one-cell stage. WT (B-B') and aSyn-expressing cells (C-C') were imaged at 2 dpf. D) Mitochondrial density was higher in aSyn-expressing cells (WT: 226.2 ± 19.1 mitos/μm; aSyn: 302.0 ± 29.8 mitos/μm; n ≥ 20 axons, p=0.031). E) Mitochondrial morphology was quantified as the ratio of length to width in individual mitochondria. Values were binned and the frequency distribution was plotted on a histogram. Mitochondria in aSyn-expressing axons were more spherical in wild-type axons, with fewer mitochondria exhibiting a high length/width ratio. F) Large, swollen mitochondria occupied the spheroids in dystrophic aSyn-expressing axons. G) Mitochondrial transport was evaluated along 50-μm axonal segments every second. Kymographs (distance over time) were generated to evaluate mitochondrial motility, defined as the percentage of mitochondria that moved within a 6-minute time-lapse movie. Overall mitochondrial transport was significantly reduced in aSyn-expressing axons (WT % motile: 27.4 ± 2.7%; aSyn: 15.05 ± 3.4%; n ≥ 20 axons; p = 0.0061). F) A higher percentage of distance traveled by motile mitochondria was in the retrograde direction (WT % retrograde distance: 44.61 ± 5.07%; aSyn: 62.17 ± 6.06%; n ≥ 52 mitos, p = 0.0300). G) Motile mitochondria spent less time moving in the anterograde direction (WT: 36.44 ± 4.80%; aSyn: 17.39 ± 4.05%; n ≥ 52 mitos; p = 0.0063), and a greater percentage of time paused than in wild-type axons (WT: 41.25 ± 4.43%; aSyn: 54.82 ± 4.85%; n ≥ 52 mitos; p = 0.0478).

PGC-1α expression mitigates toxicity in aSyn-expressing sensory neurons

Because the mitochondrial phenotype in aSyn-expressing axons was early and dramatic, we hypothesized that mitochondrial dysfunction was directly involved in degeneration. We therefore investigated whether improved mitochondrial function could prevent degeneration in aSyn-expressing sensory neurons. The transcriptional co-activator PGC-1α has a number of regulatory roles in mitochondrial biogenesis and ROS detoxification (Wu et al., 1999; St-Pierre et al., 2006), and PGC-1α overexpression is protective in multiple models of neurodegeneration (St-Pierre et al., 2006; Keeney et al., 2009; Shin et al., 2011; Mudò et al., 2012). We co-expressed PGC-1α and aSyn in peripheral sensory neurons (Figure 3.9A), and saw that PGC-1α robustly protects against aSyn toxicity between 2 and 3 dpf (Figure 3.9B-E).

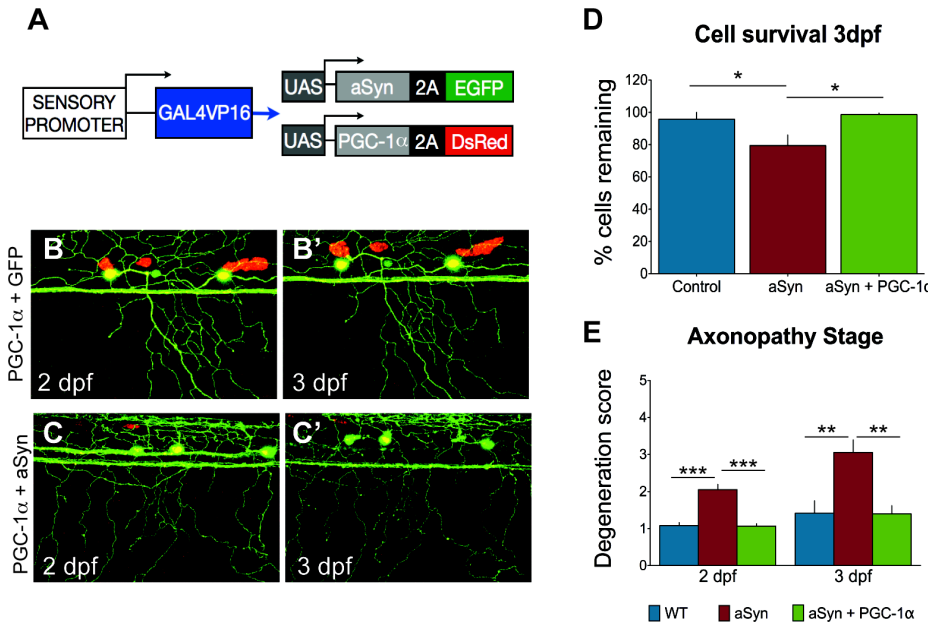


Figure 3.9 PGC-1a mitigates aSyn toxicity.

A) Transgenes coinjected to express PGC-1α and aSyn in sensory neurons. Cells were imaged between 2 and 3 dpf (B-C). D) Cell survival at 3 dpf was higher in aSyn-expressing cells that also expressed PGC-1α (aSyn: 79.36 ± 6.62%; aSyn+PGC-1α: 98.61 ± 0.95%; n ≥ 18; p = 0.0129). E) PGC-1α prevented axonopathy at 2 dpf (aSyn degeneration score: 2.05 ± 0.14; aSyn+PGC-1α: 1.07 ± 0.07; n ≥ 15; p<0.0001). At 3 dpf, PGC-1α-expressing axons were still protected at a level equivalent to wild-type (WT: 1.42 ± 0.34; aSyn: 3.05 ± 0.35; aSyn+PGC-1α: 1.40 ± 0.22; n ≥ 10; one-way ANOVA with Newman-Keuls post-test, p=0.0052). Wild-type and aSyn data are replotted from Figure 3.7.

DISCUSSION

We have expressed human wild-type aSyn in zebrafish peripheral sensory neurons, and observed aggregate formation and moderate cell death. Cell death was often preceded by axonal dystrophy, which was accompanied by aberrations in mitochondrial morphology and transport. The transcriptional co-activator PGC-1α prevented both cell death and axonopathy in aSyn-expressing neurons, suggesting that regulation of mitochondrial biogenesis and ROS production may be therapeutically relevant in vivo.

Wild-type human aSyn has been expressed in mice (Masliah et al., 2000; van der Putten et al., 2000; Fleming et al., 2004), flies (Feany and Bender, 2000; Auluck et al., 2002), and worms (Lakso et al., 2003) in an effort to understand the relevance of this protein to Parkinson disease (PD). None of these model systems recapitulates all aspects of disease, but all have strengths that can be exploited to interrogate various aspects of aSyn toxicity (Fernagut and Chesselet, 2004; Chesselet, 2008; Lim and Ng, 2009). The limitations of the model we describe include its rapid onset, high levels of gene expression, and specificity to peripheral sensory neurons, none of which characterize human pathophysiology in PD. However, these very limitations are also strengths of the system. Embryonic and larval zebrafish are increasingly recognized as a promising model organism for neurodegeneration research because early and robust phenotypes permit high-throughput analysis of potential therapeutic targets in a living vertebrate system (Tomasiewicz et al., 2002; Bandmann and Burton, 2010). Moreover, given the optical transparency of zebrafish and the superficial location of peripheral sensory neurons, this model is uniquely poised to identify and interrogate compartment-specific degeneration pathways involved in aSyn toxicity.

Approximately 20% of aSyn-expressing peripheral sensory neurons died by 3 dpf in this model. This relatively mild phenotype is consistent with reports in other systems, in which overexpression of wild-type aSyn often has limited overt toxicity, but increases cellular vulnerability to other insults (Gitler et al., 2009; Kim et al., 2011). The mild phenotype can be exploited to identify insults that exacerbate aSyn toxicity. In wild-type neurons, for example, axon transection resulted in compartment-specific degeneration of the distal axon, and the cell body and proximal axon remained intact. In aSyn-expressing neurons, however, axon transection increased cell death, suggesting that selective injury

of the axonal compartment can cause more global intracellular damage in aSyn-expressing cells. Calcium influx after axotomy has been observed in a number of cell types (George et al., 1995; Ziv and Spira, 1995; Adalbert et al., 2012; Avery et al., 2012), and aSyn was recently shown to impair mitochondrial calcium buffering in HeLa cells (Calì et al., 2012). If this defect also occurs in neuronal mitochondria, axotomy could lead to mitochondrial calcium overload, which would trigger opening of the mitochondrial permeability transition pore (mPTP) and cell death (Marambaud et al., 2009; Zündorf and Reiser, 2011). Alternatively, axotomy-induced cell death in aSyn-expressing cells could reflect disruptions in redox homeostasis after injury. Axotomy of the optic nerve often leads to retrograde degeneration of retinal ganglion cells (RGCs) following the accumulation of oxidative DNA damage (Al-Abdulla and Martin, 1998; Martin et al., 2003). Exogenous antioxidant treatment can prevent this axotomy-induced death (Castagné and Clarke, 1996; Hall et al., 1996). We have recently observed an increase in ROS production in wild-type neurons after axotomy (K.C.O and A.S., unpublished observations). In vitro, aSyn overexpression has been shown to sensitize cells to exogenous oxidants (Giasson et al., 2000; Sherer et al., 2002; Parihar et al., 2008). It is therefore possible that aSyn expression in our system impairs ROS detoxification after axonal injury, leading to oxidative damage that culminates in cell death.

Axonopathy precedes cell death

In post-mortem studies, aSyn aggregates are often observed in the axon prior to the cell body (Braak et al., 1999; Galvin et al., 1999), a phenotype that has also been observed in some disease models (Marui et al., 2002; Orimo et al., 2008; Schulz-Schaeffer, 2010; Volpicelli-Daley et al., 2011; Boassa et al., 2013). Early aggregation, consistent with a predominantly synaptic localization of aSyn, may cause early dysfunction at the presynaptic terminal, causing defects in neurotransmission long before cell death. In

multiple models of PD, both toxin-induced (Herkenham et al., 1991; Orimo et al., 2008; Li et al., 2009a; Cartelli et al., 2010; Arnold et al., 2011; Kim-Han et al., 2011; Mijatovic et al., 2011) and genetic (Li et al., 2009b; Decressac et al., 2012), axon degeneration is observed prior to cell death, and in a higher percentage of cells. This has raised the question of whether PD represents a “dying-back” of dopaminergic neurons (Hornykiewicz, 1998), with synapse loss initiating a retrograde degenerative process that leads to cell death. We observed early axon pathology in aSyn-expressing cells, with focal swellings or widespread beading in the axon, before cell death. A higher percentage of cells exhibited axonopathy than cell death, suggesting that axon degeneration might lead to cell death in this model. However, time-lapse imaging revealed that although axonal varicosities were observed early, axonal fragmentation was not stereotyped, and did not always occur prior to death of the cell body. By contrast, after transection, Wallerian degeneration of the distal axon proceeded with the same, stereotyped kinetics in aSyn-expressing axons as in wild-type cells. The early axonopathy observed in uninjured axons therefore does not cause a “functional” axotomy, and the fragmentation that later occurs is not prevented by WldS. Together, these results suggest that aSyn-induced axon degeneration is not Wallerian-like. They also suggest that degeneration in this model is not a “dying-back” process in which axon degeneration is required for cell death. Nevertheless, the early axonopathy could be associated with significant functional impairment, and likely represents an important therapeutic target.

Our characterization of the relationship between axonal fragmentation and cell death in this model does not rule out the possibility that independent, compartment-specific degeneration pathways are activated by aSyn. Indeed, dopaminergic neurons in JNK2/3 double-knock-out mice do not die after MPTP administration, but their axons degenerate,

suggesting that separate mechanisms underlie degeneration in the two compartments in a PD model (Ries et al., 2008). Likewise, WldS is protective against axon degeneration but not cell death after systemic MPTP treatment (Hasbani and O'Malley, 2006; Antenor-Dorsey and O'Malley, 2012) or application of 6-hydroxydopamine (Sajadi et al., 2004). Retrograde axonal degeneration is therefore not required for cell death in these acute models, but may benefit from independent protection. In our system, WldS delayed the early axonopathy caused by aSyn, and had no effect on cell death, consistent with the aforementioned toxin studies. However, in cells that died, WldS-expressing axons were not preserved. Because WldS protection is dose-dependent (Mack et al., 2001), it is possible that aSyn toxicity was initiated before levels were sufficient to provide lasting protection. Future studies with inducible aSyn expression could address this question.

Mitochondrial dysfunction and axon degeneration

Mitochondrial dysfunction may be upstream of axon degeneration in aSyn-expressing cells. At 2 dpf, we observed changes in mitochondrial density and morphology that were consistent with mitochondrial fragmentation, even in the absence of axonal dystrophy. This phenotype is consistent with recent in vitro studies indicating that aSyn associates directly with mitochondria, causing mitochondrial fragmentation that is associated with respiratory chain dysfunction and impaired calcium homeostasis (Chinta et al., 2010; Kamp et al., 2010; Nakamura et al., 2011; Butler et al., 2012). In mouse dopaminergic neurons, mitochondrial fragmentation causes selective degeneration of the axonal compartment, causing motor deficits that occur before (Pham et al., 2012) or in the absence of (Lee et al., 2012) nigral cell death. It is possible, then, that aSyn increases mitochondrial fragmentation in vivo. This could cause impairments in redox homeostasis and ATP synthesis that disproportionately sensitize the axonal compartment to further insults, such as injury, oxidant stress, or aSyn aggregation (Gu et al., 2010).

The early mitochondrial transport deficits we observed in aSyn-expressing axons could also be pathologically relevant. Mitochondrial motility was reduced, and motile mitochondria in aSyn-expressing cells favored retrograde transport towards the cell body. Deficits in anterograde transport of mitochondria are associated with synaptic dysfunction and degeneration (Stowers et al., 2002; Weihofen et al., 2009; Misko et al., 2010; Misko et al., 2012). Mitochondrial transport deficits have been reported in the MPTP model (Cartelli et al., 2010; Kim-Han et al., 2011), and in cells expressing the A53T mutant form of aSyn (Xie and Chung, 2012). The transport impairment we observed could therefore underlie later dysfunction. Alternatively, reduced motility could be a protective response to mitochondrial dysfunction. PINK1 and parkin orchestrate the transport arrest of depolarized mitochondria (Wang et al., 2011; Cai et al., 2012; Liu et al., 2012), which inhibits further network impairment. The increased retrograde transport in aSyn-expressing axons could then represent trafficking of damaged mitochondria to lysosomes, where mitophagy occurs. Transport arrest and mitophagy of damaged mitochondria are critical to quality control in the mitochondrial network.

A better understanding of the mitochondrial dysfunction in this model may give insight into PD pathogenesis. Many genes associated with hereditary PD converge on mitochondrial function and quality control (Cardoso, 2011; Sai et al., 2012), and both genetic and pharmacologic models of PD implicate mitochondrial dysfunction in pathogenesis (Cassarino et al., 1997; Przedborski and Jackson-Lewis, 1998; Exner et al., 2012; Van Laar and Berman, 2012). In our model, focal varicosities in severely beaded axons were occupied by swollen, rounded mitochondria. A similar phenotype has been observed in mice expressing a disease-associated form of human aSyn (A53T) (Martin et al., 2006a; Chinta et al., 2010). In these cells, focal axonal swellings

often contained mitochondria and aSyn aggregates (Martin et al., 2006a). Mitochondrial swelling is consistent with opening of the mitochondrial permeability transition pore (mPTP), which is sufficient to induce axon degeneration in some cell types (Barrientos et al., 2011). Opening of the mPTP is induced by calcium overload in the mitochondria (Haworth and Hunter, 1979; Gunter et al., 1994), and is facilitated by ROS accumulation (Costantini et al., 1996; Kowaltowski et al., 1996; Vercesi et al., 1997). Normal pacemaking through L-type calcium channels in dopaminergic neurons causes oxidant stress and may lower the threshold for mPTP formation (Guzman et al., 2010; Surmeier et al., 2011; Goldberg et al., 2012), which may underlie the selective vulnerability to dopaminergic neurons to cell death in PD. Indeed, mitochondria isolated from the rat striatum are more sensitive to calcium influx than cortical mitochondria (Brustovetsky et al., 2003). Mitochondria at the presynaptic terminal may be particularly vulnerable (Brown et al., 2006), consistent with early synapse loss in PD. Loss-of-function mutations in PINK1, Parkin, and DJ-1, all of which are associated with PD, lower the threshold for mPTP opening (Guzman et al., 2010; Gandhi et al., 2012; Gautier et al., 2012; Giaime et al., 2012). In vitro studies indicate that inhibition of mPTP formation is protective against MPP^+ toxicity (Thomas et al., 2012) and against cell death caused by A53T mutant aSyn (Smith et al., 2005). Our data suggest that mPTP formation may also be relevant to aSyn toxicity in vivo.

PGC-1α is protective against aSyn toxicity

The transcriptional co-activator PGC-1α has critical roles in mitochondrial biogenesis and ROS scavenging, and may be a therapeutically relevant target in the treatment of neurodegenerative disease (Anderson and Prolla, 2009; Handschin, 2009; Zheng et al., 2010). Defects in PGC-1α activity were recently reported in fibroblasts from patients with

early-onset, parkin-deficient PD (Pacelli et al., 2011), and genome-wide association studies of identified reduced expression of many PGC-1α-regulated genes in tissues from PD patients (Zheng et al., 2010). We found that overexpression of mouse PGC-1α protects against aSyn toxicity in both the axon and the cell body. Others have reported that it protects mouse dopaminergic neurons from MPTP toxicity (St-Pierre et al., 2006; Mudò et al., 2012). This effect appears to be mediated by upregulation of ROS detoxification programs, including increased expression of mitochondrial superoxide dismutase (SOD2) (St-Pierre et al., 2006). Siddiqui and colleagues recently reported that aSyn associates with PGC-1α during oxidative stress, inhibiting these protective effects; however, overexpression of PGC-1α reestablished protection (Siddiqui et al., 2012). PGC-1α and its downstream target genes may therefore be relevant therapeutic targets in the treatment of synucleinopathies (Tsunemi and La Spada, 2012).

Conclusions

The current results show that expression of human wild-type aSyn induces aggregate formation and degeneration in a living vertebrate system. Mitochondrial fragmentation and mPTP formation may be relevant to the early axonopathy observed in this model, as mitochondrial protection by PGC-1α provided robust protection. The highly compartmentalized nature of peripheral sensory neurons, and the accessibility of the peripheral arbors, permits the longitudinal study of compartment-specific degeneration and protection. This system may therefore yield new insights into the vulnerability of the axonal compartment to aSyn toxicity, and will allow further study of the relationship between axon degeneration and cell death in disease.

MATERIALS AND METHODS

Fish

Fish were raised on a 14 h/10 h light/dark cycle at 28.5°C. Embryos were kept in a 28.5°C incubator. Experiments were approved by the Chancellor's Animal Research Care Committee at the University of California, Los Angeles.

Transgenes

A plasmid endoding aSyn and the viral T2A cDNA sequence cloned into pDsRed-Monomer N1 vector (Clontech) has been described elsewhere (Prabhudesai et al., 2012), and was cloned into the p3E entry vector of the Tol2/Gateway zebrafish kit (Kwan et al., 2007). The T2A sequence causes ribosomal “skipping” and eventual cleavage of the transcript (Donnelly et al., 2001) to generate two proteins from a single open reading frame, resulting in stoichiometric expression of the gene of interest and the fluorescent reporter (Tang et al., 2009). The T2A-DsRed cDNA was cloned into the p3E entry vector of the Gateway system (Invitrogen), downstream of a multiple cloning site (MCS). Because GFP expression is brighter than monomeric DsRed and is therefore preferable for axon imaging, the T2A sequence was also cloned into the p3E entry vector between a MCS and GFP. ASyn was then cloned into the MCS to generate p3E-aSyn-2A-GFP. WldS or mouse PGC-1a (Hanai et al., 2007) (gift from Dr. Shintaro Imamura) was inserted into the p3E-MCS-T2A-DsRed plasmid. In all constructs, the *CREST3* enhancer (gift of H. Okamoto; (Uemura et al., 2005) in the p5E entry vector drove expression of Gal4 and 14XUAS (Köster and Fraser, 2001) in pME, and these were recombined with one of the p3E donor vectors to generate the following transgenes:

A: *CREST3*:Gal4:UAS:GFP
B: *CREST3*:Gal4:UAS:aSyn-T2A-GFP

C: *CREST3*:Gal4:UAS:DsRed
D: *CREST3*:Gal4:UAS:WldS-T2A-DsRed
E: *CREST3*:Gal4:UAS:PGC-1a-T2A-DsRed

To visualize mitochondria, a cox8 mitochondrial targeting sequence was added to DsRed and cloned into the Gateway system to generate UAS-mitoDsRed-polyA (mitoDsRed; gift from the Kohler laboratory). This was coinjected with Plasmid A or B above so that the *CREST3* enhancer drove expression of GFP (+/- aSyn) and mitoDsRed in the same neurons. Approximately 15 pg of each transgene was injected into embryos at the one-cell stage for transient, mosaic transgene expression in sensory neurons, and embryos were screened at 1 and 2 dpf for reporter expression. Because DsRed maturation proceeds more slowly than GFP, robust expression of DsRed reporter transgenes was not observed until 2 dpf, so this was the earliest time point used for all experiments.

Immunohistochemistry

At 48hpf, embryos were dechorionated and fixed with 4% paraformaldehyde in PBS, pH 7.4, at 4°C overnight. Fixed embryos were cryoprotected with 30% sucrose and embedded into OCT Compound (Electron Microscopy Sciences) for frozen sectioning. 10mm sections were produced using a cryostat (Leica CM3050) and bonded to glass slides. Sections were washed with PBS, blocked with 10% normal goat serum, and then incubated with anti-alpha synuclein mouse IgG primary antibody (BD Biosciences) at 1:500 dilution at 4°C in a humidified chamber overnight. Slides were again washed in PBS and then incubated with Alexa 594-conjugated goat anti-mouse IgG (Invitrogen) secondary antibody at 1:500 dilution for 2hrs at room temperature and with 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear staining. Single-channel images were

obtained with a fluorescence microscope (Eclipse e400, Nikon) and merged using Adobe Photoshop software.

Imaging

Embryos were dechorionated, anesthetized in 0.01% tricaine, mounted in 1.2% low-melt agarose (Promega) in sealed chambers (O'Brien et al., 2009), and imaged on a heated stage with a 20X air objective on a confocal microscope (Zeiss LSM 510), using a 488 nm laser line for GFP and 543 nm for DsRed. Cell death was initially quantified by observing the same cohort at 2, 3, and 4 dpf; however, in pilot studies a moderate amount of developmental cell death was observed in GFP-expressing control embryos by 4 dpf. Further studies were therefore restricted to 2-3 dpf. Cell death was initially quantified in cells expressing only GFP or aSyn-2A-GFP. The counts were also performed in embryos co-injected with a DsRed reporter transgene, to allow later comparison with WldS and PGC-1a-expressing cells.

For time-lapse analysis of axon degeneration and cell death, embryos were imaged every 20-60 minutes for up to 12 hours. Images were compiled into projections and movies with QuickTime software. A staging system for axonal pathology was developed, and axons were quantified at 2 and 3 dpf.

Mitochondrial imaging: To determine the effect of aSyn expression on mitochondrial density and morphology, mitoDsRed-expressing embryos were imaged at 2 dpf using a 40X oil objective and 3X digital zoom. Mitochondrial transport was visualized by time-lapse imaging of a single optical section using only the 543 nm laser, at a frequency of ~1 Hz, for 6 minutes.

Mitochondrial morphology and transport

In the green channel, all axons within an image were traced using ImageJ software. Line length was calibrated to convert pixels to distance, and the Measure plugin was used to quantify the total axon length. Density was calculated as mitochondria/total axon length. Mitochondrial morphology was calculated as the ratio of length to diameter. Line length was calibrated to convert pixels to distance, and all mitochondria within an image were quantified. Mitochondrial motility was defined as the percent of total mitochondria that moved in a 50 μm axon segment during a 6-minute movie, and was quantified using the Kymograph macro for ImageJ. A mitochondrion was considered to be moving only if it traveled at least 2 μm at a speed of at least 0.1 μm/s (Misgeld et al., 2007). Speed was calculated as the slope of distance (x) over time (y, in pixels) on the kymograph, and direction was determined by the sign of the slope. Mitochondrial transport behaviors were characterized by quantifying the percentage of time that motile mitochondria spent paused or moving in the anterograde or retrograde direction.

Axon transection

GFP- and aSyn-2A-GFP-expressing axons were cut using a Zeiss 710 microscope equipped with a multiphoton laser (O'Brien et al., 2009). Embryos were imaged with a 25X water objective and 488/543 nm laser scanning to identify the axonal region of interest, then 1-5 scans of the two-photon laser (tuned to 910 nm) were used to transect an axonal region of interest at 100X digital zoom.

Data analysis

Data were analyzed with GraphPad Prism software. Unpaired t-tests were used to evaluate changes in mitochondrial morphology and transport between WT and aSyn-

expressing cells, and to quantify cell death in GFP- and aSyn-2A-GFP-expressing cells. Minimal significance was set at $p<0.05$. One-way ANOVA and planned, unpaired Student's t-tests were used to evaluate the effect of aSyn on cell death and axon degeneration, and the ability of PGC-1α or WldS to prevent those effects. One-way ANOVA was followed by the appropriate post-test to correct for multiple comparisons.

ACKNOWLEDGMENTS

We thank Meghan E. Johnson and Carla Koehler for the mitoDsRed construct, and Dr. Shintaro Imamura for the mouse PGC-1α. KCO was supported by a training grant from the UCLA Training Program in Neural Repair (NINDS T32 NS07449:13). This work was supported by grants to AS from the National Institutes of Dental and Craniofacial Research (RO1 DE018496) and the American Parkinson Disease Association Pilot Fund (20082501).

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Chapter 4. Targeted Ablation of Peripheral Sensory Neurons

As discussed in the previous two chapters, I have identified a role for mitochondria in both injury- and aSyn-induced degeneration. In this chapter, I will describe two other tools I have used to cause tissue-specific, temporally inducible degeneration in zebrafish peripheral sensory neurons. These tools could be used to complement the work described in previous chapters, and they may also have broader utility in other projects in the lab.

NITROREDUCTASE NFSB: PRODRUG-DEPENDENT APOPTOSIS

Apoptosis can be induced in a spatially and temporally specific manner in zebrafish through prodrug-dependent cell ablation. This technique combines the spatial selectivity of tissue-specific gene expression with the temporal specificity of prodrug application. The enzyme nfsB, purified from *E. coli* (McCalla et al., 1978), mediates bacterial sensitivity to nitrofuran antibiotics (McCalla et al., 1978). When expressed in the zebrafish pancreas (Pisharath et al., 2007), heart, or liver (Curado et al., 2007), nfsB has no effect on development or survival. When the prodrug metronidazole is added to the water, however, nfsB-expressing cells die within 24 hours (Curado et al., 2007; Pisharath et al., 2007; Pisharath and Parsons, 2009). The mechanism of death is reported to be apoptosis induced by DNA cross-linking (Curado et al., 2007). When fused to a fluorescent reporter, nfsB-expressing cells can be monitored before and during cell death.

We acquired a transgenic line of zebrafish expressing UAS–nfsB-mcherry in all cells (Tg(UAS-E1b:Eco.NfsB-mCherry)c264, Zfin) (Davison et al., 2007). I crossed these fish to the *isl1(ss)*:Gal4-UAS-GFP stable line (Sagasti et al., 2005) used in Chapter 2, and observed GFP and nfsB-mcherry expression in peripheral sensory neurons (Figure 4.1). I monitored these cells over several days to confirm that nfsB expression itself was not toxic to peripheral sensory neurons. I added 20 mM metronidazole (MTZ) at various time points to determine whether selective ablation of these cells could be achieved. As in other zebrafish tissues, I saw that degeneration could be induced by metronidazole in nfsB-expressing sensory neurons (Figure 4.1). To confirm that MTZ was not toxic in wild-type cells, I exposed Isl1(*ss*):Gal4-UAS-GFP embryos to the drug, and saw no degeneration. This confirms the utility of the prodrug/nitroreductase system in inducing massive degeneration in a spatially and temporally controlled manner.

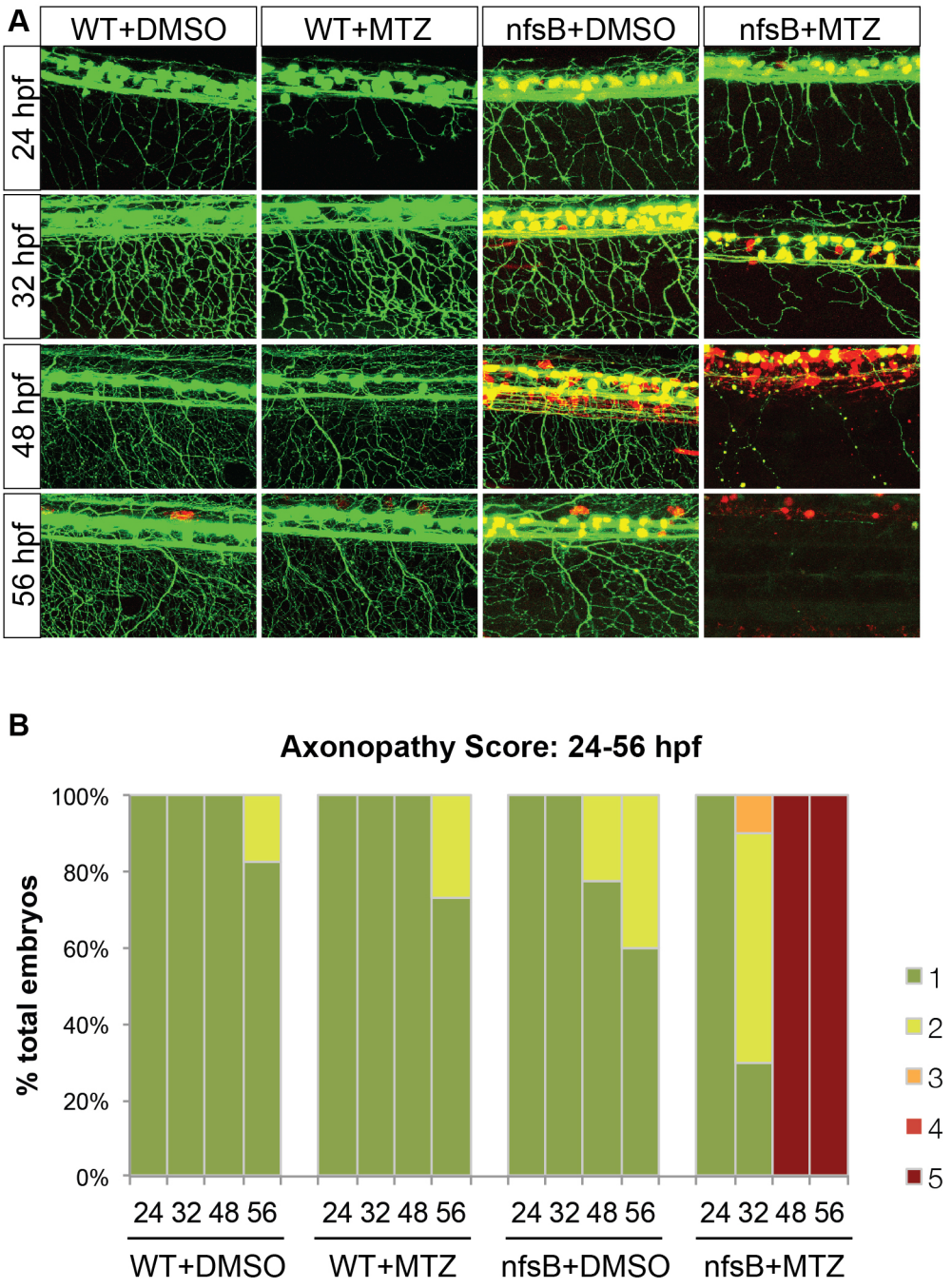

Figure 4.1 Prodrug-dependent degeneration of zebrafish peripheral sensory neurons.

Transgenic *isl1(ss)*:Gal4-UAS-GFP fish expressing GFP in peripheral sensory neurons were crossed to each other ("WT") or to transgenic fish expressing UAS-nfsB in all cells ("nfsB"). Embryos were collected and treated with 20 mM metronidazole (MTZ) at 8 hours post-fertilization (hpf). A) At 24, 32, 48, and 56 hpf, axons were imaged. B) Axon pathology was scored using the five-point system described in Chapter 3. A percentage of axons in all groups displayed mild beading by 56 hpf. MTZ did not increase this effect in WT cells. By 32 hpf, a majority of MTZ-exposed axons in nfsB-expressing cells exhibited a beaded morphology, with a small percentage undergoing fragmentation (stage 3). By 48 hpf, axons in all nfsB-expressing embryos had entirely degenerated (stage 5).

Complications

One difficulty with the system is the fact that the onset of cell death is not synchronous across embryos, so the system could not be used to detect very mild protective effects. The timing of degeneration is even less synchronous if time-lapse imaging is required, because en embryo embedded in agarose is not exposed to the same concentration of the drug as an embryo bathed in it. This results in much slower degeneration in agarose than would be observed if embryos had been in the MTZ solution throughout the imaging period (Figure 4.2, compare B and C).

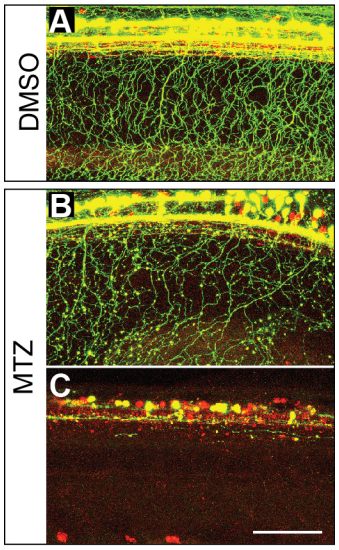

Figure 4.2 Agarose mounting delays MTZ-induced axon degeneration in nfsB-expressing cells.

Cells expressing GFP and nfsB-mcherry were bathed in MTZ or DMSO starting 24 hpf. Eight hours later, embryos were either mounted in 1.2% agarose supplemented with MTZ, or left in MTZ solution. The following day, no degeneration was observed in DMSO-exposed embryos (A). MTZ-exposed embryos that had been mounted in agarose showed marked beading (B) but little overt fragmentation. Axons from cells that had been bathed in MTZ overnight had completely degenerated (C). Scale bar: 100 μm.

WldS-expressing axons undergo prodrug-dependent degeneration

Despite these limitations, the nfsB/MTZ system can be used to screen for robustly protective phenotypes. To determine whether WldS protected axons in this model, I

crossed nfsB-expressing fish to a transgenic line expressing WldS in peripheral sensory neurons (Martin et al., 2010). Axons expressing both nfsB and WldS underwent degeneration (Figure 4.3), though slightly more slowly than axons not expressing WldS (compare to Figure 4.1). It is possible that WldS would be more protective if MTZ were added at a later time point, after higher levels of WldS have been expressed.

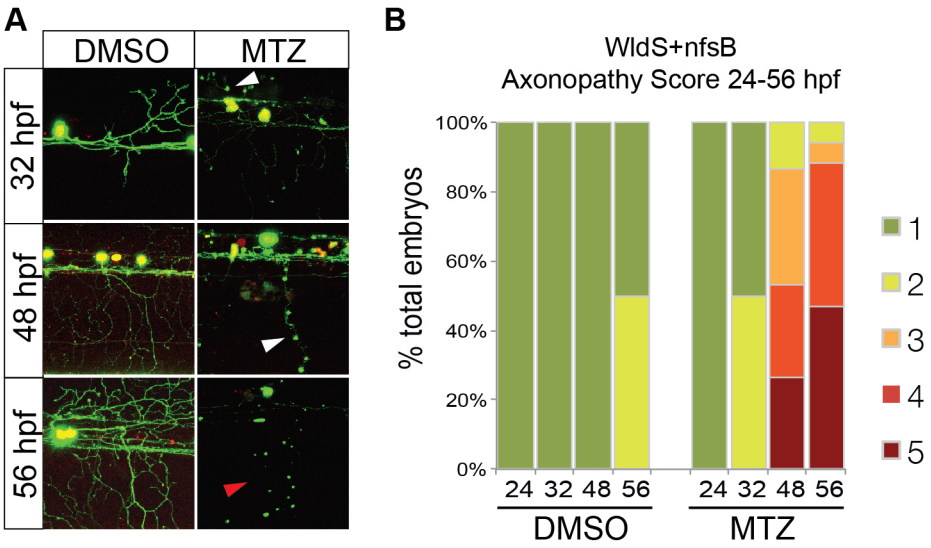

Figure 4.3 WldS does not prevent prodrug-dependent degeneration.

A) A transgenic line of embryos expressing WldS in peripheral sensory neurons was crossed to fish expressing UAS-nfsB-mcherry in all cells. Embryos were exposed to either DMSO or metronidazole (MTZ) at 8 hpf, then imaged at various time points. By 32 hpf, cells expressing nfsB exhibit beading (white arrowheads), and by 48 hpf, all cells exhibit axonopathy (stage 2-5). Clearance of axonal debris (i.e., progression to stage 5) is slightly delayed in WldS-expressing cells (compare to nfsB+MTZ in Figure 4.1).

Discussion

The robust degenerative phenotype of prodrug-dependent apoptosis provides a promising model that could be used to screen for protective compounds. Because both cell death and axon degeneration are so severe, the model could be used to identify factors that provide compartment-specific protection. Given the fact that cell death induction can vary by several hours, such a screen would likely yield many false-

negative results because subtle protective phenotypes would not be discerned. Nevertheless, any positive findings could then be applied to other models. The system could also be used to explore other aspects of degeneration, such as clearance of axonal fragments. Because so many cells undergo degeneration, a vast amount of debris is generated. This model could be used to identify the cell types responsible for phagocytosis of that debris. Other studies in our lab have also explored the coordinate development of axons and skin cells in embryonic zebrafish (O'Brien et al., 2012). By inducing degeneration at different time points—potentially even before axonal outgrowth—this model could be used to explore the consequences for skin cell development.

KILLERRED: PHOTOACTIVATABLE ROS PRODUCTION

KillerRed is a photosensitizer derived from a hydrozoan homolog of GFP (Bulina et al., 2006b). Upon exposure to green light, its phototoxicity is at least 1000-fold higher than that of GFP, an effect mediated by ROS generation (Bulina et al., 2006a; Carpentier et al., 2009). When the protein is localized to mitochondria, and photoactivated in vitro, it causes mitochondrial depolarization and mPTP formation (Shibuya and Tsujimoto, 2012), and cell death proceeds by a combination of apoptotic and non-apoptotic pathways (Bulina et al., 2006b; Shibuya and Tsujimoto, 2012; Shirmanova et al., 2013).

We acquired a transgene in which KillerRed was fused to two cox VIII-derived mitochondrial targeting sequences (pKillerRed-dMito, Evrogen). I cloned this transgene into the Gateway system downstream of the CREST3 enhancer and Gal4-UAS. I coinjected it with a transgene driving DsRed expression in the cytoplasm (Figure 4), and

observed expression in peripheral sensory neurons (Figure 4.4). It is notable that the mitochondrial targeting was not as effective in this transgene than in the mitoDsRed and mito-roGFP transgenes described in Chapter 2, though the sequence was verified (Laragen).

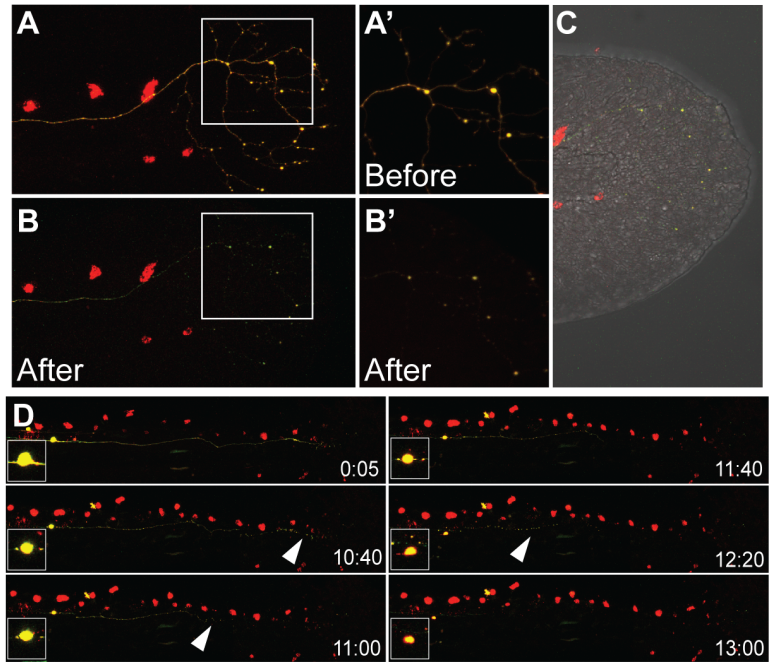

Figure 4.4 Photoactivation of KillerRed by green light causes degeneration

GFP- and KillerRed-expressing sensory neurons were exposed to arc lamp irradiation with green light for 25 minutes. Photoactivation was accompanied by photobleaching of the red protein (compare A and B). C) Skin of the tail was not damaged. D) The cell was imaged at 20-minute intervals for 13 hours after photoactivation. Inset is magnification of the cell body. Cell body morphology changes several hours after injury, indicative of apoptosis. Retrograde axonal fragmentation is observed in the exposed axon.

Although the Evrogen literature indicates that arc lamp irradiation is more efficient than laser irradiation, the latter has been used successfully in vitro (Shirmanova et al., 2013), and I hoped to selectively induce ROS production in a small region of the axon. I therefore imaged cells using a 63X water objective and exposed a region of interest to repeated scans with a 543 nm laser. In most cases, laser exposure induced neither

photobleaching nor phototoxicity (data not shown). I therefore attempted arc lamp photoinactivation, and found that in some cases, severe tissue damage was generated after even limited exposure (Figure 4.5). Similar non-specific toxicity has recently been reported in *C. elegans* expressing mitochondrial KR, with developmental defects following photoactivation (Shibuya and Tsujimoto, 2012).

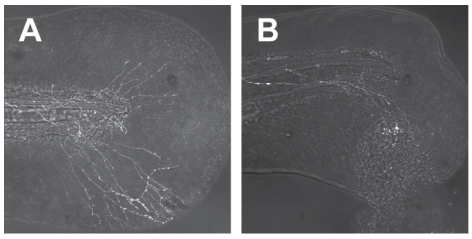

Figure 4.5 Arc lamp irradiation can cause tissue damage in KR-expressing embryos.

A) Tail fin of a 2dpf embryo expressing KR in a peripheral sensory neuron with an axonal arbor in the skin. B) After 25 minutes of arc lamp irradiation using a 63X objective, dramatic tissue damage had occurred.

Discussion

KillerRed phototoxicity is less appropriate for a chemical screen than prodrug-induced apoptosis, because the phenotype is more variable, and induction is more labor-intensive. Nevertheless, if optimized to maximize ROS production in a limited region, without causing damage to the embryos, the system could be used to further explore the role of ROS in compartment-specific neurodegeneration. For example, it could be used to address the question of whether increased ROS production is sufficient to induce Wallerian-like degeneration, or whether the calcium influx after axotomy is also required. Likewise, the ability of WldS to protect against ROS-induced degeneration could be explored. The model could be also used to test the hypothesis that ROS production after

axotomy mediates the increased level of cell death observed in injured aSyn-expressing neurons. Is moderate photoactivation of KillerRed sufficient to increase aSyn toxicity? Does PGC-1α increase the threshold for KR phototoxicity? Though further optimization of the KR model is required before these questions could be addressed, it could provide a powerful tool in future studies of the contribution of ROS production to degeneration.

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Discussion

Integration of results

In this thesis, I have described multiple models of axon degeneration in zebrafish peripheral sensory neurons. Within two of those models, I explored the relevance of mitochondrial transport and function to axon degeneration and protection. In the axotomy model, I saw that mitochondrial transport was neither necessary nor sufficient for axonal integrity. Mitochondrial dysfunction, by contrast, correlated with degeneration. In the model of synucleinopathy, changes in mitochondrial morphology were consistent with early and progressive dysfunction. These morphological changes were accompanied by a reduction in overall mitochondrial flux, and an increase in retrograde transport of motile mitochondria. These results suggest that mitochondrial transport "defects" reported in various disease models may often represent efforts to limit trafficking of depolarized mitochondria. That PGC-1α is protective in both models raises the possibility that aberrant redox homeostasis underlies many states of axonal vulnerability, and may be a promising target for early pharmacologic intervention.

Mitochondrial morphology and axonopathy

I found that mitochondria were slightly elongated in WldS-expressing axons, and dramatically so in PGC-1α-expressing cells. This phenotype is associated with a resistance to depolarization and mitophagy (Wang et al., 2012). By contrast, aSyn-expressing mitochondria were small and rounded, consistent with increased fragmentation, and eventually swelled to fill the increasingly dystrophic axons. These

studies provide in vivo support for in vitro studies indicating that aSyn interacts with mitochondria and thereby promotes fragmentation (Kamp et al., 2010; Monti et al., 2010; Nakamura et al., 2011; Zhu et al., 2012). Mitochondrial fragmentation in the absence of primary dysfunction can lead to defects in energy production and redox homeostasis (Benard and Rossignol, 2008). It may therefore represent an early event in pathogenesis.

Time-lapse imaging of aSyn-expressing neurons indicated that cell death in this model is not a stereotyped process of retrograde, “dying-back” degeneration initiated in the axon, as has been proposed in the past (Hornykiewicz, 1998). Nevertheless, the early axonopathy observed in these cells suggests that the axonal compartment is particularly vulnerable to aSyn toxicity. Indeed, axonal swelling has been reported in post-mortem studies of PD brains (Galvin et al., 1999), and in other neurodegenerative disease models, such as ALS (Nikić et al., 2011) and Alzheimer disease (Adalbert et al., 2009). Axonal dystrophy associated with amyloid plaque formation does not rapidly lead to fragmentation (Adalbert et al., 2009). Likewise, in the aSyn model, I saw that axonal beading preceded degeneration by at least several hours, and many dystrophic axons did not undergo cell death in the time frame I monitored. In the EAE mouse model of ALS, in vivo imaging of axon degeneration revealed that focal axonal swelling always precedes fragmentation, but is occasionally reversible (Nikić et al., 2011). The axonopathy I observed might therefore be a harbinger of intracellular dysfunction, and its early onset might allow detection and intervention before degenerative pathways have been irreversibly activated.

I found that aSyn-expressing cells that were axotomized were more likely to die than uninjured cells. Retrograde, axotomy-induced cell death has been observed before,

most notably in retinal ganglion cells after optic nerve transection (Bonfanti et al., 1996; Castagné and Clarke, 1996; Cenni et al., 1996). In those studies, cell death had both apoptotic and non-apoptotic features, and was associated with increased ROS production in the injured axon. It is possible that peripheral sensory neurons, which normally do not undergo axotomy-induced cell death, have robust ROS detoxification mechanisms in place that prevent ROS accumulation in the cell body after injury. Cells that express aSyn, by contrast, may have a higher baseline level of ROS production, such that additional insults could overwhelm their protective pathways. Consistent with this hypothesis, overexpression of mutant aSyn increases sensitivity to rotenone in vitro, leading to ROS-dependent cell death (Sherer et al., 2002; Ma et al., 2011).

My studies of WD in wild-type axons in Chapter 2 support the hypothesis that mitochondrial ROS generation underlies axonal vulnerability after injury. However, in those experiments I observed roGFP2 oxidation only in the distal axon, which later degenerated. This result would appear to rule out the hypothesis that ROS production in the proximal axon underlies axotomy-induced cell death in aSyn-expressing neurons. However, it is possible that in wild-type axons, rapid turnover of mitochondria in the proximal axon prevents ROS accumulation. Mitochondria that have been exposed to the insult can be transported back to the cell body to undergo fusion or mitophagy, and new mitochondria are transported to the injury site. Because mitochondrial transport is reduced in aSyn-expressing axons, such turnover would be impaired. Studies with roGFP2 in aSyn-expressing axons could test the hypothesis that basal ROS production, coupled with impaired transport, underlies axotomy-induced cell death in vulnerable neurons.

Mitochondrial transport deficits after injury and in aSyn-expressing axons

In Chapter 2, I evaluated mitochondrial transport in peripheral sensory neurons both during development and after injury. After two-photon laser transection, I observed abrupt mitochondrial transport arrest, as has previously been reported in flies (Avery et al., 2012). Consistent with a critical role for mitochondrial transport in axon protection, I did not observe the transport arrest in the proximal portion of the axon, which remains connected to the cell body and does not degenerate. In neurons overexpressing WldS, however, this correlation between transport and protection was not preserved. Mitochondria in WldS-expressing axons did not exhibit increased baseline transport of mitochondria, and acute transport arrest was observed immediately after axotomy. Transport then recovered, but only transiently: despite persistence of the transected axon, virtually all mitochondria were stationary 24 hours after injury. Preservation of mitochondrial transport is therefore unlikely to be the mechanism by which WldS protects axons. However, as described in Chapter 1, mitochondrial transport is affected by a number of intracellular changes, including calcium influx and mitochondrial depolarization. Because calcium influx is known to occur immediately after axotomy in vitro (Glass et al., 1994; Adalbert et al., 2012), it is likely that the acute transport arrest in both wild-type and WldS-expressing axons was mediated by calcium. In separate but related work in our lab, Dr. Mauricio Vargas is studying the role of calcium in Wallerian degeneration, and has observed a rapid and transient rise in axonal calcium after injury in both wild-type and WldS-expressing axons (manuscript in preparation). His findings support the hypothesis that mitochondrial transport arrest after injury occurs in two phases, with only the first being mediated by an adaptive response to calcium influx.

I hypothesized that a change in the $\Delta\Psi_m$ might underlie a second phase of transport arrest in wild-type axons, and that WldS might prevent that change. If the persistent

transport arrest represents a PINK1-mediated effort to sequester depolarized mitochondria, PINK1 knock-down should preserve transport after axotomy. Morpholino knock-down confirmed this hypothesis. Moreover, if PINK1-mediated transport arrest is protective mechanism, then PINK1 knock-down should accelerate WD. Consistent with this hypothesis, the transport recovery in PINK1-deficient axons was accompanied by a more rapid onset of fragmentation.

The dissociation between mitochondrial transport and axon degeneration in WldS-expressing or PINK1-deficient axons suggests that transport preservation is neither necessary nor sufficient for protection. Instead, oxidation of proteins in the mitochondrial matrix correlates with axon degeneration after injury, and with WldS-mediated protection. I also saw that overexpression of PGC-1α, which increases baseline expression of ROS detoxifying enzymes such as SOD2 (St-Pierre et al., 2006), delayed both mitochondrial oxidation after injury and axon degeneration. This result provides further support for the hypothesis that mitochondrial function, rather than transport, is critical to axonal integrity.

That PGC-1α expression could delay degeneration of the distal axon after injury raised the possibility that it might confer even greater protection in a degeneration model in which physical continuity with the cell body was retained. The aSyn model described in Chapter 3 provided the opportunity to test that hypothesis. I saw that PGC-1α overexpression robustly protected both axons and cell bodies from aSyn toxicity. This result supports a model in which aSyn toxicity is mediated by mitochondrial dysfunction, possibly in association with oxidative damage.

PGC-1α: a relevant therapeutic target?

PGC-1α has many roles in cellular metabolism, and its dysregulation has been associated with multiple disease states, including Parkinson disease and Huntington disease (Zheng et al., 2010; Clark et al., 2011; Pacelli et al., 2011; Tsunemi and La Spada, 2012). As such, considerable effort is being put forth to determine whether elevation of PGC-1α is a promising avenue for drug development (Handschin, 2009). Ideally, therapeutic activation of PGC-1α would be tissue-specific, to avoid broad effects on insulin sensitivity, cardiac function, and enzymatic processes in the liver (Anderson and Prolla, 2009; Handschin, 2009). PGC-1α coactivates many transcription factors, and post-translational modifications underlie its binding specificity. By altering its interactions with its various binding partners, a specific and potent effect could theoretically be achieved.

Even without a targeted approach, promising results have been reported with PGC-1α activation. The antioxidant compound resveratrol increases activity of the NAD^+-dependent histone deacetylase SIRT1, which is required for nuclear translocation and activation of PGC-1α during oxidative stress (Anderson et al., 2008). Resveratrol increases both activation and expression of PGC-1α in a SIRT1-dependent manner (Mudò et al., 2012). When the *Thy1* promoter is used to drive overexpression of PGC-1α in mice, dopaminergic neurons are partially protected from MPTP toxicity (Mudò et al., 2012). Resveratrol mimics this protective effect of PGC-1α both in vivo and in vitro, and leads to upregulation of mitochondrial antioxidant enzymes SOD2 and thioredoxin (Trx2) (Mudò et al., 2012).

A separate but related approach to targeting downstream effectors of PGC-1α-mediated protection is to administer agonists of the PPAR-γ receptor, which PGC-1α co-activates. Such agonists are already widely available as the insulin-sensitizing agents rosiglitazone and pioglitazone. Both of these compounds have been shown to be protective against MPTP-induced cell death in the SNc (Breidert et al., 2002; Schintu et al., 2009). It is noteworthy that oral administration of pioglitazone, while protective of cell bodies in the SNc, did not prevent dopamine depletion in the striatum (Breidert et al., 2002). This may be the result of regional availability, or it may reflect increased vulnerability in the striatum. Indeed, in PGC-1α knock-out mice, neurodegenerative lesions are prominent in the striatum, consistent with a higher demand for ROS detoxification at the dopaminergic terminal (Lin et al., 2004).

"Two-hit" hypothesis of axon degeneration

Various animal models implicate the combined dysfunction of mitochondrial calcium handling and ROS accumulation in the etiology of PD. Some investigators have therefore proposed a "two-hit" model of pathogenesis, in which a combination of insults is required for degeneration (Brookes et al., 2004). Cytosolic metabolism of dopamine produces ROS (Hauptmann et al., 1996; Hornykiewicz, 1998), which may provide a "first hit" that sensitizes dopaminergic neurons to further insults. Likewise, L-type calcium channels, which are implicated in WD (George et al., 1995), are abundant on the presynaptic terminal of SNc dopaminergic neurons. Their activation through normal pacemaking activity increases basal levels of oxidant stress in these cells (Guzman et al., 2010; Goldberg et al., 2012). This relationship between ROS production and calcium influx may ultimately lead to mPTP formation and degeneration. Incidentally, loss-of-function mutations in the PD-associated proteins DJ-1, PINK1, and Parkin all increase

neuronal vulnerability to oxidative stress, and all have been associated with impaired calcium buffering and a reduced threshold for mPTP opening (Piccoli et al., 2008; Gandhi et al., 2009; Guzman et al., 2010; Akundi et al., 2011; Gautier et al., 2012; Joselin et al., 2012).

The “two-hit” model need not be limited to a relationship between ROS production and calcium homeostasis. ROS-mediated oxidation of proteins increases their aggregation (Giasson et al., 2000; Souza et al., 2000; Sherer et al., 2002). These aggregates may have directly toxic effects, and/or they may cause axonal transport blockade that functionally severs the axon from the cell body (Gunawardena and Goldstein, 2001; Gunawardena et al., 2003; Falzone et al., 2010). Indeed, when Gilley and Coleman (2010) identified Nmnat2 as a putative axon survival factor, they determined that inhibition of protein synthesis in the soma, but not the axon, was sufficient to induce degeneration. Proper Nmnat2 distribution required functional axonal transport (Gilley and Coleman, 2010). Protein aggregates that impede such transport could therefore initiate axon degeneration through Nmnat2 depletion, among other effects.

Future directions

In future studies of WD, I will evaluate roGFP2 oxidation in PINK1 knock-down mitochondria. The two-step model of transport arrest suggests that roGFP2 oxidation should rise more steeply in PINK1 morphants. I will determine whether this is the case. I I will also use morpholino knock-down of zebrafish *parkin* to determine whether the effects I have seen in PINK1 morphants are in fact mediated by the PINK1/parkin pathway. To determine whether the protective effect of PGC-1α is, in fact, mediated by ROS detoxification, I will overexpress superoxide dismutase (SOD) in the cytoplasm

(SOD1) or the mitochondria (SOD2), to determine whether a higher level of ROS scavenging in either compartment is sufficient to delay degeneration. This would provide further evidence of ROS involvement in WD, and could determine whether mitochondrial or cytoplasmic ROS are selectively required for axon degeneration.

Future studies in the aSyn toxicity model could further investigate the relationship between aSyn toxicity and mitochondrial function. For example, is roGFP2 more oxidized in these neurons? As I proposed above, roGFP2 oxidation after axotomy could be monitored to determine whether ROS production was responsible for axotomy-induced cell death in aSyn-expressing neurons. I hypothesized that the mitochondrial transport deficit in aSyn-expressing axons may reflect protective PINK1-mediated transport arrest. Does PINK1 knock-down accelerate aSyn-induced degeneration? Does parkin overexpression attenuate it? Future studies in this model could also explore the relationship between aSyn aggregation, mitochondrial dysfunction, and degeneration. For example, does PGC-1α expression prevent aggregation, or is its protective effect independent of it? Such studies could determine whether mitochondrial function was up- or downstream of aggregation in vivo.

Conclusions and reflections on the model system

In the preceding chapters, I have described multiple forms of degeneration in larval zebrafish neurons, and I have discussed some of the ways in which they might be used to study degenerative processes. Although Wallerian degeneration itself is uncommon, Wallerian-like degenerative pathways, as defined by WldS protection, are highly conserved across disease models (Coleman and Freeman, 2010). WD and WldS can

therefore be used as experimental tools to elucidate the pathways that regulate axonal integrity.

Some have argued that the failure to produce disease-modifying treatments for PD Is due to a failure of the current animal models, none of which faithfully recapitulate the natural course of disease (Waldmeier et al., 2006). By that argument, the study of peripheral sensory neurons at all is not likely to identify promising therapeutic targets, to say nothing of the use of embryonic zebrafish and Wallerian degeneration. I disagree. I propose, instead, that the failure stems from the fact that we do not fully understand those diseases in the first place. In particular, we have little grasp of the earliest events in pathogenesis, which are certainly not represented in post-mortem tissue from patients who have already met the criteria for a clinical diagnosis. If we insist that our animal models reproduce a late disease phenotype, we are unlikely to discover, let alone prevent, the earliest steps in neurodegeneration (Coleman and Perry, 2002; Conforti et al., 2007; Chesselet, 2008). As disease pathways are more fully delineated, rational drug development is more likely to succeed.

The larval zebrafish is increasingly recognized as a valuable tool in neurodegeneration research, because as an optically transparent vertebrate, it is well suited to high-throughput screens (Bretaud et al., 2004; Flinn et al., 2008; Bandmann and Burton, 2010; Pienaar et al., 2010; Guella et al., 2011; Xi et al., 2011). My studies suggest that, for the same reasons, this model also has (admittedly much lower-throughput, but no less essential) utility in interrogating the cell biological basis of degeneration. By studying degenerative insults in peripheral sensory neurons, I have identified early changes in the axonal compartment that precede axonal fragmentation and cell death. Axon pathology is known to be an early event in PD, but the relevance of that pathology is not well

understood. By studying aSyn toxicity in the axonal compartment in vivo, we may identify the earliest stages of pathogenesis, and identify promising targets for therapeutic intervention.

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