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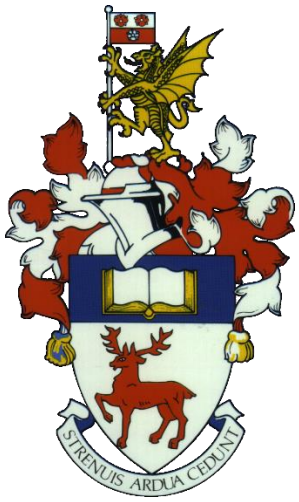

by

Katy Stubbs

Thesis for the degree of Doctor of Philosophy

June 2015

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF NATURAL & ENVIRONMENTAL SCIENCE

Biological Science

Thesis for the degree of Doctor of Philosophy

INVESTIGATING NEURODEGENERATION IN A *DROSOPHILA* MODEL OF TAUOPATHY

Kathryn Laura Stubbs

Axonal degeneration occurring in disease is thought to be similar to that occurring after acute injury, known as Wallerian degeneration. Wallerian degeneration is an active process that can be delayed by the expression of the slow Wallerian degeneration (Wld^S) protein. In this project I sought to investigate whether Wld^S could delay axonal degeneration in a model of neurodegenerative disease: tauopathy. Tauopathy is characterised by neuronal dysfunction and degeneration occurring as a result of changes to the microtubule associated protein tau. Using *Drosophila* co-expressing $htau^{0N3R}$ and Wld^S, neuronal dysfunction was investigated in larvae. However, Wld^S did not improve tau-mediated deficits in axonal transport, synaptic alterations and locomotor behaviour, indicating Wld^S does not improve tau-mediated neuronal dysfunction. Furthermore, co-expression in the adult system, in which both neuronal dysfunction and degeneration occur, did not improve locomotor behaviour nor delay the onset or slow the progression of axonal swellings indicative of axonal degeneration. A significant increase in lifespan was observed, however as this was not accompanied by delayed axonal degeneration this is likely to be an off target effect. The failure of Wld^S to delay tau-mediated axonal degeneration suggests that a different mechanism controls axonal degeneration in disease than in injury. Supporting this, following axotomy $htau^{0N3R}$;Wld^S axons displayed robust protection on a par with Wld^S axons. Interestingly, subsequent bystander protection against tau-mediated axonal swellings was observed in injured $htau^{0N3R}$;Wld^S axons. This further supports that different mechanisms control axonal degeneration in chronic disease and acute injury.

Mitochondria play an important role in neuronal dysfunction and degeneration in tauopathy, but how this contributes to axonal degeneration remains unclear. Expression of $htau^{0N3R}$ results in mitochondrial mislocalisation in the larval model of neuronal dysfunction. Restabilising the microtubule cytoskeleton using the drug NAP restored axonal transport but did not rescue mitochondrial mislocalisation, indicating that the mislocalisation is not simply due to the loss microtubule stabilisation by tau.

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DECLARATION OF AUTHORSHIP

I, ...

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Investigating neurodegeneration in a *Drosophila* model of tauopathy

I confirm that:

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3. Where I have consulted the published work of others, this is always clearly attributed;
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Acknowledgements

First and foremost I would like to thank my supervisors Dr Amrit Mudher and Professor Hugh Perry, for their support and guidance over the last few years. I feel privileged to have been supervised by such passionate, dedicated scientists – thank you for your encouragement and for never giving up on me. I would also like to thank the all the academic members of staff with whom I've had the pleasure of working with; the SoNG community especially for the stimulating discussions I have had with many of you. A special thank you to the Gerald Kerkut Trust for the financial support and for giving me this opportunity, I have enjoyed being a part of Gerald's legacy.

Huge thanks to members of the Mudher lab past and present, Cath, MT, Shmma, Kirstin, Kelly, Casey, Luisa and Megan, also to Dr Herman Wijnen, Karolina and Miguel. I have greatly enjoyed working with you all, your help on all things fly-related and companionship during the long hours in the lab are very much appreciated. I especially want to thank Megan for being such a great lab buddy, for teaching me everything, putting up with my fly rants and generally being all round awesome!

Thank you to all those that have helped me in the lab, especially with microscopy. Anton Page, Hans Schuppe, Jo Bailey, John Chad, Katrin Deinhardt and Mark Willet, this thesis would not be so pretty if it wasn't for all of you!

There are way too many people to name everyone individually, so just a big blanket thank you to the biosci PhD community, past and present. I have been lucky to make so many friends during my time here. Thank you for the lunchtime laughs, pub o'clock Fridays and for being generally fantastic!

To my wonderful BFFL, housemate, confidant and all round rock Sarah; I cannot express enough how much I value your friendship and support. I look forward to when we are old and can finally open our bakery. Helen, thank you for being someone I know I can call anytime and for the deep discussions and shenanigans. Rhys, thank you for being you, for being supportive even when you were halfway round the world. To all my friends who have put up with me constantly banging on about Alzheimer's – thank you – but I'm never going to stop!

And last but not least, the parents. I am blessed to be your child, thank you for your love and support through everything.

Definitions and Abbreviations

6-OHDA	6-hydroxydopamine
AAD	acute axonal degeneration
AD	Alzheimer's disease
ANOVA	analysis of variance
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid-beta
CD38	cluster of differentiation 38
cdk	cyclin dependent kinase
CMAP	compound muscle action potential
CMT	Charcot-Marie-Tooth disease
CNS	central nervous system
CsA	cyclosporin A
dae	days after eclosion
DAI	diffuse axonal injury
DLK	dual leucine kinase
dpi	days post injury
DRG	dorsal root ganglion
Drp1	dynamin related protein 1
DTI	diffusion tensor imaging
EAE	experimental autoimmune encephalomyelitis
EGTA	ethylene glycol tetraacetic acid
EPSP	Excitatory postsynaptic potential
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FITC	fluorescein isothiocyanate
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GAD	gracile axonal dystrophy
GFP	green fluorescent protein
GSK3	glycogen synthase kinase 3
hiw	Highwire
HRP	horseradish peroxidase
htau	human tau
IKK	IκB kinase
IOP	intra-ocular pressure
JIP1	JNK interacting protein 1
JNK	c-Jun N-terminal kinase
KLC	kinesin light chains
MAPK	mitogen activated protein kinases
MAPT	microtubule-associated protein tau gene
MARCM	mosaic analysis with a repressible cell marker

MARK2	MAP/microtubule affinity-regulating kinase 2
MND	motor neuron disease
MOG	myelin oligodendrocyte glycoprotein
mPTP	mitochondrial permeability transition pore
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
MTS	mitochondrial targeting sequence
NA	nicotinic acid
NAD	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
Nam	nicotinamide
Nampt	nicotinamide phosphoribosyltransferase
NAP	davunetide
NFT	neurofibrillary tangles
NH_2	N-terminal fragment of human tau
NLS	nuclear localising sequence
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
NMN	nicotinamide mononucleotide
Nmnat	nicotinamide mononucleotide adenylyl transferase
OPA1	optic atrophy 1
OreR	Oregon R
ORN	olfactory receptor neuron
PAD	phosphatase activating domain
PARP1	poly (ADP-ribose) polymerase 1
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
Phb2	prohibitin 2
PHF	paired helical filaments
PHR1	PAM-Highwire-Rpm-1
PIPES	piperazine-N,N'-bis
PMN	progressive motorneuron neuropathy
PMP22	peripheral myelin protein of 22kDa
PNS	peripheral nervous system
PSD95	postsynaptic density protein 95
ROS	reactive oxygen species
RT	room temperature
Sarm	sterile alpha and Armadillo motif
SCG	superior cervical ganglion
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SMA	spinal muscular atrophy
SOD	superoxide dismutase

TBI	traumatic brain injury
TEM	transmission electron microscopy
TH	tyrosine hydroxylase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UAS	upstream activating sequence
Ube4b	ubiquitin conjugation factor E4 B
UPS	ubiquitin proteasome system
VCP	valosin containing protein
vGFP	vesicular GFP
VNC	ventral nerve cord
wae	weeks after eclosion
WldS	slow Wallerian degeneration
Wnd	Wallenda
wpi	weeks post injury
WT	wild type

Chapter 1: Introduction

1.1 General introduction

How neuronal cells die in neurodegenerative diseases has eluded scientists for many years, however evidence suggests that axonal degeneration precedes neuronal death in a variety of conditions. Furthermore, the changes which occur in degenerating axons in disease are remarkably similar to those occurring in injury situations, leading many to suggest that a common mechanism may be responsible. Historically this was based purely on morphological observations; however the discovery of mediators of axonal degeneration has enabled the investigation of the molecular events governing the breakdown of axons in injury situations. The further investigation of these molecular mediators in disease could yield important mechanistic insights into these diseases and highlight potential therapeutic strategies.

The aim of the work presented in this thesis was to investigate axonal degeneration in chronic neurodegenerative disease. Knowledge of the molecular mediators controlling Wallerian degeneration after injury is increasing, beginning with the discovery of the Wld^S mutant mouse in which degeneration was significantly delayed. Whether a common mechanism underpins degeneration in injury and disease is not clear, and translation of knowledge from injury into disease models will yield important insight into this question. Tauopathies are neurodegenerative diseases which share many common features with Wallerian degeneration, and so investigating the role of axonal degeneration in this model of disease is of particular interest. An ideal model system in which to investigate similarities between axonal degeneration in injury and disease is the fruit fly, *Drosophila melanogaster*. Significant contributions to the understanding of both Wallerian degeneration and tauopathy have been made using this simple model organism, making it ideal for this work

1.2 Wallerian degeneration

The morphological changes occurring in axons after a physical injury were first described over 150 years ago by Augustus Waller. Upon cutting the glossopharyngeal nerves of frogs, he noted the characteristic fragmentation and breakdown of the injured nerves (Waller, 1850), observations that held true for subsequent researchers, leading to the description being named after him – Wallerian degeneration.

1.2.1 Morphological changes

There are 3 distinct stages to Wallerian degeneration; acute axonal degeneration (AAD) occurring in the minutes following the injury, followed by a latent period of around 30 hours, before finally the rapid and heterogeneous fragmentation of the distal axon. AAD occurs within 30 minutes of axotomy, when both the proximal and distal axon retract 200-300μm from the cut site (Figure 1-1 A) (Kerschensteiner et al., 2005). This is followed by a latent stage in which distal axons remain stable and are still capable of conducting evoked action potentials along their length. During this time, intra-axonal changes begin to occur, such as the breakdown of microtubules and neurofilaments, mitochondrial swelling and fragmentation of the endoplasmic reticulum (Figure 1-1 B) (Vial, 1958, Webster, 1962, Schlaepfer and Micko, 1978). These changes are followed by the sudden and rapid onset of granular fragmentation of the distal axon which is complete within a few hours.

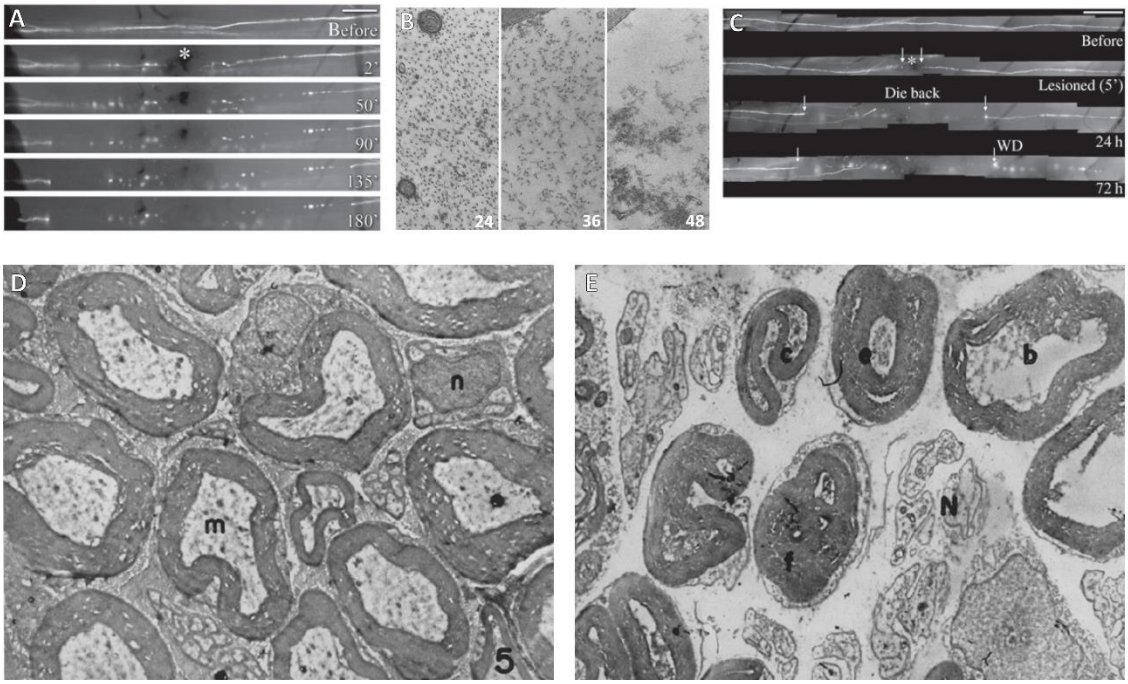


Figure 1-1. Morphological changes in Wallerian degeneration. [A] Immediately after injury, AAD occurs and axonal ends retract from the cut site over the course of minutes (Kerschensteiner et al., 2005). [B] In the following latent period, intra axonal changes such as the breakdown of microtubules and neurofilaments occurs (Schlaepfer and Micko, 1978). [C] After the latent period, the distal axonal fragment undergoes rapid and asynchronious fragmentation (Kerschensteiner et al., 2005). Transverse section through intact axons [D] and axons 48h after injury shows the gross morphological aberrations in ensheathing myelin and the breakdown of intraxonal components (Vial, 1958).

1.2.2 Cellular responses to axonal degeneration

The presence of degenerating axons within the nervous system elicits a response from the surrounding cells. In mammals, this response differs in the peripheral nervous system (PNS) compared with the central nervous system (CNS), due to the different cells present in each situation.

In the PNS, axons are ensheathed in myelin, which is provided by Schwann cells. Following injury, Schwann cells rapidly react, reducing their synthesis of myelin (White et al., 1989), with the sheaths becoming segmented (Stoll et al., 1989). Myelin is cleared by a number of means, with Schwann cells degrading their own myelin, phagocytosing surrounding myelin debris and also presenting myelin to macrophages (Stoll et al., 1989). Whilst resident macrophages proliferate and phagocytose myelin (Mueller et al., 2003), circulating macrophages are recruited (Perry et al., 1987), which is aided by the release of chemokines and cytokines (Shamash et al., 2002). Together, this cellular activity clears the debris resulting from the injury, which is thought to enable subsequent axonal regeneration to occur.

By contrast, clearance of debris in the CNS is less efficient, with this suggested to contribute to the reduced capability for regeneration following axonal injury in the CNS. Oligodendrocytes ensheath axons in myelin in the CNS, with their survival dependent upon contact-mediated signals (Barres et al., 1993). Following injury and the loss of these signals, oligodendrocytes have been observed to initiate apoptosis or to enter an atrophy-like resting state (Barres et al., 1993). Whilst the Schwann cells in the PNS proliferate in response to injury (Morris et al., 1999), the oligodendrocytes of the CNS do not, and additionally demonstrate little phagocytic activity (Vaughn & Pease, 1970). Furthermore, there is little evidence of recruitment of circulating macrophages to sites of CNS injury (Vargas & Barres, 2007), due to the blood brain barrier remaining largely intact post-injury. Whilst circulating macrophages are not recruited to the site of injury, the surrounding glial cells in the CNS are. Following injury, nearby microglia are activated, begin proliferating and migrate towards the area of degeneration, extending processes towards the dying cells (Jensen et al., 1994, Bechmann & Nitsch, 1997, Petersen & Daily, 2004). Additionally, astrocytes are also observed to be activated following injury, becoming hypertrophic, and both microglia and astrocytes have been observed to phagocytose debris (Jensen et al., 1994, Bechmann & Nitsch, 1997, Petersen & Daily, 2004).

The response to injury by the surrounding cells shares similarities between *Drosophila* and mammalian systems, which is perhaps unsurprising considering the similarities in the organisation of the *Drosophila* nervous system. As in vertebrates, *Drosophila* neurons are bundled into axonal tracts, wrapped in glia and surrounded by circulating immune cells in the hemolymph. Investigations of the antennal lobes in the *Drosophila* CNS have indicated that different types of glia exist in *Drosophila*: cortex, surface and neuropil glia, which are further divided into ensheathing and astrocytic glia (Doherty 2009). Astrocytic glia extend their processes deeply into the neuropil and to synaptic regions and display similar molecular markers to mammalian astrocytes. The ensheathing glia have been observed to extend their membranes to surround individual antennal lobe glomeruli, with this type of glia responsible for the engulfing of axonal debris following injury (Doherty 2009). In uninjured flies, the glia surround the borders of the antennal lobe however, following injury the glia increase their surface area, extend their membranes towards the degenerating axons and upregulate the expression of an engulfing receptor, called Draper (MacDonald et al., 2005). Like astrocytes in the mammalian CNS, *Drosophila* CNS glia do not proliferate in response to injury, but are recruited specifically to degenerating axons, and can engulf debris (MacDonald et al., 2005).

How do the glia and surrounding cells recognise that axons are degenerating? Are there signals released by degenerating axons as part of a programmed destruction? Or does phagocytosis simply occur in response to the presence of degenerating material and axonal debris?

1.2.3 Processes underlying Wallerian degeneration

Calcium (Ca^{2+}) plays a key role in the breakdown process of Wallerian degeneration, as demonstrated by a lack of axonal degeneration occurring in neurons cultured in Ca^{2+} free media, with the addition of Ca^{2+} triggering degeneration (Schlaepfer and Bunge, 1973). Following injury, Ca^{2+} influx occurs through L-type Ca^{2+} channels, resulting in the activation of calpains and other Ca^{2+}-activated proteases (George et al., 1995). AAD can be blocked using calpain inhibitors indicating a pivotal role for calpains at this early stage (Kerschensteiner et al., 2005).

Cytoskeletal breakdown occurs in Wallerian degeneration however different processes appear to control the breakdown of neurofilaments and microtubules. The breakdown of neurofilaments is caused by calcium influx and calpain activation, as Ca^{2+} chelation (EGTA)

and inhibition of calpain (calpeptin) prevent the breakdown of neurofilaments (Park et al., 2013b). However, the breakdown of microtubules is not caused by calcium, as Ca^{2+} free buffer, addition of EGTA or calpeptin do not prevent the breakdown of microtubules (Schlaepfer and Bunge, 1973, Park et al., 2013a)

Ca^{2+} influx after injury has wider reaching effects than just calpain activation, as the delay in axonal degeneration mediated by Ca^{2+} chelation is superior to that mediated by calpain inhibition (George et al., 1995, Zhai et al., 2003). Ca^{2+} influx also increases proteasome activity, as inhibition of the proteasome (MG132) can delay Wallerian degeneration, protecting neurofilaments from breaking down but having no effect upon microtubule breakdown (Park et al., 2013a).

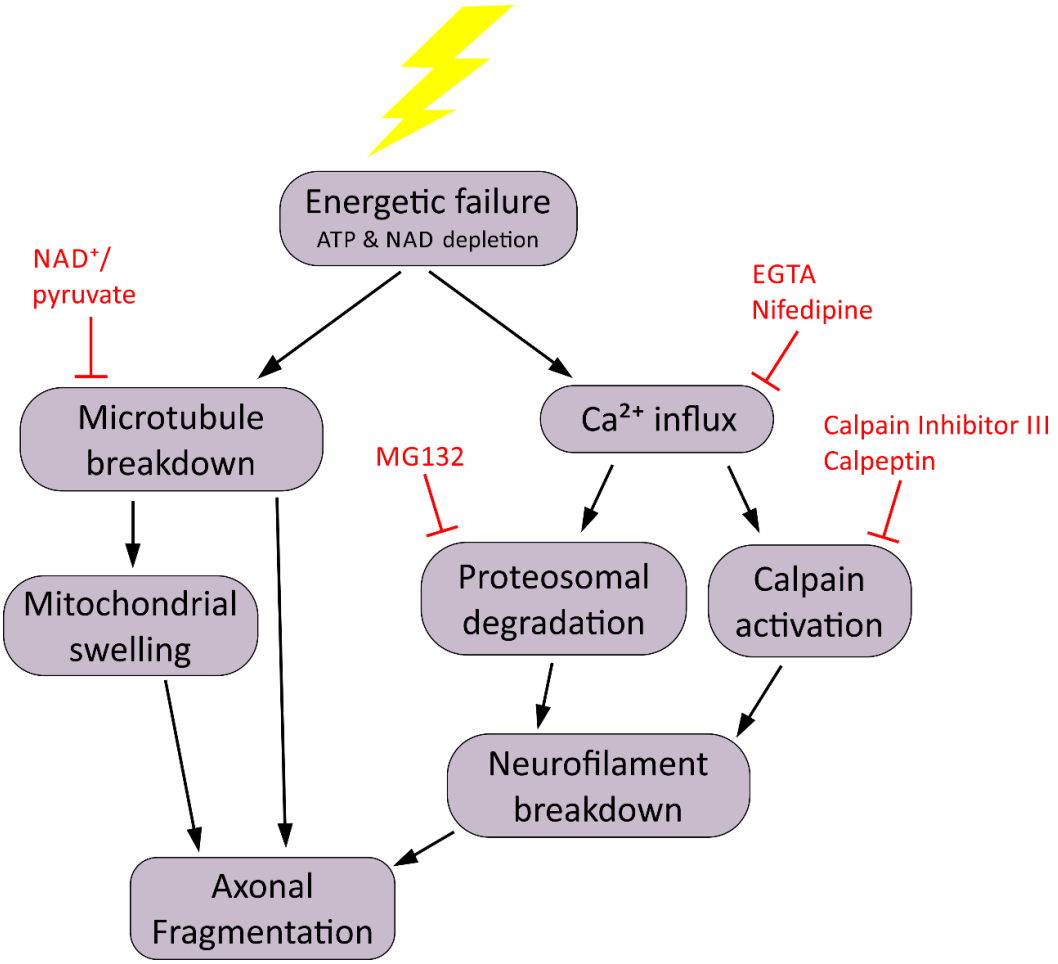


Figure 1-2. Sequence of events occurring in Wallerian degeneration. After injury, reductions in ATP and NAD^+ levels results in energetic failure, causing microtubule breakdown and leading to mitochondrial swelling. Moderate protection of microtubules and mitochondria occurs with supplementation of NAD^+/pyruvate. Waves of Ca^{2+} influx activate calpains and increase proteasome activity leading to neurofilament breakdown. This can be blocked by Ca^2 chelation (EGTA) or Ca^{2+} channel blockers (nifedipine) which prevent Ca^{2+} influx. Calpain inhibition has a moderate ability to delay degeneration, as does inhibition of the proteasome (MG132).

However, not all degenerative processes are mediated by calcium, as evidenced by the lack of protection of microtubules by the addition of EGTA or culturing in Ca^{2+} free buffer. Following injury, energy failure within the axon is observed, with a drop in NAD^+ and ATP levels (Wang et al., 2005, Park et al., 2013a). Ca^{2+} chelation does not prevent the drop in NAD^+ or ATP indicating this decline is independent of Ca^{2+} influx (Figure 1-2) (Park et al., 2013a). Energetic failure is thought to be responsible for the breakdown of microtubules, as supplementing cultures with NAD^+ and pyruvate after injury increased the number of microtubules present within axons (Park et al., 2013a). The breakdown of microtubules is thought to be responsible for mitochondrial swelling, as like microtubules, mitochondria are not protected by Ca^{2+} chelation or proteosome inhibition (Park et al., 2013a).

Wallerian degeneration shares some similarities with apoptosis, such as blebbing of the membrane and exposure of phosphotidylserine, as well as loss of mitochondrial membrane potential (Sievers et al., 2003). However the absence of caspase activation in Wallerian degeneration (Finn et al., 2000) combined with the lack of axon protection when proapoptotic proteins (Bax and Bak) were knocked out (Whitmore et al., 2003) suggests that different signals initiate death in the axon than in the cell body. Furthermore, whilst proteasome inhibition has been shown to delay axonal degeneration, is has no effect on apoptosis of the cell body (Zhai et al., 2003), indicating different programmes control death in different cellular compartments. Wallerian degeneration was considered a passive response to injury; the separated axon fragment wasted away as it was no longer receiving trophic support from the neuronal cell body (Lubinska, 1977). However this view was changed by the serendipitous discovery of the slow Wallerian degeneration (Wld^S) mutant mouse, which indicated that Wallerian degeneration was in fact an active process.

1.3 Slow Wallerian degeneration - Wld^S

Whilst investigating the role of macrophage recruitment in the breakdown process of Wallerian degeneration, a group in Oxford came across a mouse strain in which injured axons remained intact (Figure 1-3), with the distal axon stump capable of conducting evoked potentials for over 10 days after sciatic nerve axotomy (Lunn et al., 1989). Further investigation of this strain revealed the protective phenotype extended to the CNS (Perry et al., 1991) and was intrinsic to the axon (Perry et al., 1990, Glass et al., 1993).

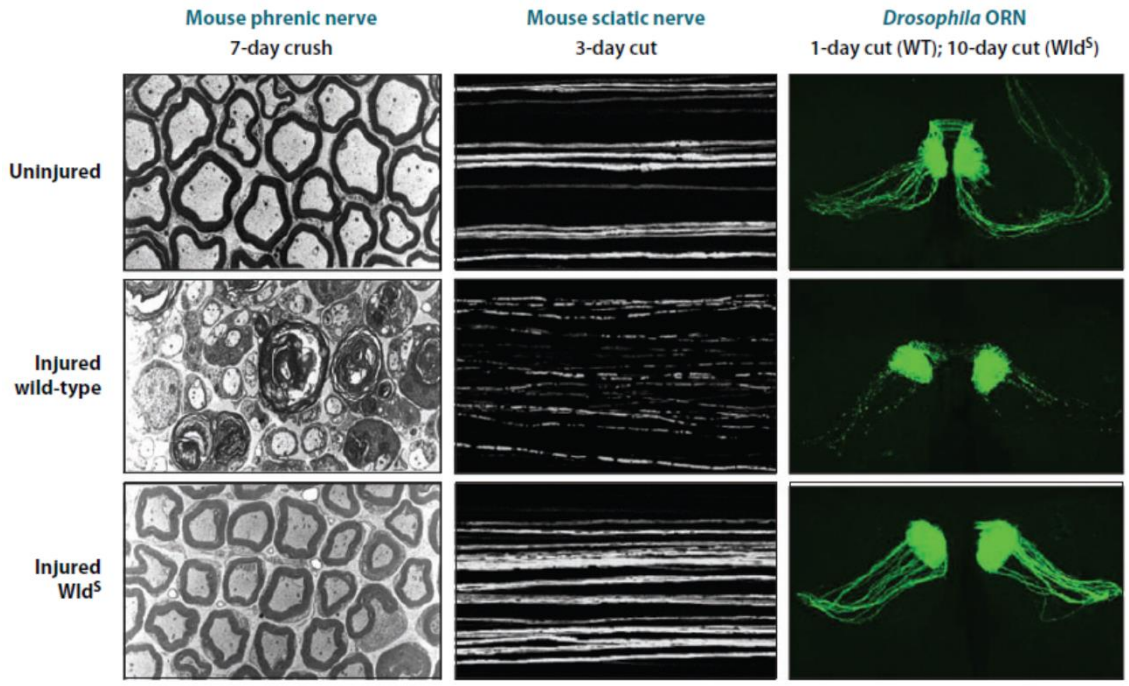


Figure 1-3. Wld^S protection of injured axons. Electron micrographs of transverse sections of injured mouse phrenic nerves indicate the structural preservation of the axon in Wld^S animals. Longitudinal images from injured mouse sciatic nerve display the lack of fragmentation in Wld^S animals. Images from Wld^S transgenic *Drosophila* indicate the robust preservation following injury and the conservation of the mechanisms underpinning Wallerian degeneration. Taken from (Coleman and Freeman, 2010)

The slow Wallerian degeneration (Wld^S) phenotype was found to be controlled by an autosomal dominant gene which mapped to mouse chromosome 4 (Perry et al., 1990, Lyon et al., 1993). It emerged that a spontaneous mutation had occurred in this mouse strain, resulting in an 85kb tandem triplication on mouse chromosome 4 (Coleman et al., 1998). This mutation results in the production of a chimeric fusion protein, Wld^S which consists of the full length of nicotinamide adenylyl transferase 1 (Nmnat1) and the N-terminal portion of Ube4b, linked together by an 18 amino acid sequence from the *Nmnat1* 5' untranslated region (Figure 1-4) (Conforti et al., 2000, Mack et al., 2001). It was confirmed as the genetic source of the protective phenotype by the generation of a transgenic mouse which fully recapitulated the phenotype (Mack et al., 2001). Axonal protection after injury was also observed in *Drosophila* transgenic for Wld^S, indicating that the mechanisms underpinning Wallerian degeneration were conserved between *Drosophila* and mammals (Figure 1-3) (Hoopfer et al., 2006, MacDonald et al., 2006).

The protection mediated by Wld^S is dose dependent, as demonstrated by the generation of transgenic lines in which the level of Wld^S protein expression was found to correlate with the level of axon protection (Mack et al., 2001). Wld^S provides a functional as well as a

morphological protection of the distal axon fragment, with Wld^S axons able to conduct evoked action potentials for days compared to hours usually (Tsao et al., 1994, Moldovan et al., 2009). The protection extends to synapses, and neurotransmitter release can be evoked up to five days after injury compared to 12–20 h in WT axons (Ribchester et al., 1995). However there appears to be additional complexity in the mechanisms controlling synaptic loss than axon loss. Whilst age does not alter the protective capacity of Wld^S in axons (Crawford et al., 1995), older mice show little protection of synapses in the PNS (Gillingwater et al., 2002) but robust protection of synapses in the CNS (Wright et al., 2010).

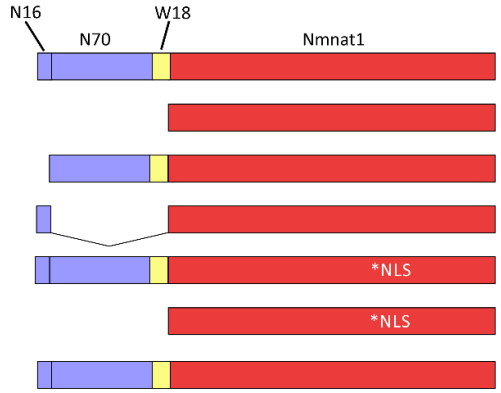
1.3.1 How and where does Wld^S protect?

How does the chimeric fusion protein Wld^S result in axon protection? And where in the neuron does it exert this effect? To answer this, researchers looked at the component parts of the Wld^S protein. Wld^S contains Nmnat1 which is the final enzyme in the NAD^+ salvage pathway (Figure 1-6), and Ube4b, which is part of the ubiquitin proteasome system. Only the 70 N-terminal amino acids of Ube4b (N70) are included in the Wld^S protein, with this region lacking the catalytic domain of the full protein. Therefore modulation of ubiquitination and proteasome function is an unlikely mediator of the Wld^S phenotype.

However, overexpression of Nmnat1 alone does not produce axonal protection comparable to Wld^S. In dorsal root ganglion (DRG) cultures, lentiviral mediated overexpression of Nmnat1 has been found to result in varying degrees of axonal protection after injury (Araki et al., 2004, Conforti et al., 2007, Sasaki et al., 2009b). However, in cultures from Nmnat1 transgenic mice no axonal protection was seen after injury, despite higher levels of Nmnat1 activity than in Wld^S mice (Conforti et al., 2007). Furthermore, overexpression of Nmnat1 showed no protection after sciatic nerve lesion, indicating that Nmnat1 cannot substitute for Wld^S (Conforti et al., 2007, Yahata et al., 2009). If Nmnat1 expression alone does not result in axonal protection, then the N70 domain in Wld^S must be augmenting the normal function of Nmnat1 to provide axonal protection.

One such possibility was that this region caused Wld^S (and therefore Nmnat1) activity to be redistributed outside the nucleus. Initially, studies indicated that Wld^S was predominantly found in the nucleus (Mack et al., 2001) however others reported Wld^S present in axons and associated with mitochondria (Wang et al., 2005, Beirowski et al., 2009, Yahata et al., 2009, Avery et al., 2012) (Figure 1-5). Mutations within the nuclear localising sequence (NLS) of

Wld^S resulted in higher levels of Wld^S being detected in neurites and an enhanced axonal protective phenotype, indicating that Wld^S functioned outside of the nucleus to provide protection (Beirowski et al., 2009).



	Level of Axonal Protection	
	Mouse	Drosophila
Wld^S	++++	++++
Nmnat1	-	++
Wld^S-ΔN16	-	++
N16::Nmnat1	n.d.	++++
Wld^S-ΔNLS	+++++	n.d.
cytNmnat1	++++	n.d.
Enzyme dead Wld^S	-	-/+

Figure 1-4. The Wld^S protein and its derivations. Wld^S consists of 70 amino acids from Ube4b (N70) linked to Nmnat1 by 18 amino acids from the 5' UTR of Nmnat1 (W18). In *Drosophila*, Nmnat1 alone provides low level protection compared to Wld^S, and removal of the N16 from Wld^S reduces protection to the level of Nmnat1 (Wld^S-ΔN16). Fusion of N16 to Nmnat1 results in protection comparable to Wld^S (N16::Nmnat1). In mice, comparable protection can be achieved with cytoplasmic Nmnat1 (cytNmnat1) and enhanced protection occurs when a mutated NLS is introduced to Wld^S. + indicates protection; - indicates lack of protection; n.d., indicates not determined *in vivo*. Adapted from Coleman & Freeman, 2010.

Whilst the N70 portion of Wld^S does not have ubiquitination activity, it may still associate with binding partners, which could redistribute Nmnat1 activity out of the nucleus. In a screen for Wld^S binding partners, it was found that Wld^S binds valosin containing protein (VCP), through the first 16 N-terminal residues (N16) (Laser et al., 2006). Further investigation in *Drosophila* revealed that deletion of the N16 reduced axonal protection to the level produced by Nmnat1 and that fusion of N16 to Nmnat1 resulted in protection indistinguishable to Wld^S (Avery et al., 2009). Similarly, deletion of the N16 in Wld^S mice also resulted in loss of axonal protection (Conforti et al., 2009) indicating that the N16 was an essential component in Wld^S, and that it likely functioned by redistributing Nmnat1 activity out of the nucleus (Figure 1-4). This idea was further supported by evidence from transgenic mice containing a mutated NLS in Nmnat1 (cytNmnat1), which resulted in higher levels of protection than Wld^S (Figure 1-4) (Sasaki et al., 2009a). In addition, mutations in the NLS of Nmnat1 combined with the addition of the axonal targeting sequence of amyloid precursor protein (APP) also resulted in higher levels of protection than Wld^S (Babetto et al.,

2010). Together this indicates that redistributing Nmnat1 activity out of the nucleus is crucial for axonal protection and supports a local mechanism of protection by Wld^S/Nmnat1.

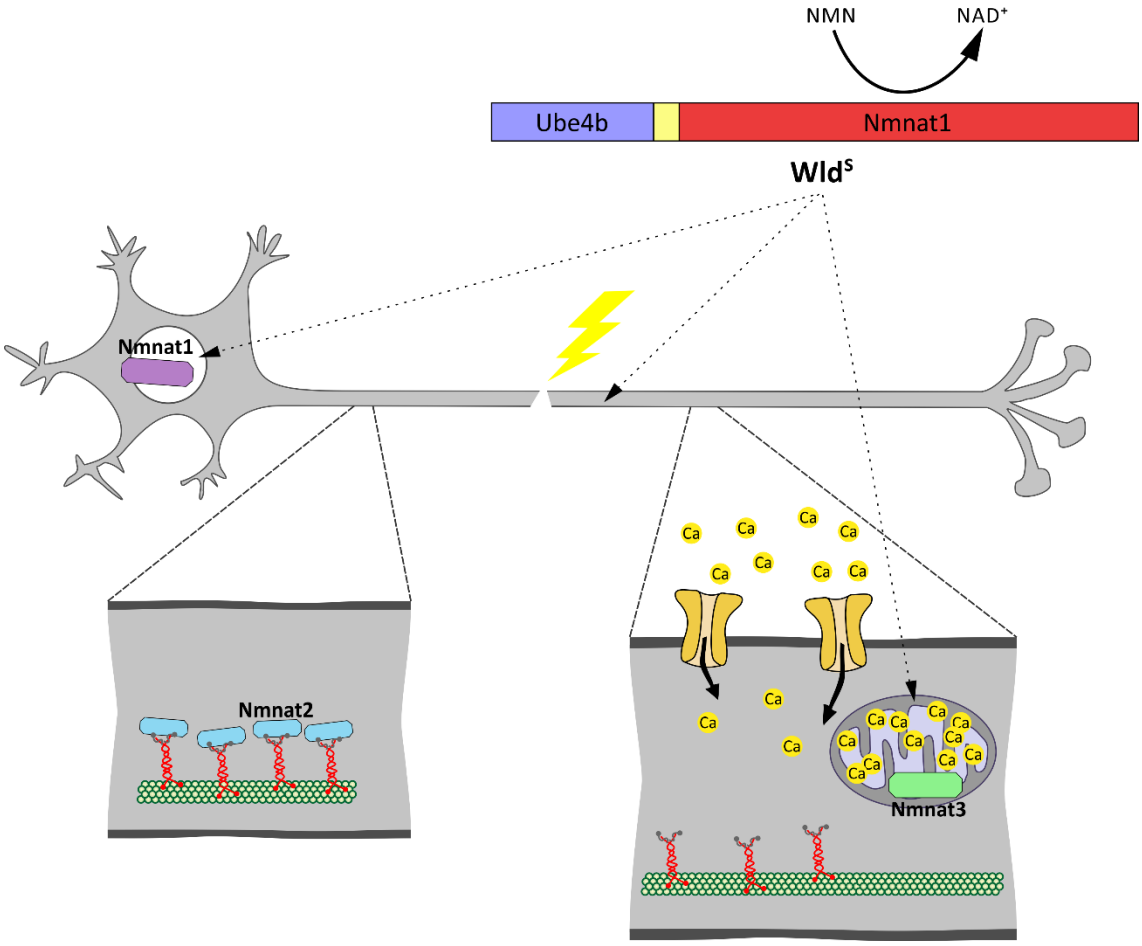


Figure 1-5. Mechanism of Wld^S protection. The mammalian Nmnat isoforms differ in their subcellular localisations; Nmnat1 in the nucleus, Nmnat2 in the cytoplasm and Nmnat3 in mitochondria. Wld^S has been detected in the nucleus, cytoplasm and mitochondria, and whilst the precise location of activity is still under debate, the presence of Wld^S within the axon is required for protection. Protection is thought to be mediated through compensating for the loss of Nmnat2 after injury, maintaining NAD^+ levels/preventing NMN build-up and by enhancing mitochondrial Ca^{2+} buffering capacity.

Nmnat1 is one of three mammalian isoforms, with each differing in their cellular localisation: Nmnat1 in the nucleus, Nmnat2 in the Golgi complex and Nmnat3 in mitochondria (Figure 1-5) (Berger et al., 2005, Lau et al., 2010). Whilst overexpression of Nmnat1 and Nmnat2 result in axonal protection lesser than Wld^S, overexpression of Nmnat3 results in axonal protection comparable to Wld^S *in vivo* (Avery et al., 2009, Yahata et al., 2009, Fang et al., 2012), leading many to suggest that Wld^S could be localised to mitochondria where it boosts Nmnat3 activity. This is supported by a number of studies which demonstrated Wld^S to be enriched within mitochondria (Beirowski et al., 2009, Yahata et al., 2009, Babetto et al.,

2010, Avery et al., 2012). However, mitochondria have been shown to be non-essential for Wld^S-mediated axonal protection, by experiments which created mitochondria free axons through disruptions of the mitochondrial adaptor proteins Milton or Miro. Knock down of Milton results in the loss of mitochondria from axons, but does not reduce Wld^S-mediated protection up to 24h post crush injury (Kitay et al., 2013). Whilst mutant Miro resulted in a reduction in Wld^S mediated protection after axotomy, the level of axonal preservation observed was far superior compared to injured WT axons (Avery et al., 2012), indicating that mitochondria are not essential for Wld^S mediated protection.

Whilst the precise cellular location where Wld^S mediates its protection remains unclear, the consensus is that it protects within the axon through a local mechanism. No causative link has been shown between gene expression or proteomic changes and Wld^S mediated protection, furthermore the cytoplasmic site of action does not support a protective mechanism by these means. So what is Wld^S doing within the axon to provide axonal protection?

NAD^+ and axonal degeneration

Nmnat is the final enzyme in the NAD salvage pathway, and Wld^S mice have 4-fold higher Nmnat1 activity in their brains than controls, but no corresponding alteration in NAD^+ levels (Mack et al., 2001). However after injury, Wld^S DRG cultures do not display the rapid decline in NAD^+ levels that occurs in wild type cultures following transection (Wang et al., 2005). Therefore whilst Wld^S does not increase steady state NAD^+ levels, it may delay the onset of degenerative processes by maintaining NAD^+ levels after injury. Further supporting this, Nmnat2 has the shortest half-life of all Nmnat isoforms, with levels dropping after injury in both WT and Wld^S superior cervical ganglion (SCG) cultures (Gilley and Coleman, 2010). Therefore, Wld^S could be protective by compensating for the loss of Nmnat2 activity within the axon after injury.

The role of NAD^+ in axon protection is contentious. Exogenous application of NAD^+ (0.1-1mM) to DRG cultures prior to transection was found to delay axonal degeneration, but application at the time of transection did not (Araki et al., 2004). In contrasting results, application of higher doses of NAD^+ (1-20mM) at the time of transection and up to 5 hours after delayed axonal degeneration (Wang et al., 2005). However, others have found no protective effect of exogenous NAD^+ application (1mM) upon axonal degeneration in DRG cultures after transection (Conforti et al., 2007). Furthermore, increasing NAD^+ levels

through knocking out NAD^+ consumers, PARP1 and CD38, had no protection after injury in both *in vitro* DRG cultures and *in vivo* sciatic nerve lesions, despite 2-fold higher NAD^+ levels (Sasaki et al., 2009b).

If higher NAD^+ levels do not correlate with axonal protection, then is the enzymatic activity of Nmnat1 in Wld^S required for protection? This has been investigated using enzyme dead versions of Wld^S, through the introduction of a point mutation which reduces NAD^+ biosynthesis (Figure 1-4). *In vitro,* enzyme dead Wld^S has been found to have significantly reduced or even no axonal protection (Araki et al., 2004, Conforti et al., 2007), and no protection *in vivo*, in both *Drosophila* and mice (Avery et al., 2009, Conforti et al., 2009). This indicates that the enzymatic activity of Nmnat1 in Wld^S is required for axonal protection however it does not correlate with NAD^+ levels. Further support for this comes from work in which cytNmnat1 neurons were treated with FK866, which inhibits Nampt (Figure 1-6) resulting in a reduction in NAD^+ levels. Despite this reduction, strong axonal protection after injury was observed, indicating a disconnect between NAD^+ levels and Nmnat-mediated axonal protection (Sasaki et al., 2009b).

A potential explanation for this disconnect is that Nmnat1 in Wld^S functions to remove a toxic metabolite. In the NAD salvage pathway in vertebrates, Nmnat1 converts NMN to NAD^+ (Figure 1-6) and evidence shows that levels of NMN increase *in vivo* after sciatic nerve lesion in WT axons, but not in Wld^S axons. Additionally, inhibiting Nampt using FK866 *in vitro* resulted in axonal protection after injury, which was reversed by supplementation with NMN (Di Stefano et al., 2015). This would suggest that preventing the rise in NMN after injury, either by removing it using Wld^S or inhibiting its production using FK866, is responsible for axon protection. However, the overall story for NMN remains murky, as some have reported that FK866 reversed Wld^S-mediated protection (Conforti et al., 2009), whilst others have seen no effect of FK866 on cytNmnat1-mediated protection (Sasaki et al., 2009a). Also, the length of protection afforded by FK866 inhibition of Nampt was lesser than Wld^S indicating that Wld^S may be having multiple effects within the axon.

What is clear from work to date is that for the full Wld^S protective phenotype, the biosynthetic activity of Nmnat1 and its localisation within the axon are essential. What Wld^S does within the axon to bring around axonal protection remains under investigation and debate, but it is likely to be affecting multiple pathways and processes.

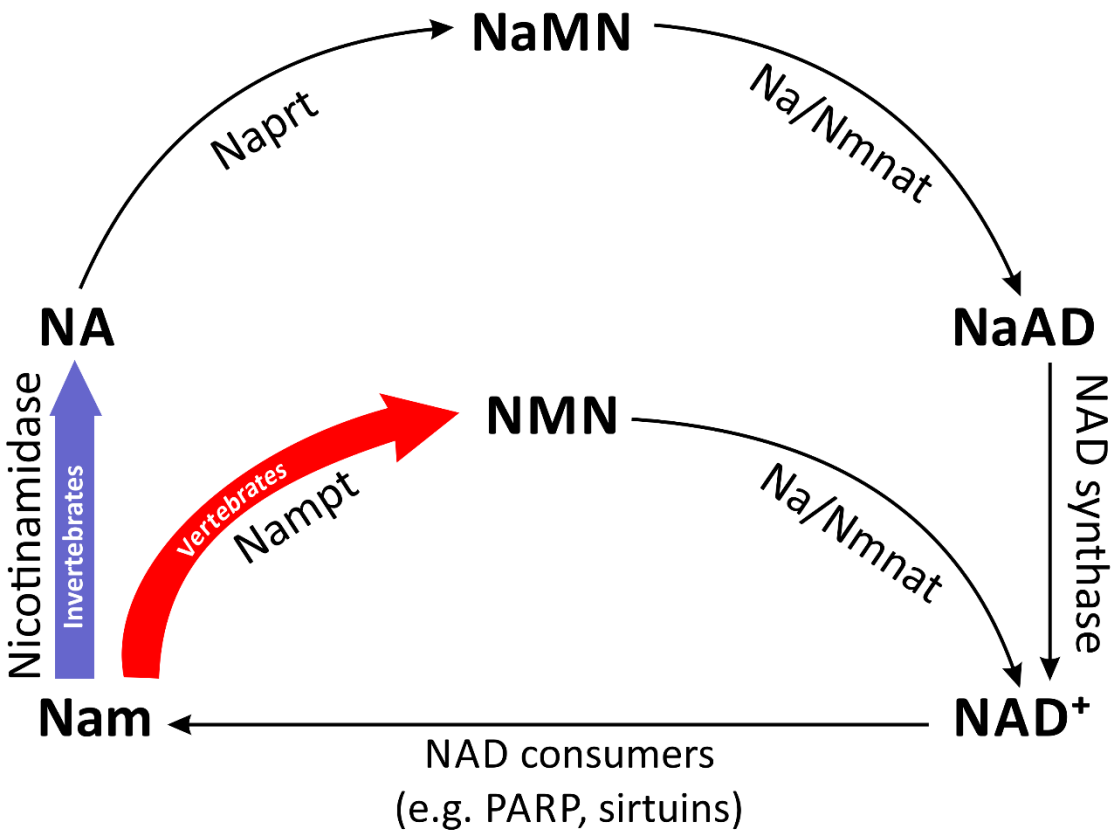


Figure 1-6. NAD salvage pathway. In vertebrates, nicotinamide (Nam) is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (Nampt) before the final conversion to nicotinaminde adenine dinucleotide (NAD^+) by nicotinic acid/nicotinaminde mononucleotide adenylyl transferase (Na/Nmnat). In invertebrates, Nam is converted to nicotinic acid (NA) by nicotinamidase (D-NAAM in *Drosophila*) then to nicotinic acid mononucleotide (NaMN) by nicotinic acid phosphoribosyl transferase (Naprt). NaMN is then converted to nicotinic acid dinucleotide (NaAD) by Na/Nmnat prior to conversion to NAD^+ by NAD synthase. NAD^+ is used as a co-enzyme in a number of reactions such as redox reactions. NAD^+ has a wide range of roles, from being consumed by sirtuins and DNA ligases to being used as a precursor of cyclic ADP-ribose.

Loss of an essential survival factor?

It has been suggested that Wld^S compensates for the loss of an essential survival factor, thereby delaying axonal degeneration. Investigation of Nmnat isoforms revealed that Nmnat2 has the shortest half-life of around 4 hours, whilst Nmnat1, Nmnat3 and Wld^S show little turnover in 72 hours (Gilley and Coleman, 2010). In addition, Nmnat2 levels drop rapidly after injury, suggesting that axonal injury disrupts its transport into the axon (Figure 1-5) and that its levels dropping below a threshold is a trigger for degeneration (Gilley and Coleman, 2010).

Nmnat2 has also been implicated in axon development, as Nmnat2 knockout mice display defects in axon extension in both the peripheral and central nervous system (Gilley et al., 2013). These defects can be rescued using Wld^S, providing further support to the idea that Wld^S compensates for loss of Nmnat2 (Gilley et al., 2013).

Further support for Nmnat having a role as an essential survival factor comes from work in *Drosophila*. In *Drosophila* there is a single isoform of Nmnat (dNmnat), and when this is lost spontaneous degeneration occurs (Zhai et al., 2006, Fang et al., 2012). This degeneration can be rescued by the expression of dNmnat and also the 3 murine Nmnat isoforms (Fang et al., 2012). Furthermore, examination of development in flies expressing loss-of-function mutant dNmnat in their eyes revealed no alterations of eye morphology and normal synapse formation, with the degenerative phenotype manifesting progressively in adult flies (Zhai et al., 2006). This indicates that in *Drosophila*, dNmnat is not required for neuronal development and differentiation, but is required for the maintenance of mature neurons.

Changes to mitochondrial function?

The association between Wld^S and mitochondria has led many to suggest that mitochondria are a key site for Wld^S-mediated protection (Beirowski et al., 2009, Yahata et al., 2009, Babetto et al., 2010, Avery et al., 2012). In addition, energetic failure during Wallerian degeneration is a key event; therefore Wld^S may be protective by preventing this. Indeed, ATP depletion does not occur in Wld^S neurons (Yang et al., 2015) and mitochondria from Wld^S neurons have increased ATP synthesis rates (Yahata et al., 2009) and maintain ATP synthesis after injury (Godzik and Coleman, 2015). Wld^S may function to delay the opening of the mitochondrial permeability transition pore (mPTP), a catastrophic event triggering downstream processes which result in degeneration. Cyclosporin A (CsA) binds to a subunit of the mPTP and prevents it opening, and CsA treatment of WT neurons results in axonal protection after injury comparable to Wld^S. Furthermore, treatment of Wld^S neurons with CsA does not further delay axonal degeneration, suggesting that both CsA and Wld^S act upon the mPTP (Barrientos et al., 2011). In addition, treatment of Wld^S cultures with atractyloside, which triggers opening of the mPTP, resulted in axonal degeneration comparable to WT (Barrientos et al., 2011). Together, this indicates that Wld^S provides protection by somehow delaying the opening of the mPTP. As opening of the mPTP is correlated with decreased ATP synthase activity (Alavian et al., 2014), could the continued supply of NAD^+ in the axon through Wld^S drive the continued synthesis of ATP and prevent opening of the mPTP?

Another suggestion is that Wld^S alters Ca^{2+} buffering within the axon (Figure 1-5). Whilst some groups have reported that Wld^S mitochondria can withstand higher levels of Ca^{2+} before losing their membrane potential (Avery et al., 2012, Shen et al., 2013), others have not found this to be the case (Barrientos et al., 2011). Following axotomy, Ca^{2+} levels rise rapidly however in Wld^S axons there is only a small transient rise in intracellular Ca^{2+} initially (Avery et al., 2012), and no later rise in intracellular Ca^{2+} (Adalbert et al., 2012). Increased intracellular Ca^{2+} is thought to be responsible for halting mitochondrial motility after injury, as the mitochondrial adaptor proteins Miro and Milton are Ca^{2+} sensitive. However in injured Wld^S axons, mitochondrial motility persists (Avery et al., 2012, O'Donnell et al., 2013), further supporting lower intracellular Ca^{2+} levels in Wld^S axons immediately following injury. It has been suggested that the maintenance of mitochondrial motility is key to Wld^S-mediated protection, however knocking down *pink1* also results in increased mitochondrial motility after injury but with no axonal protective effect (O'Donnell et al., 2013). Furthermore, Wld^S protection is maintained in axons devoid of mitochondria (Kitay et al., 2013), albeit at a lower level (Avery et al., 2012), indicating that mitochondria are not essential for Wld^S-mediated protection, but may be one of many mechanisms affected by Wld^S.

Chaperone activity?

Some groups have suggested that Nmnat and Wld^S are protective through their chaperone activity. This idea came from investigations of endogenous *Drosophila* Nmnat (dNmnat). Whilst enzyme dead versions of Wld^S and Nmnat1 are not protective in mammalian systems, enzyme dead dNmnat was just as protective as active dNmnat in *Drosophila* (Zhai et al., 2006). Additionally, enzyme dead dNmnat was shown to have chaperone activity indicating that this function was independent of NAD^+ synthesis (Zhai et al., 2008). However, whilst enzyme dead Wld^S has similar levels of chaperone activity to dNmnat, it does not delay axonal degeneration in mice *in vivo* indicating that chaperone activity of Wld^S does not contribute to the axonal protective phenotype (Conforti et al., 2007).

1.4 Other molecular mediators of Wallerian degeneration

The discovery of Wld^S indicated that there was an active programme of events which occurred in an axon post-injury, and paved the way for investigation of the endogenous mediators of this process. Since then a number of molecular mediators of Wallerian degeneration have been described.

1.4.1 PHR1/hiw and DLK/Wnd

The E3 ubiquitin ligase PHR1 (PAM-Highwire-Rpm-1) plays a conserved role in axonal and synaptic development (Schaefer et al., 2000, Wan et al., 2000, Bloom et al., 2007) and was more recently described to accelerate regenerative responses following injury (Xiong et al., 2010), therefore representing an ideal candidate for regulating injury responses. Null mutants of *Highwire* (*hiw*), the *Drosophila* homolog of PHR1, were found to delay axonal degeneration up to 20 days post-axotomy (Xiong et al., 2012). They also reported that levels of Wallenda (Wnd), a mitogen-activated protein kinase kinase kinase (MAP3K; also known as dual leucine kinase [DLK]), were increased in *hiw* null animals (Xiong et al., 2012), contrasting with previous reports in which loss of DLK/Wnd provided axon protection (Miller et al., 2009). DLK/Wnd can activate c-Jun N-terminal kinase (JNK), with this thought to be a mediator of axonal degeneration, as JNK inhibitors are capable of delaying axonal degeneration (Miller et al., 2009). However this is not the only downstream process that PHR1/hiw affects to bring about axonal degeneration. PHR1/hiw has been found to affect levels of Nmnat, with loss of PHR1/hiw resulting in increased levels of Nmnat2/dNmnat (Xiong et al., 2012, Babetto et al., 2013). Knockdown of Nmnat2/dNmnat abolishes PHR1/hiw-mediated axonal protection post injury (Xiong et al., 2012, Babetto et al., 2013) by a pathway independent of Wnd, as knockdown of Wnd did not reduce the protection mediated by dNmnat overexpression (Xiong et al., 2012). Furthermore, the modulation by PHR1/hiw is specific for Nmnat2, as in mice only levels of Nmnat2 increase when PHR1 is knocked out (Babetto et al., 2013), and in *Drosophila*, loss of hiw resulted in an increase in murine Nmnat2 and Nmnat1 or Nmnat3 when each isoform was co-expressed (Xiong et al., 2012).

Together, this work adds weight to the hypothesis which states that the loss of an essential survival factor is a trigger for axonal degeneration, as the evidence shows PHR1/hiw specifically target Nmnat2/dNmnat for degradation after injury (Figure 1-7). However, this could still be viewed as a passive mechanism, as it is not known whether the activity of PHR1/hiw is upregulated after injury, or whether it continues as normal and it is the loss of continued supply of Nmnat2/dNmnat from the cell body combined with this that results in levels falling below a threshold and triggering degeneration. Furthermore, the protection mediated by loss of PHR1/hiw is modest in comparison to Wld^S, indicating that this is not the only injury response pathway.

1.4.2 GSK3 and IKK

Other mediators of the injury response were identified using an image based screening assay on DRG neurons treated with a large library of compounds. Inhibitors of glycogen synthase kinase 3 (GSK3) and IκB kinase (IKK) were found to reduce axonal degeneration after transection, however to a lower degree than proteasome inhibition achieves (Gerdts et al., 2011). It was found that the kinases play an early role in the response to injury, as addition of the inhibitors was protective if added at the time of transection, but not when added 2h post injury (Gerdts et al., 2011). Whilst protection of neurofilaments was observed, no effect on microtubule breakdown was seen and, in addition to the lower protection mediated than that by Wld^S (Gerdts et al., 2011), indicates that these stress kinases are just one component of the response to injury.

1.4.3 Sarm

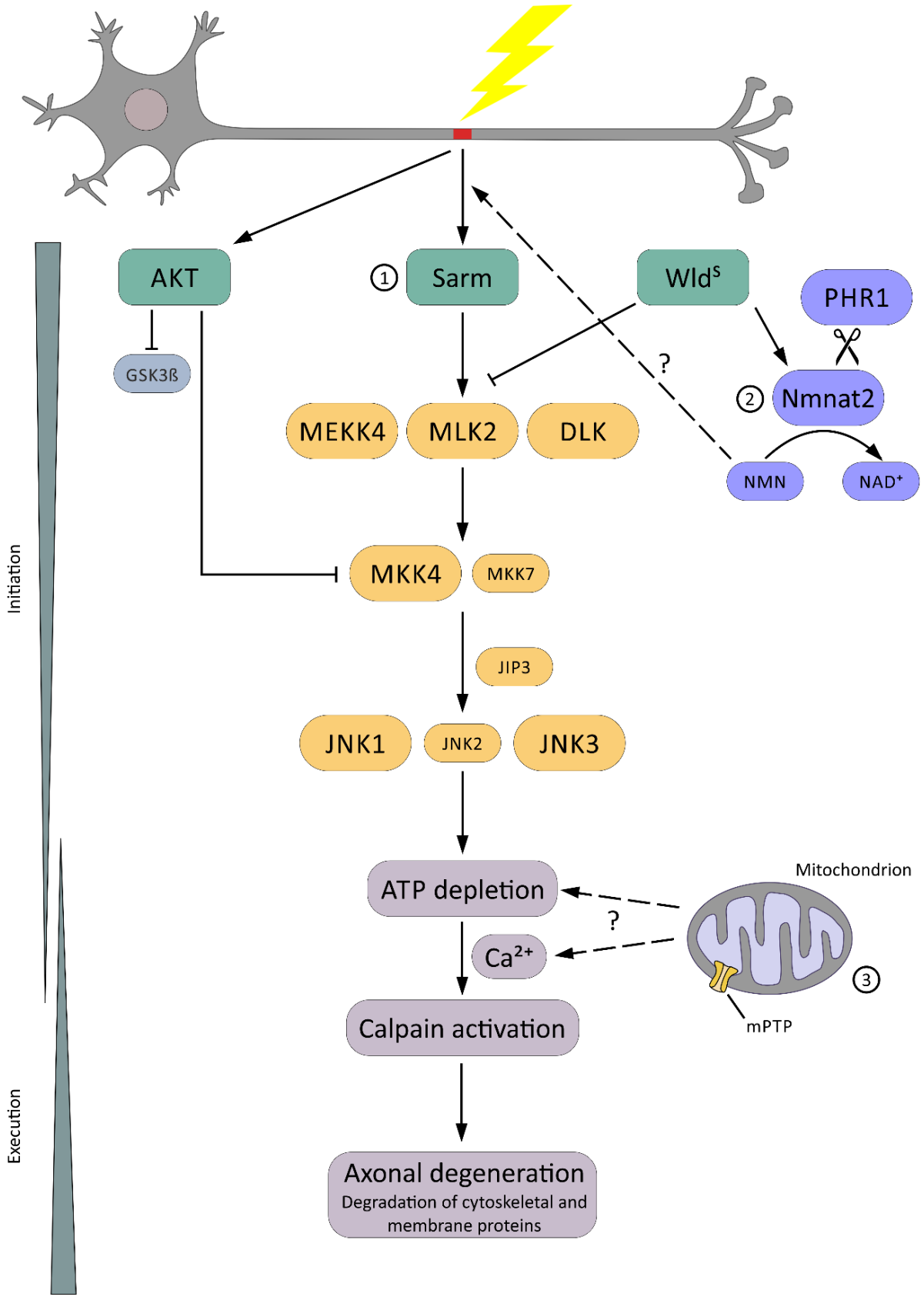
Sarm was the first endogenous molecular mediator found which when lost provided protection on a par with Wld^S, and was a crucial discovery in the understanding of Wallerian degeneration as an active process. A forward genetic screen using clonal mosaic analysis (MARCM) in *Drosophila* enabled the characterisation of both viable and lethal mutants (Figure 1-13), from which three mutant alleles were found to delay axonal degeneration up to 50 days post-axotomy, comparable to Wld^S (Osterloh et al., 2012). All three mutant alleles were found to affect a single gene, dSarm (*Drosophila* sterile alpha and Armadillo motif), which is evolutionarily conserved with homologs in *C. elegans* (TIR1) to mice (Sarm1) (Mink et al., 2001, Liberati et al., 2004, Meijer et al., 2004, Kim et al., 2007). The axonal protective phenotype was also found to be conserved, with knockout of the mouse homolog, Sarm1, demonstrated to robustly delay axonal degeneration comparable to Wld^S both *in vitro* and *in vivo* (Osterloh et al., 2012).

The *Sarm* gene encodes a protein containing an Armadillo/HEAT (ARM) domain, two sterile alpha motifs (SAM) and a Toll/Interleukin-1 Receptor homology (TIR) domain, and is preferentially expressed in neurons (Kim et al., 2007). As a Toll-like receptor adaptor protein, Sarm functions in innate immune responses and when knocked out in neurons provides protection against a range of bacterial and viral infections (Hou et al., 2013, Mukherjee and Chakrabarti, 2013). The protein contains a mitochondrial targeting sequence (MTS) but this is not thought to be essential for its axonal protection, as expression of Sarm1

lacking the MTS restored injury induced degeneration in Sarm1$^{-/-}$ neurons (Gerdts et al., 2013). Overexpression of Sarm1 does not result in spontaneous axonal degeneration, indicating it requires an injury signal to trigger downstream degenerative processes (Gerdts et al., 2013). A constitutively active version of Sarm1 (SAM-TIR) results in spontaneous degeneration which cannot be blocked by apoptotic inhibitors but can be significantly reduced by Ca^{2+} chelation (Gerdts et al., 2013), further supporting that this pathway is distinct from apoptosis in controlling axonal degeneration.

Sarm functions at the time of injury, as if it is removed up to two hours after the injury, axonal protection still occurs but after this time loss of Sarm has no effect. It also has a local mechanism of action, as in microfluidic devices in which proximal and distal axons can be manipulated separately, activation of Sarm1 in distal axons did not cause degeneration in proximal axons (Gerdts et al., 2015). Evidence indicates that Sarm is a key upstream mediator of axonal degeneration, signalling through a MAPK pathway to bring around axon destruction (Figure 1-7). Knockdown of key components in this pathway, such as DLK which has been previously implicated in the response to axonal injury, resulted in axon protection with the activation of pathway mediators, such as the MAPK kinase MKK4, being blocked in Sarm1$^{-/-}$ neurons (Figure 1-7) (Yang et al., 2015). ATP depletion after injury was found to be downstream of the Sarm1-MAPK pathway, as knockdown of components at each stage prevented the loss of ATP similar to loss of Sarm1 but upstream of calpain activation, which was blocked by loss of Sarm1 or JNK inhibition (Yang et al., 2015). Whilst the use of Ca^{2+} chelators and calpain inhibitors delayed the morphological breakdown of axons, they did not prevent ATP depletion, indicating that Sarm signals through the MAPK cascade to trigger a local energy deficit and the activation of calpains leading to the morphological breakdown of the axon (Yang et al., 2015).

Figure 1-7 (next page). The axon death pathway. [1] Acute axonal injury initiates a cascade of events mediated through Sarm and MAPK signalling. This pathway results in ATP depletion and then calpain activation through an increase in intracellular Ca^{2+}, leading ultimately to axonal degeneration through degradation of cytoskeletal and membrane proteins. [2] The modulation of Nmnat2 levels post injury has also been implicated, with the reduction in Nmnat2 by PHR1 leading to a rise in NMN. The build-up of NMN leads to axonal degeneration, with recent evidence putting the increase in NMN levels upstream of Sarm. [3] Mitochondria are implicated in both the initiation and execution of Wallerian degeneration. It is unclear whether Sarm localises to mitochondria and whether this localisation is required for its pro-degenerative function. The mPTP is likely to function in the execution of degeneration, and mitochondria are a potential source of Ca^{2+} release into the cytosol.


AKT
1
Sarm
Wld^S
PHR1
GSK3ß
?
2
Nmnat2
MEKK4
MLK2
DLK
NMN
NAD$^+$
Initiation
MKK4
MKK7
JIP3
JNK1
JNK2
JNK3
ATP depletion
Mitochondrion
?
Ca^{2+}
3
Calpain activation
mPTP
Execution
Axonal degeneration
Degradation of cytoskeletal and membrane proteins

Interestingly cytNmnat1, which phenocopies Wld^S, also prevented the activation of the MAPK pathway and subsequent ATP depletion, calpain activation and morphological breakdown of axons (Yang et al., 2015). This indicates that whilst Wld^S/cytNmnat1 is a gain of function mechanism, its protection appears to be mediated through inhibition of the endogenous 'axon death' pathway (Figure 1-7). Whilst the role of NAD^+ in Wld^S mediated protection remains contentious, recent evidence indicates that maintaining NAD^+ levels after injury is essential to $Sarm1^{-/-}$ protection, as depleting NAD^+ in injured $Sarm1^{-/-}$ axons reverts the protective phenotype and causes degeneration to occur (Gerdts et al., 2015).

Like expression of Wld^S, loss of Sarm has been found to provide resistance to many forms of injury other than physical transection, such as mitochondrial poisons (Summers and Diantonio, 2014, Yang et al., 2015), chemotherapy (Yang et al., 2015) energy deprivation (Kim et al., 2007) and excitotoxcitiy (Massoll et al., 2013). However, whether the protective effects of Wld^S expression or loss of Sarm extend to chronic neurodegenerative conditions remains to be seen. Axonal degeneration has been implicated in a number of neurodegenerative diseases and shares many similarities with axonal degeneration occurring after injury. Understanding the molecular processes underpinning axon loss in disease could be important in the search for therapeutics, and the increasing knowledge of the axon death pathway in injury provides the tools with which to investigate the similarities and differences between injury and disease.

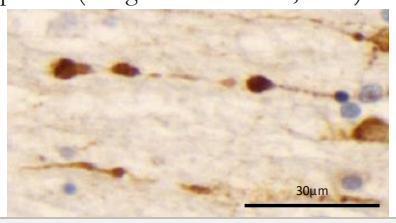
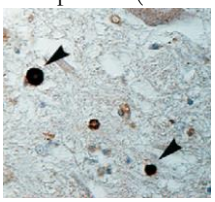
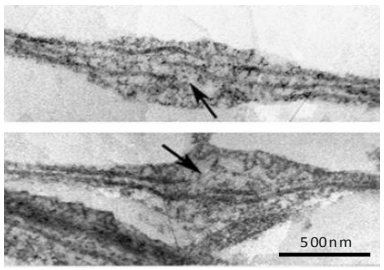
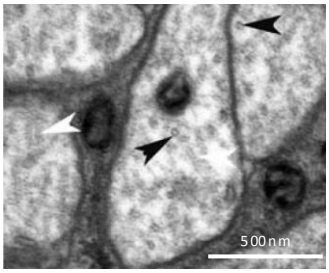
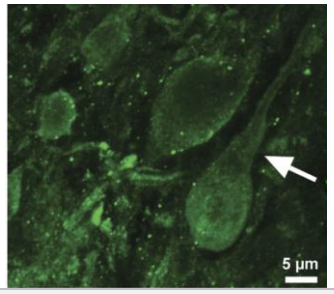
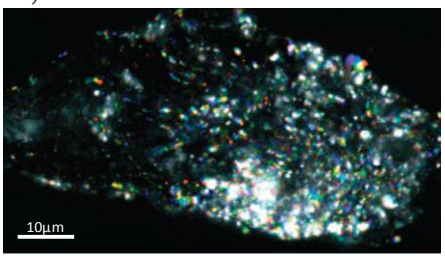
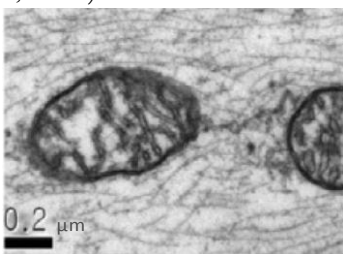
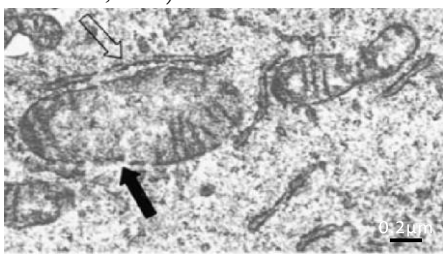
1.5 Axonal degeneration in neurodegenerative disease

In the first description of Wallerian degeneration, Augustus Waller noted:

> *"It is impossible not to anticipate important results from the application of this inquiry to the different nerves of the animal system. But it is particularly with reference to nervous diseases that it will be most desirable to extend these researches." (Waller, 1850)*

The idea that axonal degeneration shares similarities whether it is caused by injury or disease has permeated the world of degenerative research ever since. Morphological similarities between axons that have been injured and those in disease had added weight to this idea, with axonal degeneration in disease often referred to as 'Wallerian-like' degeneration.

Table 1-1. Axonal degeneration is morphologically similar in injury and disease.

Injury	Disease
Swellings	
APP positive axonal swellings from a young TBI (18M) patient (Tang-Schomer et al., 2012) 	APP postitive axonal swellings from the spinal cord of an early onset AD patient (Wirths et al., 2007) 
Cytoskeletal breakdown	
Broken microtubules following stretch injury *in vitro* (Tang-Schomer et al., 2012) 	Reduced microtubule density in axons from *Drosophila* expressing human tau (Cowan et al., 2010) 
Axonal transport disruption	
Swellings containing the fast axonal transport marker APP (Beirowski et al., 2010) 	Stationary vesicles appear white in swellings from *Aplysia* cultures expressing human tau (Shemesh et al., 2008) 
Mitochondrial morphology	
Swollen mitochondria following sciatic nerve lesion (Park et al., 2013a) 	Swollen mitochondria from AD cybrid cells (Trimmer et al., 2000) 
White matter changes	
Decreased fractional anisotropy observed by DTI in the white matter of mild TBI patients compared to control (Bazarian et al., 2007)	Decreased fractional anisotropy detected in the white matter of mild cognitive impairment and AD patients compared to controls (Huang et al., 2007)

In the CNS there are some clear morphological similarities. In human cases of traumatic brain injury (TBI), a common feature is diffuse axonal injury (DAI) leading to axonal degeneration. In DAI, axonal transport is disrupted causing the appearance of multiple swellings along the length of the axon, after which the axon degenerates by Wallerian degeneration (Johnson et al., 2013). Animal models of CNS injury recapitulate key features of human TBI, such as the appearance of axonal swellings prior to axonal degeneration (Beirowski et al., 2010). Furthermore, axonal swellings are observed in a number of models of neurodegenerative disease, such as Alzheimer's (Table 1-1) (Lin et al., 2003, Tsai et al., 2004, Wirths et al., 2007).

As discussed previously, cytoskeletal breakdown is a key event in Wallerian degeneration, and in DAI the breakage of microtubules is thought to contribute to the formation of axonal swellings due to the disruption of transport at the breakpoint and the accumulation of cargo (Tang-Schomer et al., 2012). Cytoskeletal breakdown is implicated in neurodegenerative diseases such as AD, as changes to the microtubule associated protein tau result in destabilised microtubules and transport impairment (Cowan et al., 2010, Quraishe et al., 2013).

Diffusion tensor imaging (DTI) enables the investigation of microstructural changes in the brains of patients, with changes in the underlying brain structure altering the diffusion of water through the brain. Through these techniques, further similarities have been drawn between degenerative changes occurring in injury and disease. Changes in white matter in both TBI (Bazarian et al., 2007) and Alzheimer's (Huang et al., 2007) are thought to represent underlying axonal pathology, with DTI sensitive enough to pick up white matter changes in minor cases of TBI (Mayer et al., 2010) and mild cognitive impairment (Huang et al., 2007).

Axon loss is a prominent feature in degenerative diseases of the peripheral nervous system, such as motor neuron disease (MND). In MND axonal degeneration has been traditionally viewed as a 'dying back' mechanism due to denervation at the synapse preceding a retrograde spread along the axon. Some argue that this makes dying back distinct from Wallerian degeneration, however synaptic denervation occurs early after axotomy and despite the axon degenerating anterogradely from the cut site, the direction of degeneration is now thought to be inconsequential to the underlying molecular process.

The morphological similarities between degenerating axons in injury and disease could imply that similar molecular mechanisms control the breakdown in both. By understanding the

mechanisms by which axons become dysfunctional and degenerate in disease it could be possible to slow down degeneration which would be of great benefit to patients. Many people have investigated the protective capacity of Wld^S in a range of disease models, and although this has had varying success, it has been an important strategy in understanding axonal degeneration in disease.

1.5.1 Wld^S and neurodegenerative disease

One of the first investigations of Wld^S in disease was in a mouse model of MND, the *pmn* mouse in which a mutation in a tubulin chaperone results in progressive motorneuron neuropathy. When the *pmn* mouse was crossed with Wld^S, disease progression was delayed by a week as measured by motor performance and survival (Ferri et al., 2003). This coincided with a higher number of axons in *pmn;Wld*^{S} mice compared to *pmn*, as well as improvements in retrograde axonal transport. Analysis of neuromuscular junctions (NMJs) revealed that synaptic pathology was twice as great in *pmn* mice compared to *pmn;Wld*^{S} mice, indicating that Wld^S had rescued both axonal and synaptic pathology in this model (Ferri et al., 2003).

Further success has been shown in models of demyelinating diseases, such as $P0^{-/-}$ mice which are deficient in P0, an essential myelin component in the peripheral nervous system. This model recapitulates a number of inherited peripheral neuropathies in humans which are characterised by defective myelin formation and axon loss. When Wld^S was co-expressed with P0 knockout mice, a reduction in the number of degenerating axons was recorded at both 6 weeks and 3 months of age, however by 6 months this difference was no longer evident. This was reinforced by improved muscle strength and conduction properties in the double mutants at 6 weeks and 3 months of age, but as with axonal loss this effect was not observed at 6 months (Samsam et al., 2003).

Another model of demyelinating disease is the CMT1A rat, which models Charcot-Marie-Tooth disease type 1A in which peripheral myelin protein of 22kDa (PMP22) is overexpressed. CMT1A is characterised by demyelination which results loss of motor and sensory axons. When Wld^S was introduced into this model, a reduction in axonal loss was observed however this was not via changes in myelination. The reduced axonal loss was found to be concomitant with improvements in compound muscle action potentials and strength of the animals (Meyer Zu Horste et al., 2011). The results from both these studies show improvements in axon numbers without an effect on the initial myelin defect, which is

not unexpected as it is likely that Wld^S protects an axon against degeneration without affecting the individual disease pathology.

However, Wld^S does not appear to represent a cure-all mechanism, with reported failures to delay the onset of degeneration in a number of models. In superoxide dismutase (SOD1) models of MND, missense mutations in SOD1 give rise to axonal degeneration, with defects in slow axonal transport being reported in a number of models as an early feature of disease progression. In mice co-expressing Wld^S and SOD1 mutations ($SOD1^{G37R}$ and $SOD1^{G85R}$) no change in survival was observed nor was any appreciable difference in axonal or NMJ loss seen (Vande Velde et al., 2004). In contrast in another SOD1 mouse model ($SOD1^{G93A}$), Wld^S was observed to have a modest effect on survival, with lifespan extended by 10 days in $SOD1^{G93A}$ /Wld^S animals (Fischer et al., 2005). This correlated with a higher percentage of innervated motor end plates in these mice at early time points, however no effect was seen at later stages of disease. No change in axonal numbers was observed at any time point, nor was there a delay in disease onset as measured by behavioural assessments. These mixed data are hard to explain as whilst extended survival was recorded, this was not accompanied by a difference in NMJ or axonal survival at late disease stages (Fischer et al., 2005).

Wld^S has had some success in ameliorating axonal degeneration in diseases of the CNS. Global cerebral ischemia results in widespread neuronal damage ordinarily, however when it was induced in Wld^S mice, a reduction in neuronal loss was observed (Gillingwater et al., 2004). Whilst this is not a direct measure of axonal protection, it does indicate that Wld^S is broadly protective against damage within the CNS. The symptoms associated with Parkinson's disease occur due to the loss of dopaminergic neurons in the brains of sufferers, and this can be modelled using the neurotoxins MPTP and 6-OHDA which selectively destroy dopaminergic neurons. When Wld^S mice were injected with these neurotoxins, the onset of axonal degeneration was delayed by a number of days, and a higher number of Wld^S mice survived the MPTP treatment (Sajadi et al., 2004, Hasbani and O'Malley, 2006).

Table 1-2. Models of diseases in which Wld^S/Nmnat have been investigated

Disease modelled	Insult or mutation	Wld^S or Nmnat added	Axonal Protection?	Other phenotypes	Reference
Charcot-Marie-Tooth disease 1A	Overexpression of *pmp22*	Wld^S	Yes - tibial axon numbers same as control at 13 weeks	CMAP improved but not to control levels	(Meyer Zu Horste et al., 2011)
Charcot-Marie-Tooth disease 1B	*P0*$^{+/-}$ mice	Wld^S	Yes - up to 3 months	Improved CMAP, retrograde axonal transport and grip strength	(Samsam et al., 2003)
Excitotoxic injury to retina	NMDA injected	Wld^S	Yes - degeneration delayed by 2 weeks	NA	(Bull et al., 2012)
Glaucoma	Raised IOP	Wld^S	Yes - reduced loss of ONs at 2 weeks	NA	(Beirowski et al., 2008)
Gracile axonal dystrophy	Loss of *uchl1*	Wld^S	Yes - 50% reduction in axonal spheroids at 4 months	No effect on behaviour.	(Mi et al., 2005)
Hypoxic-ischaemic injury	Carotid artery ligation followed by hypoxia chamber	cytNmnat1	Yes - implied by no hyperintensity detected via MRI and reduced tissue loss at 7d	NA	(Verghese et al., 2011)
Multiple sclerosis	EAE mice - MOG innoculation	Wld^S	Yes - reduced axon loss up to 8 weeks	Behavioural onset delayed 2.5d	(Kaneko et al., 2006)
Parkinson's disease	6-ODHA	Wld^S	Yes - increased TH+ optical density at 11 days	NA	(Sajadi et al., 2004)
	MPTP	Wld^S	Yes - increased TH+ optical density at 7 days	Greater number of mice survived the MPTP treatment to 7d	(Hasbani and O'Malley, 2006)
Progressive motor neuropathy	*pmn* mice - Tbce mutation	Wld^S	Yes - no axon loss at P30	Improved locomotor behaviour at P30. Axonal transport same as controls	(Ferri et al., 2003)
Toxic neuropathy	Taxol	Wld^S	Yes - degeneration delayed by >2 weeks	Behavioural improvement - rotarod	(Wang et al., 2002)
Diabetes	Streptozotocin induced mice	Wld^S	Not measured directly - reduced retinopathy	Improved axon conductance	(Zhu et al., 2011)
FTD - Tauopathy	P301L tau mice	Nmnat1/ Nmnat2	Not meaured directly	Reduced neurodegeneration	(Ljungberg et al., 2012)
	R406W tau Drosophila	dNmnat	Not measured directly	Reduced neurodegeneration	(Ali et al., 2012)
Stroke - global cerebral ischemia	Transient occulasion of carotid artery	Wld^S	Not measured directely - increased neuron counts at 72h	NA	(Gillingwater et al., 2004)
Charcot-Marie-Tooth disease 2D	*Gars* mutation	Wld^S	No	NA	(Stum et al., 2011)
Hereditary spastic paraplegia	*Plp-/-* mice	Wld^S	No difference at 18 months when axonopathy manifests	N/A	(Edgar et al., 2004)
Motor neuron disease	*SOD1-G93A* mice	Wld^S	No	Lifespan increased (10d). 32.9% more nerve plate innervation than SOD/WT	(Fischer et al., 2005)
	SOD1-G37R mice	Wld^S	No	No change in lifespan or disease onset. Small reduction in end plate denervation at 5 months	(Vande Velde et al., 2004)
	SOD1-G85R mice	Wld^S	No	No change in lifespan or disease onset	(Vande Velde et al., 2004)
Prion disease	Scrapie strain 139A intracerebral innoculation	Wld^S	No	No effect on survival, astro- or microgliosis or neurodegeneration	(Gultner et al., 2009)
Spinal muscular atrophy	SMN2$^{+/+}$;SMNΔ7$^{+/+}$;Smn$^{+/-}$ mice	Wld^S	No	No change in birth weight, disease progression and survival.	(Kariya et al., 2009)
	SMN2;Smn$^{-/-}$	Wld^S	No (no axon loss in mutant)		(Kariya et al., 2009)

Multiple sclerosis is characterised by demyelination of CNS axons resulting in motor symptoms and axon loss. Its animal model is the experimental autoimmune encephalomyelitis (EAE) mouse in which the injection of myelin oligodendrocyte glycoprotein (MOG) triggers the immune system to destroy myelin. MOG immunisation ordinarily produces a typical time course of neurological disability becoming apparent at 10 days post immunisation and persisting for up to 8 weeks, but this was delayed in Wld^S mice along with a reduction in axon loss. Inducing EAE in the Wld^S mice also prevented the reduction in NAD^+ levels normally observed in EAE mice which, like in injury, may have delayed the onset of degeneration. These results again demonstrate that Wld^S can delay axonal degeneration without impacting on the specific disease associated pathology, as Wld^S did not impact upon the immunological response to demyelination and eventual apoptosis of affected neurons (Kaneko et al., 2006).

Axonal degeneration has been observed in prion disease, however when prion infected brain homogenate was injected into the brain of Wld^S mice no delay in axonal degeneration was seen. No effect upon disease hallmarks such as gliosis and deposition of misfolded prion protein was observed, nor upon survival time, indicating Wld^S had no effect upon disease associated processes or axonal degeneration (Gultner et al., 2009).

Expression of Nmnat isoforms has also had some success in disease models, such as tauopathy. In *Drosophila* expressing mutant human tau (R406W), overexpression of dNmnat resulted in improvements in learning and memory and locomotor behaviour, shown to be due to reductions in tau oligomers. Whilst axons weren't looked at directly, a reduction in neurodegeneration was demonstrated by lower levels of brain vacuolisation and reduced apoptosis (Ali et al., 2012). Similarly, expression of Nmnat1 and Nmnat2 was found to be protective in mice expressing mutant human tau (P301L). This was shown to compensate for transcriptional down regulation of Nmnat2 expression and resulted in reduced tau phosphorylation, gliosis and apoptosis, indicating lower neurodegeneration was occurring (Ljungberg et al., 2012). However, as axons were not looked at directly these improvements cannot be attributed to a delay in axonal degeneration, and may be occurring via another mechanism.

Whilst the ability of Wld^S to delay axonal degeneration in models of disease is varied (Table 1-2), it indicates that there are some similarities in the mechanisms controlling axonal degeneration in injury and disease. Considering that cytoskeletal breakdown is a key event in

Wallerian degeneration (Schlaepfer and Bunge, 1973, Schlaepfer and Micko, 1978, Park et al., 2013a) and is delayed by Wld^S, investigating this protection in tauopathy is particularly interesting as microtubule destabilisation has been implicated in tau-mediated axonal dysfunction and degeneration (Mudher et al., 2004, Cowan et al., 2010, Quraishe et al., 2013).

1.6 Tauopathy

Tauopathies are neurodegenerative diseases characterised by the accumulation and aggregation of the microtubule associated protein, tau. Tau pathology can be the primary pathology, such as in Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) or can co-exist with other pathologies such as with amyloid-beta (Aβ) in Alzheimer's disease (AD). Currently there exists no disease modifying therapies for neurodegenerative diseases, so understanding the underlying pathological mechanisms is highly important. As tau pathology is present in a range of neurodegenerative diseases (Table 1-3), understanding this one disease associated protein could be highly beneficial to a number of diseases.

Tau is predominantly found within the central nervous system where its primary function is microtubule stabilisation. There are six human tau isoforms produced by the alternative splicing of the *MAPT* gene, which contain either three (3Rtau) or four (4Rtau) C-terminal tandem repeat sequences (Goedert et al., 1989a, Goedert et al., 1989b, Andreadis et al., 1992), which are the microtubule binding domains (Himmler et al., 1989, Lee et al., 1989). The presence or absence of one or two inserts in the N-terminal region also differentiates between isoforms (0N, 1N or 2N respectively) (Goedert et al., 1989b).

The function of tau is regulated by its post-translational modifications, predominantly phosphorylation. Increasing levels of phosphorylation decrease the affinity of tau for microtubules, resulting in a more plastic cytoskeleton which is important during development and cell division in which microtubules are required to rapidly grow or disassemble. The phosphorylation state of tau is regulated by a number of kinases including GSK3β, cdk5 and MAPK (Hanger et al., 2009). Alterations to the phosphorylation state are thought to be key to tauopathies, leading to the aggregation and deposition of tau protein.

Table 1-3 Diseases in which tau pathology is observed. Tau pathology is found in a number of neurodegenerative diseases, either as the predominant neuropathologic feature (*) or in combination with other pathological hallmarks. Taken from (Lee et al., 2001)

Diseases with tau-based neurofibrillary pathology
Alzheimer's disease
Amyotrophic lateral sclerosis/parkinsonism–dementia complex*
Argyrophilic grain dementia*
Corticobasal degeneration*
Creutzfeldt-Jakob disease
Dementia pugilistica*
Diffuse neurofibrillary tangles with calcification*
Down's syndrome
Frontotemporal dementia with parkinsonism linked to chromosome 17*
Gerstmann-Sträussler-Scheinker disease
Hallervorden-Spatz disease
Myotonic dystrophy
Niemann-Pick disease, type C
Non-Guamanian motor neuron disease with neurofibrillary tangles
Pick's disease*
Postencephalitic parkinsonism
Prion protein cerebral amyloid angiopathy
Progressive subcortical gliosis*
Progressive supranuclear palsy*
Subacute sclerosing panencephalitis
Tangle only dementia*

1.6.1 How does tau become pathological?

Tau was identified as being the major component of paired helical filaments (PHF) in the brains of Alzheimer's patients (Goedert 1988, Wischik 1988), with tau from PHF aggregations found to be more highly phosphorylated, containing 6 to 8 moles of phosphate per mole of tau, compared to 1.9 moles from normal brains (Ksiezak-Reding et al., 1992). It is thought that excessive kinase activity paired with reduced phosphatase activity leads to the hyperphosphorylation of tau, making it insoluble and prone to aggregation. In animal models of tauopathy, inhibition of the tau kinase GSK3β reduces tau phosphorylation, improving behavioural phenotypes associated with tau dysfunction (Mudher et al., 2004) and reducing neuronal loss (Noble et al., 2005). The hyperphosphorylation of tau represents a key event in tauopathy, triggering a pathological cascade resulting in the formation of insoluble aggregates and neuronal cell death (Figure 1-8).

For a long time, the aggregation of tau in PHFs and subsequent formation of neurofibrillary tangles (NFT) was thought to be the toxic component, taking up vital space within the neuronal cell body. However a dissociation between NFT and toxicity has been described;

in mice expressing the P301L tau mutation (causative of FTDP-17), tau expression was switched off at 4 months, resulting in a reduction in tau levels, improvements in memory and prevention of neuronal loss, despite increasing levels of NFT (Santacruz et al., 2005). This study, along with others (Spires et al., 2006), indicates the dynamic nature of tauopathy; that by removing the toxic species functional improvements and prevention of degeneration can be achieved, and that the pathological process is not unstoppable once initiated. Furthermore, this work indicates that it is the events leading up to NFT formation which are toxic and not the NFTs themselves.

Tau oligomers are the intermediates between tau monomers and NFT and are largely recognised as the toxic species in tauopathy, appearing early in the disease process (Figure 1-8). In human AD brains, tau oligomers have been found at early Braak stages (stage I) whereas NFTs were only seen later at Braak stage V (Maeda et al., 2007). Others have found that in human AD brain, oligomers can be detected in neurons containing protofilaments and intraneuronal NFTs, but following neuronal death, oligomers are no longer found in the extracellular ghost NFTs (Lasagna-Reeves et al., 2012). In addition, when tau monomers, oligomers or fibrils were injected in the hippocampus of WT mice, only the oligomers were found to cause memory deficits and degeneration (Lasagna-Reeves et al., 2011).

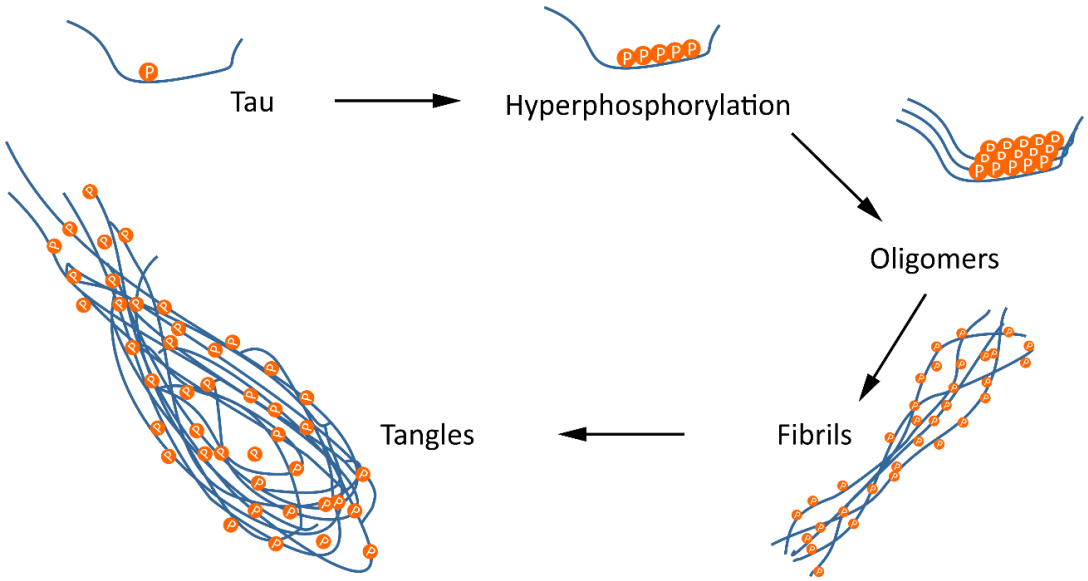


Figure 1-8. Pathological changes to tau. In tauopathy, levels of tau phosphorylation increase such that tau becomes 'hyperphosphorylated'. This leads to monomers of tau associating and forming oligomeric species. The next stage is the formation of fibrils (PHF) which further aggregate to become tangles (NFT).

Whilst both tau phosphorylation and aggregation are key pathological changes in tauopathy, the relationship between them remains unclear. Does hyperphosphorylation cause tau to become aggregate prone? In support of this, hyperphosphorylated tau isolated from human AD brains has been shown to form PHF *in vitro*, with dephosphorylation of tau removing this propensity towards aggregation (Alonso et al., 2001). However others have found that alone, high levels of tau phosphorylation aren't sufficient to cause aggregation and that others factors are required to promote the assembly of tau oligomers and fibrils (Tepper et al., 2014). Despite their controversial relationship, hyperphosphorylated tau and tau oligomers remain key players in the pathological cascade of tauopathy. But how do these changes result in neuronal dysfunction and degeneration?

1.6.2 How does tau cause neuronal dysfunction?

Axonal dysfunction

The hyperphosphorylation of tau reduces its affinity for tubulin resulting in the microtubule cytoskeleton becoming destabilised. This is supported by evidence showing lower levels of acetylated α-tubulin in AD brains, indicating microtubules are unstable (Hempen and Brion, 1996), and a reduction in microtubule density in human AD brains (Cash et al., 2003). This led to the formation of the tau-microtubule hypothesis, which states that all neuronal functions dependent on intact microtubules, such as axonal transport, will be disrupted due to the loss of normal tau function. This has been demonstrated in *Drosophila* overexpressing htau^{0N3R}, which is highly phosphorylated and results in the breakdown of microtubules. As a consequence axonal transport is disrupted in this model and behavioural deficits are observed, with this shown to be due to the reduced affinity of hyperphosphorylated tau for microtubules (Figure 1-9 D) (Cowan et al., 2010). Upon inhibition of GSK3β, a reduction in phospho-tau was observed along with rescue of cytoskeletal integrity and behavioural deficits (Mudher et al., 2004). Further support for the loss of normal function mechanism of axonal dysfunction comes from work in which the microtubule stabilising drug NAP was able to rescue axonal transport and the associated behavioural phenotypes in *Drosophila* expressing htau^{0N3R}, without any impact upon tau phosphorylation (Quraishe et al., 2013). Similarly, in cultures of *Aplysia* neurons expressing human tau, low concentration paclitaxel treatment rescued tau induced microtubule disorganisation and reinstated normal axonal transport (Shemesh and Spira, 2011).

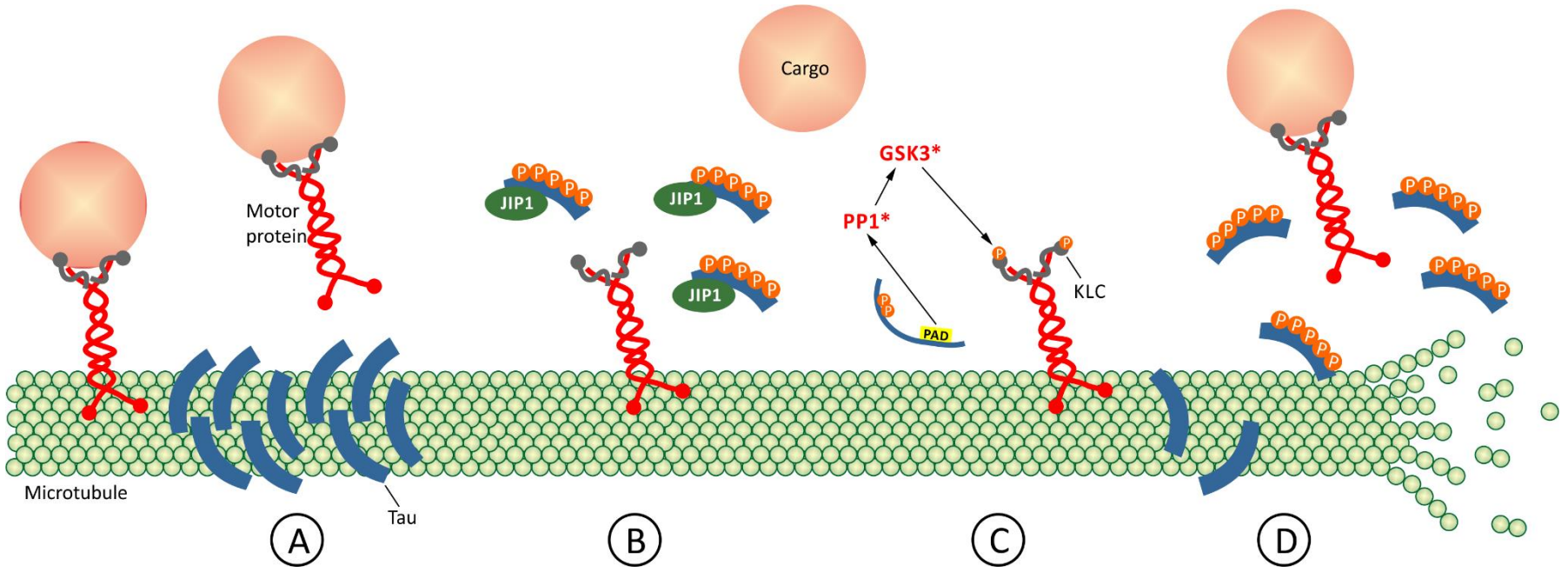


Figure 1-9. Mechanisms by which tau disrupts axonal transport. [A] Excessive binding of tau to microtubules prevents motor proteins binding (Ebneth et al., 1998). [B] Interaction between hyperphosphorylated tau and the scaffolding protein JNK interacting Protein (JIP1) disrupts binding of cargo to kinesin motors (Ittner et al., 2009). [C] Tau phosphorylation disrupts folding, causing exposure of the phosphatase activating domain (PAD), resulting in activation of PP1 which in turn dephosphorylates and thereby activates GSK3, which phosphorylates kinesin light chains (KLC) and disrupts cargo binding to motors (Morfini et al., 2002, Kanaan et al., 2011). [D] Hyperphoshorylation of tau reduces its affinity for microtubules causing it to detach, with the destabilised microtubules subsequently breaking down and preventing axonal transport (Mudher et al., 2004, Cowan et al., 2010).

Others have proposed that tau disrupts axonal transport by over-stabilising microtubules, thereby preventing motor proteins from binding (Ebneth et al., 1998). In this model, restoration of axonal transport occurs upon kinase activity, such as MARK2, and results in tau phosphorylation and reduced affinity of tau for microtubules (Figure 1-9 A). Whilst this clears the microtubules of blockages and enables kinesin motors to bind and resume axonal transport, without tau stabilisation the microtubules are unstable and breakdown (Mandelkow et al., 2004). However, the over-stabilisation of microtubules by hypophosphorylated tau is unlikely to be a disease associated mechanism, as hyperphosphorylation is known to occur in tauopathy (Ksiezak-Reding et al., 1992). Furthermore, one of the sites phosphorylated by MARK2 is S262, a site found to be phosphorylated in tau derived from PHF in AD brains (Hanger et al., 2007), supporting that loss of microtubule binding by tau is the more accurate disease mechanism.

However, loss of microtubule stabilisation is not the only way that tau is thought to disrupt axonal transport. Another mechanism has been proposed to involve a gain of function of tau, in which alterations in tau folding expose an N-terminal domain which activates a cascade resulting in the phosphorylation of kinesin light chains, thereby reducing kinesin based transport (Figure 1-9 B) (Morfini et al., 2002, Kanaan et al., 2011). Tau has also been proposed to impair the transport of specific cargo, as hyperphosphorylated tau interacts with JIP1, preventing it from interacting with kinesin motors, which JIP1 ordinarily regulates cargo binding to (Figure 1-10 C) (Ittner et al., 2009). Whilst many studies have reported axonal transport deficits caused by tau (Ishihara et al., 1999, Stamer et al., 2002, Mudher et al., 2004, Ittner et al., 2009, Kanaan et al., 2011), the precise molecular mechanisms are not fully understood.

Synaptic dysfunction

Changes in synaptic function have been reported in models of tauopathy, which is unsurprising considering synapses are reliant upon effective axonal transport. Mice expressing all 6 isoforms of human tau were found to have reduced release probability of vesicles and were unable to sustain high frequency transmission in response to stimulation (Polydoro et al., 2009). Synaptic function was also reduced in mice expressing the FTDP-17 mutation P301S, with loss of synaptic markers such as synaptophysin and α-synuclein occurring before neuronal loss was observed (Yoshiyama et al., 2007). In cultures of tau-expressing *Aplysia* neurons, decreases in excitatory postsynaptic potential amplitude were

rescued by paclitaxel (Erez et al., 2014), a microtubule stabilising drug which has previously been demonstrated to rescue axonal transport in this model (Shemesh and Spira, 2011). Disrupted vesicle recycling has also been observed in synapses overexpressing human tau, as has reductions in the presence of functional mitochondria at the synapse (Chee et al., 2005), together indicating that tau can affect pre-synaptic function in a number of ways.

An effect on the post-synapse has also been reported, with reports of reductions in dendritic spine density in mice expressing P301L tau (Rocher et al., 2010, Kopeikina et al., 2011) and missorting of tau to the somatodendritic compartment (Zempel et al., 2010). In the post-synapse, tau is thought to interact with synaptic proteins and modulate their function, such as the Src kinase Fyn. Tau is required for the targeting of Fyn to dendrites, where Fyn phosphorylates NMDA receptors resulting in the stabilisation of the receptors interaction with PSD95, a scaffolding protein in dendrites (Nakazawa et al., 2001). This interaction with Fyn is thought to be a key mechanism by which Aβ interacts with tau in AD, as demonstrated by mice expressing APP on a $tau^{-/-}$ background which display reduced excitotoxic seizures (Ittner et al., 2010). The majority of work investigating tau at the post-synapse focuses on the interaction with Aβ, therefore it is not clear whether excitotoxic damage as a result of altered protein interaction occurs in pure tauopathy. However, in mice expressing P301L human tau, disease associated phosphorylation of tau results in increased affinity for Fyn, indicating that in the absence of Aβ, the Fyn-tau interaction could be important for neurodegeneration (Bhaskar et al., 2005).

Mitochondrial dysfunction

Effective neuronal function requires the constant supply and maintenance of a mitochondrial pool at the synapse to meet the demands of synaptic transmission. Therefore deficits in synaptic transmission could be related to disruptions of axonal transport. Indeed, restoring axonal transport using paclitaxel rescued deficits in synaptic transmission (Erez et al., 2014). In *Drosophila* a reduced presence of mitochondria was observed at the pre-synapse, potentially due to disrupted axonal transport, and those that were present had a reduced membrane potential indicating reduced function. In addition, the effect of tau upon synaptic transmission was phenocopied using the mitochondrial poisons FCCP, oligomycin A and rotenone, indicating that mitochondrial dysfunction plays a role in synaptic dysfunction (Chee et al., 2005).

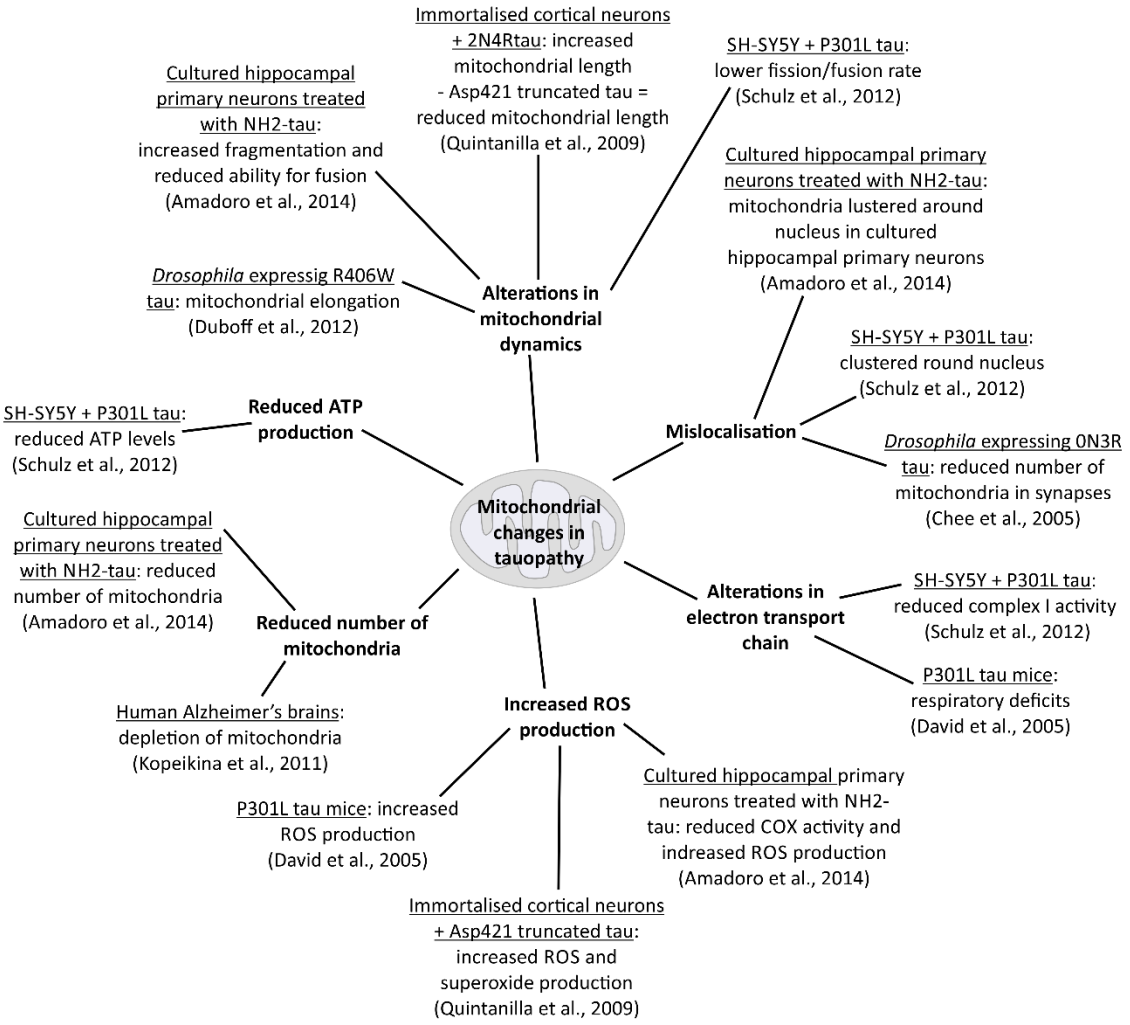


Figure 1-10. Mitochondrial changes associated with tauopathy.

Mitochondrial dynamics are an important quality control mechanism, as in short 'kiss and run' events mitochondrial membranes fuse to allow for the exchange of proteins between mitochondria. This is thought to facilitate the replenishment of stationary mitochondria with newly synthesized proteins and remove damaged proteins and mitochondria to be returned to the cell body for destruction. Phospho-tau from human AD brains has been found to interact with dynamin related protein 1 (Drp1), which helps mitochondria divide by pinching off their membranes (Manczak and Reddy, 2012). This interaction was also found in the 3xTgAD mouse model, which contains P301L tau (Manczak and Reddy, 2012), and suggests that mitochondrial dynamics could be altered in tauopathy.

Altered mitochondrial dynamics can be seen through changes in mitochondrial morphology; elongated mitochondria indicate more fusion and less fission events, and fragmented mitochondria indicate less fusion and more fission events. In *Drosophila* expressing R406W human tau, elongated mitochondria were observed. Altering the balance towards increased

fission normalised mitochondrial length and reduced the associated neurotoxicity, whilst increasing fusion increased mitochondrial length further and enhanced neurotoxicity (Duboff et al., 2012). However in cultured cortical neurons, expression of 2N4R human tau resulted in elongation with associated toxicity, and expression of Asp421 truncated tau resulted in mitochondrial fragmentation, increased reactive oxygen species (ROS) production, lower membrane potential and reduced Ca^{2+} buffering capacity (Quintanilla et al., 2009). Caspase cleaved tau has also been found to result in mitochondrial fragmentation along with increased production of ROS (Amadoro et al., 2014), indicating that perturbations in mitochondrial dynamics towards either increased fission or increased fusion are detrimental, and that different tau species may have differential effects.

Changes in mitochondrial localisation have also been observed in tauopathy. In flies (Chee et al., 2005, Duboff et al., 2012), mice (Kopeikina et al., 2011, Schulz et al., 2012, Amadoro et al., 2014) and humans (Kopeikina et al., 2011), mitochondria are found to cluster around the nucleus, with lower levels present at the synapse, perhaps due to dysfunctional axonal transport of mitochondria.

In addition to disturbances in mitochondrial localisation and dynamics, mitochondrial dysfunction is known to occur in tauopathy. Expression of human tau results in decreased mitochondrial function such as reductions in the activity of enzymes involved with oxidative phosphorylation (Schulz et al., 2012, Amadoro et al., 2014), reduced ATP production (Schulz et al., 2012) and the increased production of ROS (David et al., 2005, Quintanilla et al., 2009, Amadoro et al., 2014). Together these changes result in energetic failure and increasing levels of oxidative stress within neurons, driving neurons down the path to neurodegeneration (Figure 1-10).

Mitochondrial dysfunction can cause pathological changes to tau and is a potential feedback loop, reinforcing pathological changes in the protein. Prohibitin 2 (Phb2) is a mitochondrial scaffolding protein which stabilises OPA1, which functions in mitochondrial fusion. Loss of Phb2 was found to result in fragmented mitochondria and the phosphorylation of endogenous mouse tau at disease associated epitopes, plus the appearance of tau filaments (Merkwirth et al., 2012). This represents a mechanism by which mitochondrial dysfunction can lead to neurodegeneration through pathological alterations to tau.

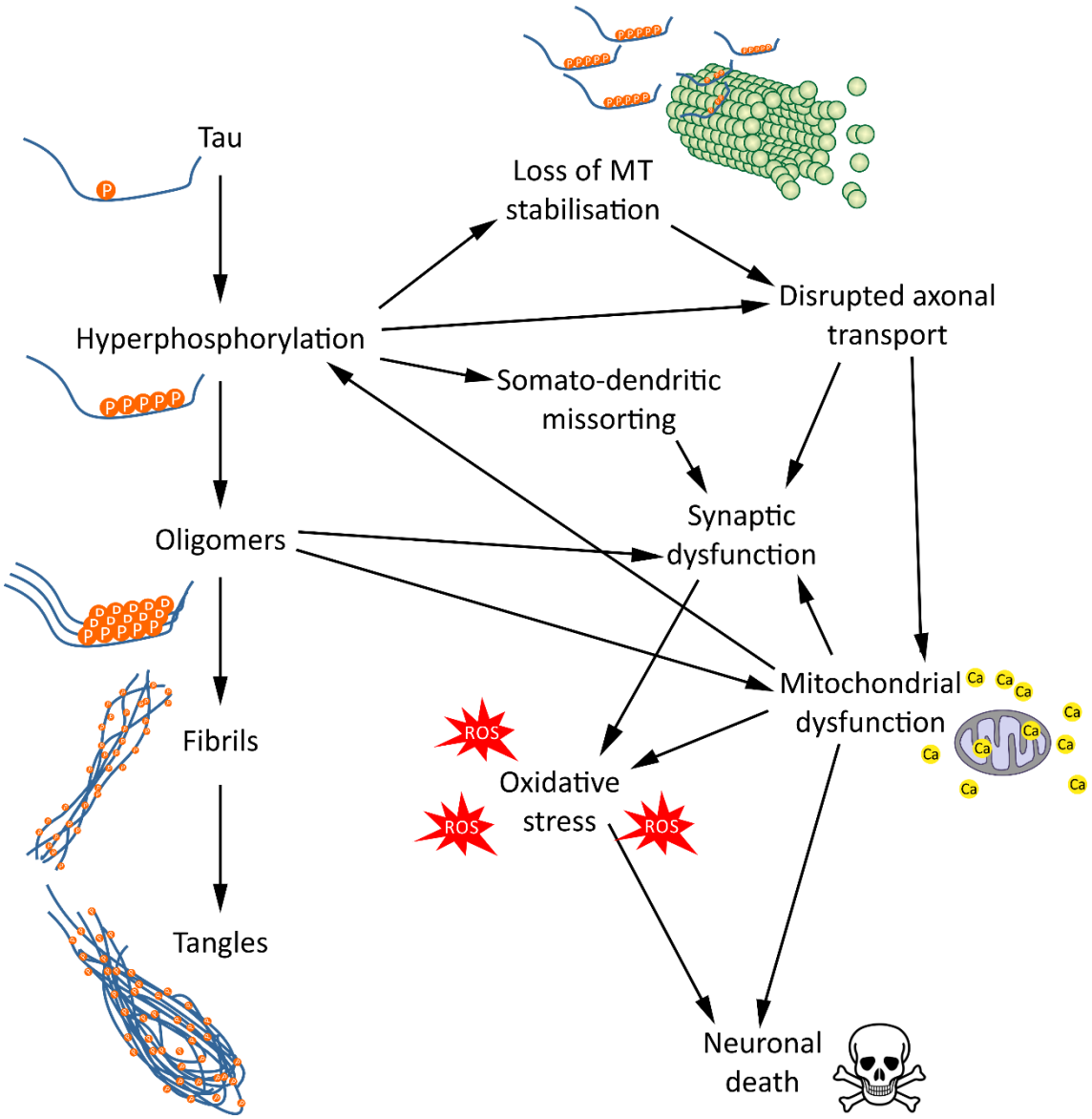


Figure 1-11. Pathological cascade of events in tauopathy. Hyperphosphorylation of tau leads to a loss of normal function, microtubule stabilisation, and also to toxic gain of function, through the formation of oligomeric species. These functional changes lead to disruptions of axonal transport and synaptic dysfunction, raising levels of oxidative stress and driving neuronal cells down a pathway to neurodegeneration.

1.6.3 How does tau cause neuronal death?

How do all the alterations in neuronal function ultimately lead to neuronal cell death? As discussed above, the widely held consensus is that small soluble oligomers of tau are the toxic species, but how do they activate degenerative processes? Does increasing neuronal

dysfunction cause a threshold to be reached after which degeneration occurs? Or are there specific processes which trigger degenerative cascades?

Synapse loss is regarded to precede neuronal loss in AD (Terry et al., 1991, Masliah et al., 2001), and this holds true in models of tauopathy, with reductions in spine density and synaptic markers appearing before neuronal loss (Yoshiyama et al., 2007, Kopeikina et al., 2013). Changes at the synapse occurring in tauopathy, such as increased affinity of tau for Fyn (Bhaskar et al., 2005), could result in excitotoxicity and contribute towards neurodegeneration. Excitotoxicity can lead to Ca^{2+} influx, which can have detrimental effects on mitochondria such as opening the mPTP in response to increased intracellular Ca^{2+}, with this being one potential way in which synaptic dysfunction in tauopathy leads to neurodegeneration. Axonal swellings have also been noted to appear early in diseases such as Alzheimer's (Stokin et al., 2005, Wirths et al., 2007), and are considered to be an indicator of axonal degeneration. These axonal swellings have also been seen in mouse models expressing WT or mutant human tau (Duff et al., 2000, Probst et al., 2000, Lin et al., 2003, Yoshiyama et al., 2007, Ittner et al., 2008, Ludvigson et al., 2011) and are morphologically similar to the swellings observed after injury to CNS axons, which are considered an early stage of Wallerian degeneration (Adalbert et al., 2009). Axonal swellings in tauopathy coincide with disrupted cytoskeletal organisation and reduced axonal transport; axonal swellings contain swirls of microtubules in which mitochondria, autophagosomes and vesicles are trapped (Lin et al., 2003, Shemesh and Spira, 2010, Ludvigson et al., 2011). How axonal swellings contribute to neurodegeneration in tauopathy is not clear, however they could indicate that Wallerian-like degeneration is occurring.

A proposed trigger for neuronal cell death in tauopathy is the re-entrance of the cell-cycle by post-mitotic neurons. The ectopic expression of cell-cycle proteins has been observed in degenerating neurons in a number of tauopathies such as AD, FTDP-17, progressive supranuclear palsy and corticobasal degeneration (Husseman et al., 2000). Inhibiting transcription factors involved with the G1/S transition reduced TUNEL staining in the brains of *Drosophila* expressing R406W human tau, supporting that cell-cycle re-entry causes neurodegeneration in tauopathy (Khurana et al., 2006). Tau phosphorylation is crucial to this process, as reducing tau phosphorylation through the expression of mutant *sgg* (*Drosophila* homolog of GSK3β), reduced staining by the S-phase marker, proliferating cell nuclear antigen (PCNA) (Khurana et al., 2006). Increases in oxidative stress mediated by phospho-tau have been implicated, as downregulation of anti-oxidant defences enhanced

vacuolisation and TUNEL positive staining, whilst increasing anti-oxidant defences reduced this neurodegeneration. Down regulation of anti-oxidant defences also caused an increase in PCNA staining, indicating increased cell-cycle re-entry with increasing oxidative stress (Dias-Santagata et al., 2007).

It is emerging that post-mitotic neurons are constantly holding the cell-cycle in check, with failure in this vigilance resulting in re-entry and ultimately apoptosis (reviewed in (Herrup and Yang, 2007)). In tauopathy, the phosphorylation of tau causes axonal and synaptic dysfunction, leading to energetic failure and increased oxidative stress, potentially all combining and resulting in failure to prevent cell-cycle re-entry with catastrophic consequences for neurons. It is not clear whether axonal or synaptic loss necessarily precedes apoptosis in tauopathy, however strategies to protect axons and synapses from degenerating could provide significant benefits.

1.7 Using *Drosophila* to investigate axonal dysfunction and degeneration in tauopathy

Axonal dysfunction and degeneration are well reported features of tauopathy, however the underlying mechanisms of degeneration are not known. Investigating the protective capacity of Wld^S in a model of tauopathy will shed light on the mechanisms controlling axonal degeneration, and indicate whether a common mechanism controls all axonal degeneration. An ideal model system in which to perform these investigations is the fruit fly, *Drosophila melanogaster,* which has been used extensively both in the study of Wallerian degeneration and tauopathy.

1.7.1 Wallerian degeneration

The *Drosophila* nervous system shares similarities with mammalian systems in its organisation and response to axonal injury. Due to these similarities, *Drosophila* have been used in a number of investigations of Wallerian degeneration, from investigating the mechanisms of degeneration after injury through to large screens to identify endogenous mediators of the injury response. *Drosophila* are well suited to the study of axonal degeneration, with a number of accessible neuronal circuits in both larvae and adult flies. Additionally, these circuits can be injured in a number of ways, from simple means, such as using forceps to crush nerves (Xiong et al., 2010) or remove antenna, to more complex and precise methods, such as laser

ablation of single axons (Figure 1-12). Whilst crude methods, such as stabbing the larval ventral cord (Kato et al., 2011) or adult brains (Leyssen et al., 2005) result in gross neuronal injury, more precise methods can be employed to injure single axons and track the degeneration occurring as a result. Laser ablation can be performed on the axon bundles in the peripheral nerves of larvae, with the nerve innervating the same segment on the other side serving as a control (Avery et al., 2012). Additionally, this method enables the investigation of changes at the neuromuscular junction, providing information on how synapses are affected by axonal injury. Investigations are temporally limited in larvae due to the formation of the puparium, however in adults, longer term investigations of axonal degeneration are possible in both the olfactory receptor neurons (MacDonald et al., 2006, Hoopfer et al., 2006) and the wing sensory neurons (Fang et al., 2012, Neukomm et al., 2014). The wing model is more accessible, and enables the live imaging of the early events occurring post-injury as well as the longer term effects, without the need for microdissection skills. However, investigations may be limited by the impermeable nature of the wing, which prevents immunohistochemical staining, to which the ORN system is better suited.

Whilst the breakdown of axons in Wallerian degeneration is similar to the breakdown of axons undergoing developmental pruning, the two were demonstrated to be distinct in experiments performed in *Drosophila*. Expression of Wld^S did not prevent the pruning of neurons in the mushroom body of the fly brain, additionally expression of dominant negative ecdysone receptor, which prevents developmental axon pruning, was not found to delay Wallerian degeneration after injury (Figure 1-12) (Hoopfer et al., 2006). Despite this difference, both forms of axon destruction involved the ubiquitin proteasome system (UPS), as expression of UBP2, a yeast ubiquitin protease, inhibits developmental pruning and delays Wallerian degeneration (Hoopfer et al., 2006).

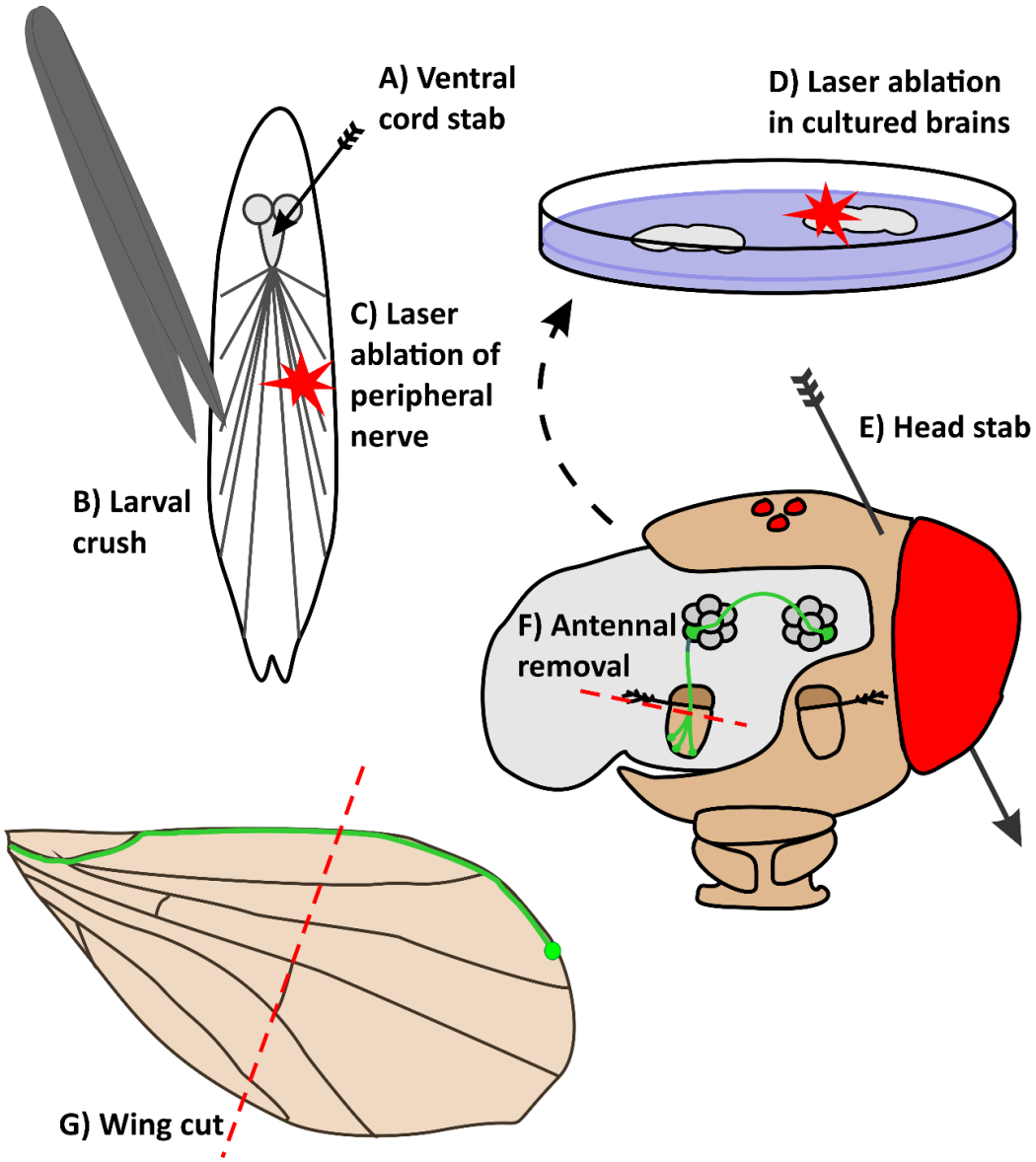


Figure 1-12. Methods of neuronal injury in *Drosophila*. [A] A crude method of injuring the larval nervous system is via a ventral cord stab (Kato et al., 2011). [B] The larval peripheral nerves can be crushed using forceps (Xiong et al., 2010), or [C] ablated using a laser (Avery et al., 2012). [D] Explanted adult brains can be laser ablated and the degeneration of axons the axons tracked in the cultured brains (Ayaz et al, 2008). [E] Like in larvae, a stab injury may be performed on adult brains for a crude injury (Leyssen et al., 2005). [F] The third antennal segment can be removed from adult flies generating an axonal injury of olfactory receptor neurons (Hoopfer et al., 2006, MacDonald et al., 2006). [G] Alternatively, a portion of the wing may be cut off, thereby injuring sensory neurons (Fang et al., 2012, Neukomm et al., 2014).

Although differences exist between vertebrates in invertebrates in the NAD salvage pathway (Fig 1-6), the mechanism by which Wld^S protects against axonal degeneration is conserved between mammals and *Drosophila*, as indicated by the robust protection exhibited by Wld^S transgenic flies after injury (Hoopfer et al., 2006, MacDonald et al., 2006). There are additional steps in the invertebrate NAD salvage biosynthesis pathway, beginning with the

conversion of nicotinamide (Nam) to nicotinic acid (NA), with this then being converted to its mononucleotide form, then the dinucleotide form prior to conversion to NAD. By contrast, in the vertebrate pathway there is no conversion step to NA, with NMN being the only intermediate between Nam and NAD. Despite the differences in the pathway, Nmnat can use both NaMN and NMN as substrates (Preiss & Handler, 1958), which may explain why Wld^S is just as protective in *Drosophila* as it is in mammals. Therefore, despite the extra steps in the invertebrate NAD salvage pathway, it appears that the alterations in this pathway which are associated with Wld^S-mediated axonal protection are conserved, meaning studies performed in *Drosophila* can provide insight into the associated mechanisms.

Drosophila have been highly useful in the understanding of how Wld^S provides axonal protection. Avery and colleagues made fly lines expressing different component parts of Wld^S to investigate which were necessary for Wld^S protection. This revealed that whilst Nmnat1 protection post injury was lower than Wld^S, fusing the N16 region of Wld^S to Nmnat1 provided comparable protection. In line with this, deletion of the N16 from Wld^S reduced axonal protection to the level of Nmnat1 after injury (Figure 1-13) (Avery et al., 2009). They also demonstrated that enzyme dead Wld^S has strongly reduced axonal protection, in support of others who had shown that the enzymatic activity of Wld^S is required for full protection after injury.

The role of mitochondria in Wld^S-mediated protection has been investigated in *Drosophila*, as localisation studies indicated an interaction between Wld^S and mitochondria. Using the genetically expressed Ca^{2+} sensor GCaMP3, it was demonstrated that Ca^{2+} influx post-injury was highly transient and of lower magnitude (~15% of control) in Wld^S larvae compared to controls (Figure 1-13). This indicated that Wld^S axons were somehow able to buffer more Ca^{2+}, which was then demonstrated using mitochondria isolated from Wld^S mice, which could buffer higher loads of Ca^{2+} (Avery et al., 2012). However, mitochondria are not indispensable for Wld^S protection, as whilst disrupting mitochondrial transport in flies expressing Wld^S reduces the presence of mitochondria in axons, significant axonal protection was apparent after injury (Avery et al., 2012, Kitay et al., 2013).

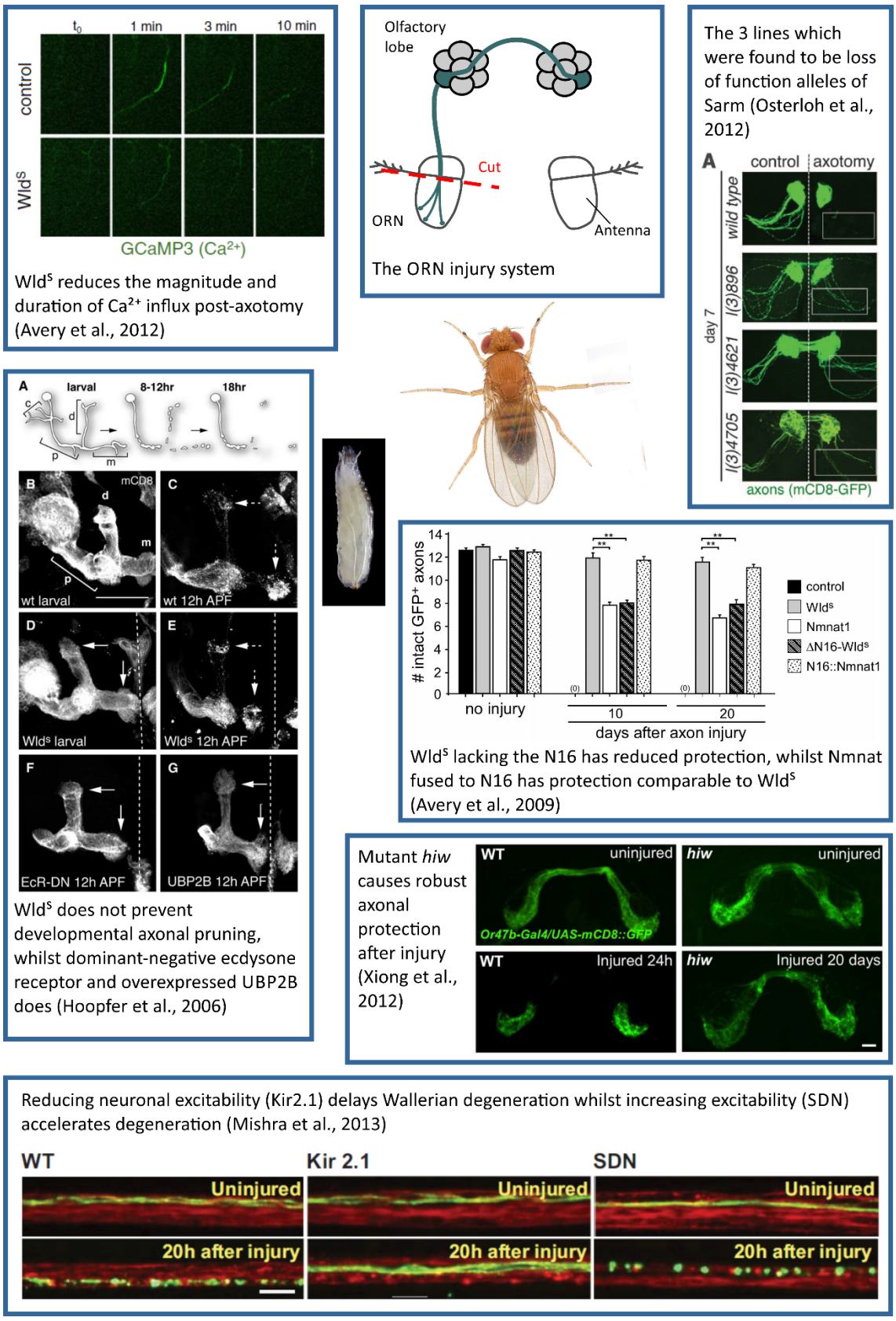


Figure 1-13. *Drosophila* models of axonal degeneration

Mediators of the injury response have also been investigated in *Drosophila*, such as DLK and PHR1. Expression of mutant *Wnd* (the *Drosophila* homolog of DLK) resulted in modest axonal protection in *Drosophila*, an effect which was also seen in mice (Miller et al., 2009). The role of PHR1 in tuning Nmnat levels after injury was first investigated in *Drosophila* (Xiong et al., 2012) before being confirmed in mice (Babetto et al., 2013). In *Drosophila* it was found that after injury, levels of dNmnat dropped, but that in mutants of *hiw* (the *Drosophila* homolog of PHR1), the decrease was prevented and axonal protection observed. In uninjured *Drosophila*, *hiw* mutants had higher levels of dNmnat whilst overexpression of *hiw* resulted in a reduction in dNmnat (Xiong et al., 2012). When the 3 murine isoforms of Nmnat were expressed on the *hiw* mutant background, only levels of Nmnat2 were found to increase (Xiong et al., 2012), an effect also observed in PHR1 knockout mice (Babetto et al., 2013). Together this indicates that the mechanisms controlling Nmnat levels are isoform specific, and support that Nmnat2 is an essential survival factor in mammals.

Other interesting work regarding Wallerian degeneration performed in *Drosophila* investigated the role of neuronal excitability in axonal degeneration. The expression of Kir2.1, which alters the resting membrane potential below the threshold required for action potential firing, was shown to delay axonal degeneration. Conversely expression of dominant negative Shaker (SDN), which results in neuronal hyperexcitability, was found to accelerate the rate of degeneration (Figure 1-13) (Mishra et al., 2013). This indicates that the excitability properties of neuronal membranes have the ability to influence axonal degeneration, with this found to be due to alterations in Ca^{2+} influx post injury. Ca^{2+} influx, as measured by GCaMP3 intensity, was significantly higher in SDN mutants after injury, and incubation of the preparation in Ca^{2+} free buffer prevented the pro-degenerative effect of SDN (Mishra et al., 2013).

Perhaps the best example of the important role *Drosophila* have played in understanding Wallerian degeneration is the discovery of Sarm. In a F_2 forward genetic screen of mosaic flies, used to circumnavigate the issues of lethality, 3 *Drosophila* lines were found to robustly delay axonal degeneration after injury on a par with Wld^S (Figure 1-13). Sequencing of these lines revealed they all affected a single gene and resulted in a loss of function allele of *dSarm*. Loss of Sarm1 in mice was also found to provide robust axonal protection, indicating that Sarm is a conserved endogenous mediator of axonal degeneration (Osterloh et al., 2012). This example highlights how useful *Drosophila* can be, as large scale screens such as this are not feasible in mammals.

1.7.2 Tauopathy

Drosophila have been well utilised in the study of tauopathy, giving important insight into the pathological mechanisms of disease. Expression of human tau throughout the *Drosophila* nervous system results in progressive neurodegeneration, indicated by increasing levels of vacuolisation in the adult fly brain. Consistent with this, increasing immunoreactivity in tau phospho-epitopes was observed as flies aged, however no evidence of filamentous tau was found. These phenotypes were more severe when R406W mutant human tau was expressed compared with the expression of full length (2N4R) htau (Wittmann et al., 2001).

Phenotypic differences caused by different tau isoforms or by mutant tau have been reported by others. Whilst 0N4R, 2N4R and R406W htau all result in the destruction of mushroom bodies (MBs), expression of 0N3R htau does not (Kosmidis et al., 2010), indicating that different tau isoforms have different roles in tauopathy (Table 1-4). In addition, the MB defects observed in flies expressing 2N4R htau were more severe than those observed in 0N4R htau flies, with the expression of a mutant form of human tau, R406W, resulting in more severe MB defects (Kosmidis et al., 2010). This has also been observed in the levels of brain vacuolisation, with R406W htau expressing flies exhibiting a greater extent of neuronal loss compared with 0N4R htau expressing flies (Ali et al., 2012). This indicates the varying levels of tau toxicity associated with the different human tau isoforms and mutations (Table 1-4). This phenotypic variation has also been observed in measures of dysfunction, such as axonal transport, with the expression of 1N4R htau reported to produce reduced levels of disruption compared with the 0N3R htau isoform (Folwell et al., 2010). The comparative ease by which different isoforms and mutations can be investigated in *Drosophila* makes this model organism particularly suited to the investigation of the role of specific phospho-tau epitopes. Additionally, by comparing behavioural phenotypes, such as olfactory learning and memory, with pathology, such as MB defects, the differential roles of isoforms in causing dysfunction and degeneration have been investigated.

The mutation of key phosphorylation sites in tau to alanine renders that site unphosphorylatable, enabling the investigation of the role of that phospho site in tauopathy. Expression of R406W htau or 2N4R htau containing S262A and S356A in MBs did not result in MB destruction, with the preservation coinciding with no learning or memory deficits (Figure 1-12). However when 2N4R htau containing S238A and T245A was expressed, while no MB toxicity was observed, flies displayed deficient olfactory learning (Kosmidis et al.,

2010). Surprisingly, flies expressing htau containing S238A and T245A displayed increased phosphorylation at other disease associated epitopes, indicating that specific phosphorylation sites cause toxicity and not general increases in phosphorylation (Kosmidis et al., 2010). Furthermore, evidence now indicates that S262 is linked to dysfunction, as S262A htau flies display no MB toxicity or learning deficits, while S238 is linked to toxicity (Papanikolopoulou and Skoulakis, 2015). Consistent with this, phosphorylation of S262 occurs earlier in the fly's lifetime than S238, with phosphorylation of S238 coinciding with increased mortality. The relevance of S238 as a disease associated site was confirmed in human tauopathy brains, in which samples from AD, Pick's disease and frontotemporal dementia were all positive for phospho-S238 whilst age matched controls were not (Papanikolopoulou and Skoulakis, 2015).

Drosophila have also been useful in understanding how tau causes neuronal dysfunction. Expression of 0N3R htau in larvae has been found to disrupt the microtubule cytoskeleton, cause axonal transport dysfunction and impair locomotor behaviour, without any evidence of neuronal loss (Table 1-4, Figure 1-14) (Mudher et al., 2004, Cowan et al., 2010). Reducing the phosphorylation of tau using inhibitors of GSK3β increases the binding of tau to microtubules and improves axonal transport and locomotor behaviour (Mudher et al., 2004, Cowan et al., 2010). This work has demonstrated the detrimental effect of the loss of function of tau caused by hyperphosphorylation, as restoring microtubules pharmacologically also rescues axonal transport and locomotor behaviour, but without any effect upon tau-phosphorylation (Quraishe et al., 2013). This model has also given insight into the effect of tau at the synapse. Expression of 0N3R htau was found to disrupt NMJs, with defects in vesicle cycling, mitochondrial mislocalisation and dysfunction, and synapses unable to sustain high frequency transmission (Figure 1-14) (Chee et al., 2005).

Work performed in *Drosophila* by the Feany lab has investigated the cascade of events triggered by pathological tau, enabling them to propose a pathway by which tau causes neuronal cell death. They discovered that in flies transgenic for R406W htau filamentous-actin (f-actin) levels were increased, and that tau interacted with f-actin and was capable of inducing bundling *in vitro*. Staining of the fly brains revealed rod shaped structures immunoreactive for actin and phospho-tau, also for cofilin and phalloidin, indicating these structures are similar to Hirano bodies which are found in AD (Fulga et al., 2007). Using the rough eye phenotype, they found that co-expressing actin with V337M htau enhanced the rough eye phenotype, whilst co-expression of cofilin (which severs actin filaments) with tau

suppressed the rough eye (Figure 1-14). Furthermore, expression of tau and actin increased the level of TUNEL staining in the brain whilst cofilin expressed with tau resulted in a reduction, indicating that the effect of tau upon actin was involved in tau toxicity (Fulga et al., 2007).

These tau induced changes to f-actin have been found to impact upon mitochondrial function. Drp1 is involved with mitochondrial fission and so is normally associated with mitochondria. However in R406W htau transgenic flies, this interaction is disrupted as Drp1 preferentially associates with f-actin, resulting in reduced mitochondrial fission and elongated mitochondria. The elongated mitochondria were associated with tau toxicity as enhancing fusion increased mitochondrial length, ROS production and enhanced neurodegeneration further, whilst enhancing fission normalised mitochondrial length and reduced degeneration. Overexpression of gelsolin (which severs actin) with tau reduced mitochondrial length, ROS production and rescues neurodegeneration (Duboff et al., 2012). These changes to mitochondria are mediated through the interaction of tau with f-actin, and lead to increased oxidative stress within neuronal cells.

The Feany lab have also investigated the role of oxidative stress in tau-mediated neurodegeneration. They found that reducing anti-oxidant defences enhanced tau toxicity whereas enhancing anti-oxidant defences reduced degeneration, as indicated by TUNEL staining (Dias-Santagata et al., 2007). Previously they have shown that areas of areas of degeneration indicated by vacuoles in the fly brain are positively stained for cell cycle markers, such as PCNA which is an S-phase marker. Furthermore, when cell cycle re-entry was blocked in R406W htau transgenic flies, reduced neurodegeneration was observed, indicating that cell-cycle re-entry can trigger degeneration (Khurana et al., 2010). When anti-oxidant defences were reduced in R406W htau flies, increased PCNA staining was observed, indicating increased oxidative stress causes neurons to ectopically express cell-cycle proteins which is associated with neuronal cell death (Dias-Santagata et al., 2007).

The work performed in *Drosophila* has provided valuable insight into the mechanisms involved in tauopathy. Flies are particularly useful for this due to the relative ease with which new lines can be generated, with work such as the investigation of specific phospho-epitopes being unfeasible in mammalian model systems.

Table 1-4. Tau isoforms and mutations and their phenotypes in *Drosophila*.

Wild type or mutant	Tau Isoform	Phenotype	Reference
wt	0N3R	Compromised MT integrity, larger axon diameter. Poor binding to MTs. Reduced binding ability of dtau to MTs. No filament formation.	Cowan et al., 2010
		No obvious defects in MB, no difference in olfactory learning. Less bound to MTs (in supernatant and pellet in MT-binding assay)	Papanikolopoulou et al., 2010
		Vesicular aggregation (More than 1N4R). Abnormal NMJ morphology; thinning inter-bouton axis and satellite boutons (not as severe as 0N3R)	Folwell et al., 2010
		No appreciable effect on MBs	Kosmidis et al., 2010
wt	0N4R	(Glial driver resulted in adult lethality when used without GAL80) Reduced lifespan, increased cell death, tau fibrils (straight)	Colodner and Feany, 2010
		Severe and specific structural defects in MB. Olfactory learning nearly abolished. Bound to MTs	Papanikolopoulou et al., 2010
		Rough eye, reduced size and retinal thickness. MB aberrations; reduced calyx size (missing completely in some)	Iijuma et al., 2010
		Rough eye, reduced size.	Iijima-Ando et al., 2010
		Poor MT binding, only hypophosphorylated tau bound (even on dtau null background)	Feuillette et al., 2010
		Normal brain morphology except for MBs; severely reduced or missing (less severe than 2N4R).	Kosmidis et al., 2010
		Age dependent deficits in learning and memory and locomotor activity (climbing) and in vacuolisation.	Ali et al., 2012
wt	1N4R	Vesicular aggregation (less than 0N3R). Locomotor abnormalities (climbing) >5 days after eclosion. Reduced lifespan	Chee et al., 2005, Folwell et al., 2010
wt	2N4R	Severe and specific structural defects in MB. Olfactory learning nearly abolished. Bound to MTs	Papanikolopoulou et al., 2010
		Neuronal death (TUNEL +ve)	Khurana et al., 2010
		Poor MT binding, only hypophosphorylated tau bound (even on dtau null background). No effect on dtau binding ability.	Feuillette et al., 2010
		Normal brain morphology except for MBs; severely reduced or missing (more severe than 0N4R).	Kosmidis et al., 2010
V337M	0N4R	Poor MT binding, only hypophosphorylated tau bound (even on dtau null background). No effect on dtau binding ability.	Feuillette et al., 2010
		Milder MB defects (than wt 4R), mainly reduced size	Kosmidis et al., 2010
		Mild rough eye. Moderate loss of bristles from notum.	Yeh et al., 2010
R406W	0N4R	Vesicle accumulation.	Falzone et al., 2010
		MB aberrations	Papanikolopoulou et al., 2010
		Reduced lifespan compared to controls. Neuronal death.	Khurana et al., 2010
		Abnormal NMJ morphology; thinning inter-bouton axis and satellite boutons	Folwell et al., 2010
		Poor MT binding, only hypophosphorylated tau bound (even on dtau null background).	Feuillette et al., 2010)
		More severe MB defects (than wt 4R), defective associate olfactory learning	Kosmidis et al., 2010
		Severe rough eye; disorganised ommatidial array, fused photoreceptors and missing bristles. Severe bristle loss from notum	Yeh et al., 2010
		Age dependent deficits in learning and memory and locomotor activity (climbing). More severe vacuolisation than 0N4R.	Ali et al., 2012
Tau^{P301L}	(0N4R?)	Rough eye	Karsten et al., 2006
		Suppression of retinal and brain toxicity.	Iijima et al., 2010
Tau^{S262A}	0N4R	Reduced tau toxicity	Iijima-Ando et al., 2010
Tau^{AP} (unphosphorylatable)	0N4R	Binds MTs, no toxicity	Feuillette et al., 2010
		No appreciable defects	Kosmidis et al., 2010
		Mild rough eye. Mild loss of bristles from notum.	Yeh et al., 2010
		Strongly binds MTs, disruption of axonal transport. Much increased vesicle pausing time (more than tau^{E14}).	Talmat-Amar et al., 2011
Tau^{E14} (phospho-mimic)	0N4R	Poor MT binding, enhanced neurodegeneration compared to tau^{wt}	Feuillette et al., 2010
		Abnormal MBs; severely reduced or missing (more severe than 0N4R).	Kosmidis et al., 2010
		Severe rough eye; disorganised ommatidial array, fused photoreceptors and missing bristles. Moderate loss of bristles from notum.	Yeh et al., 2010
		Poor MT binding, increase in vesicle pausing time compared to control	Talmat-Amar et al., 2011

MT, microtubule; MB, mushroom body; NMJ, neuromuscular junction.

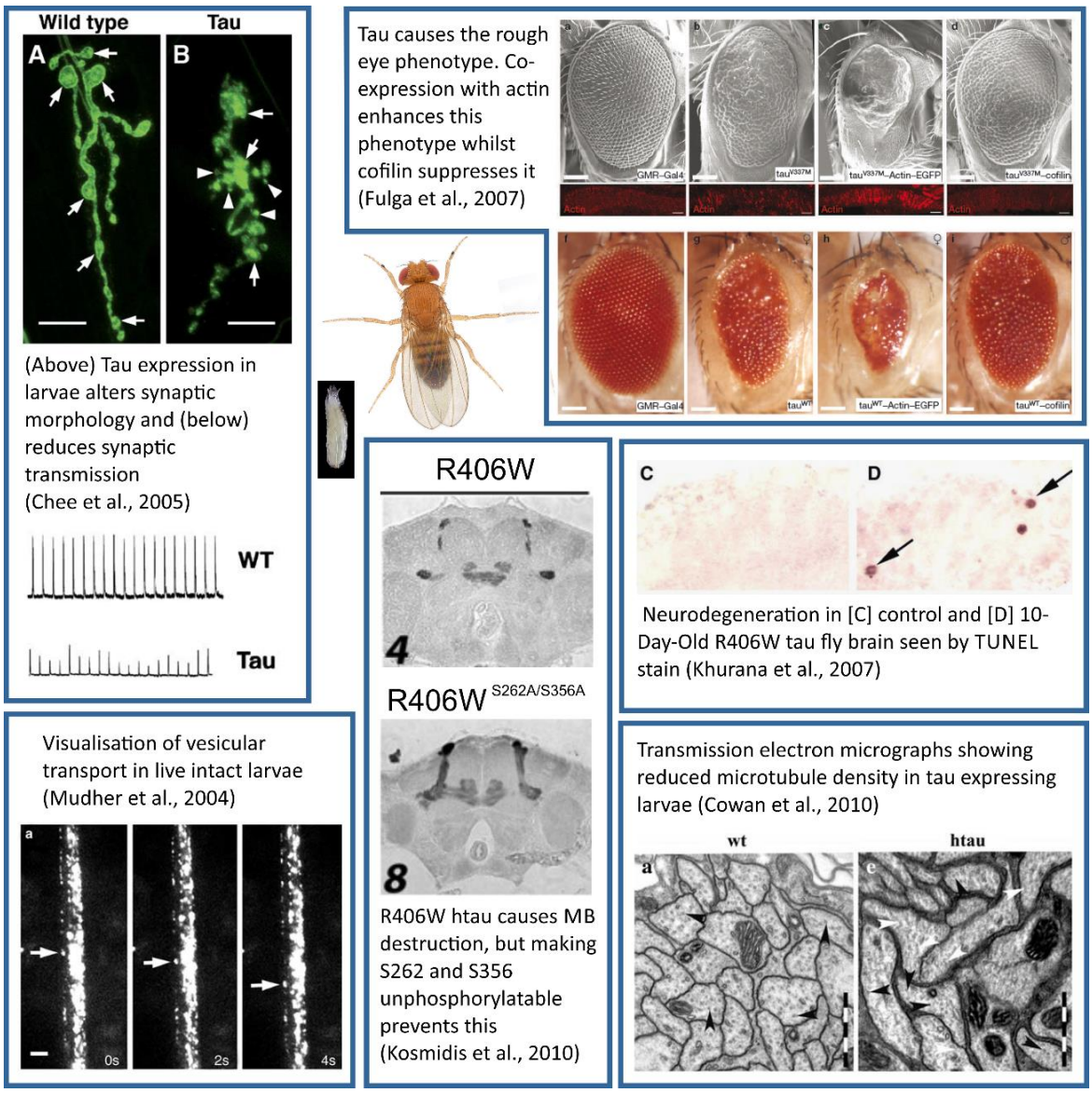


Figure 1-14. *Drosophila* models of tauopathy

1.8 Using *Drosophila* as a model system

The fruit fly, *Drosophila melanogaster*, has a rich history of use in biological research, having first being brought into the laboratory environment over 100 years ago. The creation of the GAL4/UAS system by Brand and Perrimon 20 years ago provided a system for tissue specific expression of transgenes of interest (Brand and Perrimon, 1993), and this system has been adopted by the vast majority of *Drosophila* researchers (Figure 1-15).

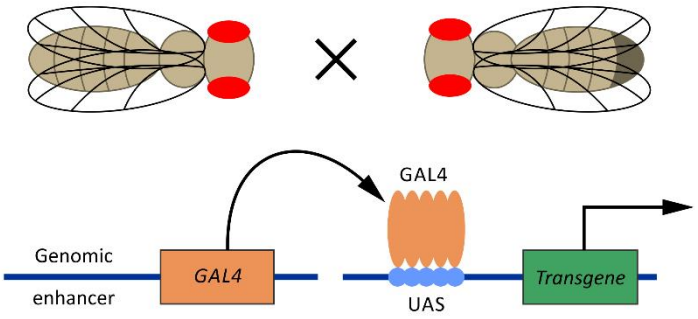
Whilst the GAL4/UAS system provides spatial control of transgene expression, it does not provide temporal control. This can be achieved through the TARGET system in which temperature sensitive GAL80 ($GAL80^{TS}$) is expressed, inhibiting GAL4 in a temperature

sensitive manner. At 18°C, $GAL80^{TS}$ is present and prevents GAL4 from binding to the UAS, thereby preventing transgene expression. But when flies are shifted to higher temperatures (29°C), $GAL80^{TS}$ is inactivated and so transgene expression occurs (Figure 1-15). This system is useful to prevent expression of potentially toxic transgenes during development, and has been used in the study of the role of specific phospho-tau species to eliminate developmental effects (Kosmidis et al., 2010, Papanikolopoulou et al., 2010).

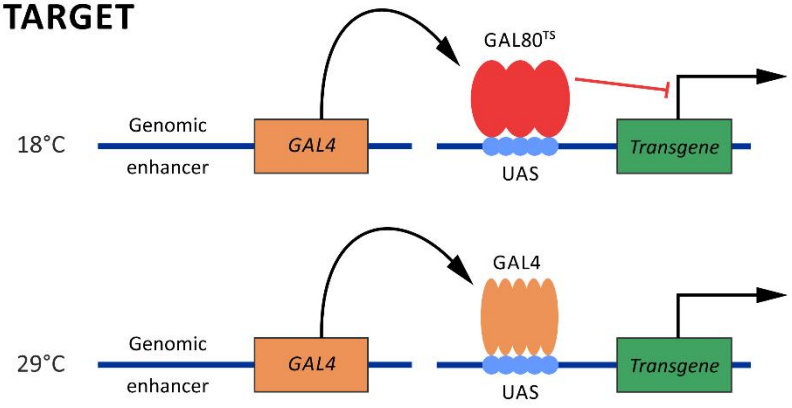
Another useful system for identifying potentially lethal mutations is the mosaic analysis with a repressible cell marker (MARCM) system. This combines GAL4/UAS, GAL80 and the FLP/FRT system to create mosaic animals. In the FLP/FRT system, a yeast flippase (FLP) catalyses recombination between recognition sites (FRT) resulting in the deletion of the intervening sequence. By placing FRT sites upstream of *GAL80* and the wild type allele on one chromosome, and upstream of UAS-GFP and the mutant allele on the other chromosome, upon Flippase activity during G2 phase recombination between the two FRT sites will occur. This results in daughter cells which are homozygous mutant or homozygous wildtype, with the homozygous mutant cells being the only ones to lack *GAL80* and so are labelled by GFP (Figure 1-15). This system was used in the screen in which Sarm was discovered as an endogenous mediator of Wallerian degeneration (Osterloh et al., 2012).

For the study of Wallerian degeneration, *Drosophila* have been particularly useful, in part due to their accessible axonal circuits and the ease with which they can be injured. In larvae, whilst some have used laser axotomy to injure the peripheral nerves (Avery et al., 2012), by far the easiest method of injury is the larval crush. By pinching a larva using forceps an axonal crush injury occurs, and it is clear when this injury has been successful as the larva is paralysed in the portion of its body below the crush site. This injury method was used in the work exploring the role of PHR1/hiw in mediating axonal degeneration (Xiong et al., 2010). However a limitation of larval injury models is the rapid developmental cycle of *Drosophila* prevents longer term study of the response to injury.

GAL4/UAS



TARGET



MARCM

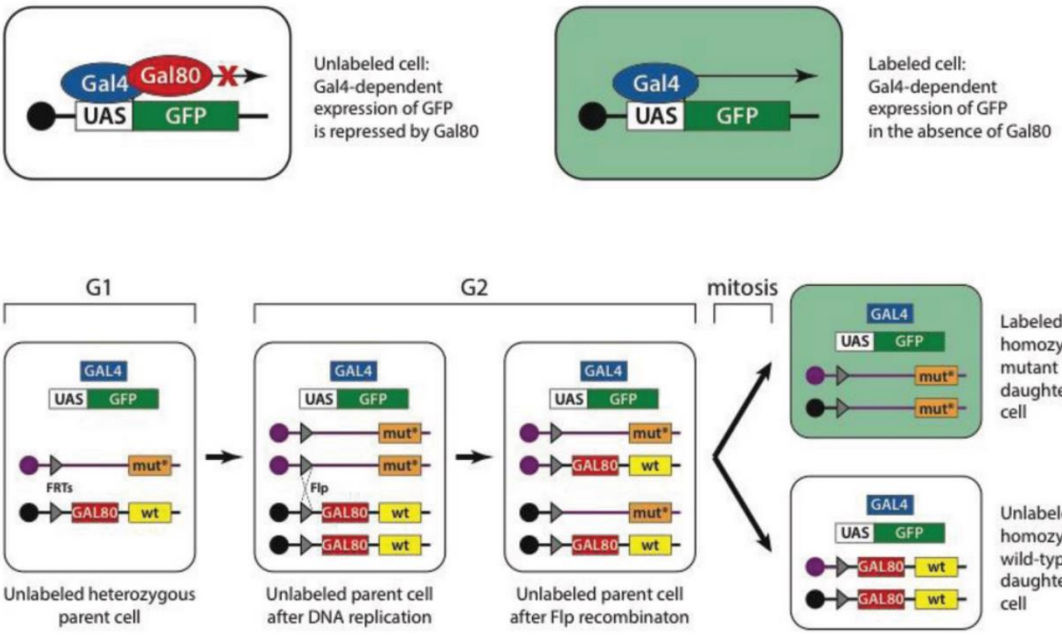


Figure 1-15. (Previous page) Genetic control of transgene expression in *Drosophila*. In the GAL4/UAS system, GAL4 is placed downstream of a genomic enhancer and the transgene of interest is downstream of an upstream activating sequence (UAS) which is activated by the binding of GAL4. The progeny resulting from this cross will only express the transgene of interest in cells expressing GAL4, therefore giving tissue specific expression of the transgene. In TARGET, $GAL80^{TS}$ inhibits GAL4 from activating transcription, however raising the temperature removes $GAL80^{TS}$ enabling expression to occur. In MARCM, recombination between FRT sites results in the production of daughter cells homozygous for the mutant allele which are labelled with GFP, whilst other daughter cells are homozygous for GAL80 and the WT allele, resulting in a mosaic fly. MARCM diagram from (Wu and Luo, 2007).

In adult flies, the ORNs are frequently used to study axonal degeneration. The fly's 3rd antennal segment contains the cell bodies of the ORNs, which project both contralateral and ipsilateral axons into discrete regions of the antennal lobe (Figure 1-12 and 1-13). Removal of this segment results in axotomy of the ORNs which degenerate by characteristic Wallerian degeneration, which can be visualised through the expression of GAL4 and membranous GFP (UAS-mCD8::GFP) in single ORN populations (Hoopfer et al., 2006, Macdonald et al., 2006). The fly wing represents another injury system; cutting the wing tip removes the cell bodies of wing sensory neurons, resulting in Wallerian degeneration (Fang et al., 2012). Both injuries are not overtly detrimental to the fly, and although the wing system appears simpler as it does not require dissection, the impermeability of the wing prevents immunostaining. Whilst the ORN system requires precise dissection skills, the brain can be stained making investigation of responses to injury possible, such as the glial response to axotomy (Macdonald et al., 2006).

Drosophila display a number of behaviours which can be used to study dysfunction within their nervous system. Expression of transgenes throughout the nervous system or limited to motor neurons enables the investigation of locomotor behaviour in both larvae and adults. Deficits in larval crawling can be investigated using an open field assay and tracking software, enabling the examination of a number of behavioural readouts (Sinadinos et al., 2012). Adult flies display a negative geotaxis response after tapping stimuli, and this has been used to investigate how climbing behaviour changes in ageing tau transgenic flies (Mudher et al., 2004). Flies also exhibit working memory which can be investigated using olfactory learning assays with transgene expression limited to the mushroom bodies. In combination with tau expression driven in the mushroom bodies of adult flies using the TARGET system, this assay has enabled the investigation of phospho-epitopes and the differentiation of those involved in dysfunction and toxicity (Kosmidis et al., 2010).

Another benefit of the *Drosophila* system is the transparent cuticle of the larva which enables live imaging, such as axonal transport through the use of GFP tagged vesicles (Mudher et al., 2004, Quraishe et al., 2013). Additionally, larvae are able to withstand dissection and survive in this state for periods up to a couple of hours, with this method used to image Ca^{2+} waves after injury (Figure 1-13) (Avery et al., 2012). This method can also be used for electrophysiological recordings, such as those performed in the investigation of the effect of tau upon synaptic transmission (Figure 1-14) (Chee et al., 2005).

Degeneration can be investigated using the adult fly brain or eye. Degeneration in the adult brain is characterised by the appearance of vacuoles which indicate that cell loss has occurred, with TUNEL staining supporting this (Figure 1-14) (Khurana et al., 2010). In the eye, expression of toxic proteins results in the 'rough eye' phenotype; neurotoxicity disrupts the normal pattern of ommatidia making the external eye surface appear rough. This phenotype lends itself to enhancer/suppressor screens, from which modulators of tau toxicity can be identified, demonstrated in work from the Feany lab (Figure 1-14) (Fulga et al., 2007). However, as the eye surface is generated in the final stages of adult development, the rough eye phenotype does not represent true neurodegeneration, despite this; the photoreceptors below the surface do display progressive neurodegeneration and so can be used to understand degenerative processes.

In the era of the 3R's (Replacement, Refinement & Reduction), *Drosophila* are an ideal model system in which to investigate the processes underpinning axonal degeneration in injury and disease. Fly husbandry has substantially lower costs associated than with mammalian work, allowing for hundreds of different genetic lines to be maintained for a fraction of the cost. The *Drosophila* lifecycle is short, taking ~10 days to produce the next generation, with a maximal lifespan of <100 days. Whilst other models, such as *C.elegans* and zebrafish can also boast these benefits, the increased complexity of *Drosophila* compared to *C.elegans* enables investigation of more complex behaviours, and in a system which is not further complicated by development, as is the case in zebrafish (Table 1-5). Together, this means that *Drosophila* are cheap, quick and easy, representing a useful model system in which to further our understanding of the intricate pathways associated with neurodegeneration.

Table 1-5. Comparison of model organisms

	C. elegans	*Drosophila*	Zebrafish	Mice
Homology with humans	40% genome homology	77% human disease genes have related genes in flies	84% of human disease genes have related genes in fish	85% genome homology in protein-coding regions
Similarity with humans	Invertebrate	Invertebrate	Vertebrate (non-mammal)	Vertebrate (mammal)
Genetic screens	Frequently utilized for high-throughput forward genetic screens	Frequently utilized for high-throughput forward genetic screens	Can be used for forward genetic screens	No
Genetic tools available	Some	Numerous	Some	Some
Used for behavioural experiments	Only display simple behaviours - associated with feeding	Many behaviours – eg. locomotion, learning and memory, sleep, courtship/mating	Many behaviours – eg. locomotion, social preference, learning and memory, anxiety	Numerous complex behaviours
Development time/lifespan	3 days from egg to adult/2-3 weeks	At 23°C - 10 days from egg to adult fly/50 days (median)	3 days from egg to larval stage, 28 days to juvenile/42 months (mean)	18-20 days from fertilisation to birth, 2 months from birth to adult/1.5-2 years
Maintenance costs	£	£	££	£££££
Ethical requirements	No ethics approval required, no restrictions on procedures	No ethics approval required, no restrictions on procedures	Home Office licence required	Home Office licence required. Restrictions on the number of animals used and the type of experiments allowed

1.9 Overview & objectives

The remarkable similarities between axonal degeneration following injury and in disease raise the idea that a common process may be underlying the breakdown in both. Whilst for a long time axonal degeneration was thought to be a passive process, the discovery of Wld^S has enabled the investigation of it as an active process. Since the initial discovery of Wld^S, knowledge of the molecular events occurring post-injury has increased and an 'axon death pathway' proposed. Furthermore, the investigation of axonal degeneration in disease has been made possible and allowed the idea of a common axon death pathway to be scrutinised.

The overarching aim of this thesis has been to investigate the mechanisms involved with tau-mediated dysfunction and degeneration, by investigating whether Wld^S is protective in tauopathy and looking into the effect of tau upon mitochondria.

I hypothesise that Wld^S protection is upstream of the tau-mediated disruptions in axonal transport and the resulting phenotypes, therefore, if true, the co-expression of $htau^{0N3R}$ and Wld^S would result in improvements in axonal transport, synaptic morphology and locomotor function, with a delay in axonal degeneration, indicating that the Wallerian-like degeneration observed in tauopathy is Wld^S-sensitive.

1. **Can Wld^S improve tau-mediated dysfunction and degeneration?**

I created a double transgenic line which co-expressed human tau ($htau^{0N3R}$) and Wld^S and investigated whether improvements were observed compared with *Drosophila* expressing $htau^{0N3R}$ alone. $Htau^{0N3R}$ was selected for this work for a number of reasons:

- Expression of $htau^{0N3R}$ using the pan-neuronal driver elav does not result in neuronal death during embryogenesis, which does occur with other isoforms of wild type (0N4R, 1N4R and 2N4R) and mutant (R406W) tau (Kosmidos et al., 2010, Pananikolopoulou et al., 2010). This will enable the study of the effect of Wld^S on progressive tau-mediated neurodegeneration.
- The 0N3R isoform is highly phosphorylated *in vivo*, therefore it recapitulates the hyperphosphorylation observed in human tauopathies. In addition, the levels of tau phosphorylation increase as the flies age when $htau^{0N3R}$ is expressed, making it an ideal model for studying progressive neurodegeneration (Sealey, unpublished observations).
- $Htau^{0N3R}$ has been demonstrated to have a stronger effect on axonal transport compared with 1N4R and mutant R406W htau, making it more suitable for the study

of axonal transport dysfunction and the associated phenotypes (Falzone et al., 2010, Folwell et al., 2010, Quraishe et al., 2013).

As cytoskeletal breakdown is implicated in both Wallerian degeneration and tauopathy, and after injury is prevented by Wld^S, I was interested to see whether Wld^S could protect cytoskeletal breakdown in tauopathy. Previous work in the $htau^{0N3R}$ model has indicated that microtubule breakdown is responsible for disrupted axonal transport, synaptic aberrations and impaired locomotor behaviour, therefore I investigated the effect of Wld^S co-expression upon these phenotypes. To investigate whether Wld^S could delay tau-mediated degeneration, I adapted the ORN model of injury and investigated tau-mediated changes in axons in uninjured flies and the effect of Wld^S co-expression on this (Chapter 3).

2. **Are $htau^{0N3R}$;Wld^S axons protected against injury induced degeneration?**

Due to the failure of Wld^S to improve tau-mediated phenotypes including axonal degeneration, I investigated whether Wld^S could delay injury induced degeneration when co-expressed with $htau^{0N3R}$. Using the ORN injury model and immunostaining, I investigated how injury and Wld^S protection modulated tau-mediated degeneration (Chapter 4).

3. **Is tau-mediated axonal transport disruption responsible for mitochondrial mislocalisation?**

To investigate how disrupted axonal transport affects mitochondrial mislocalisation, I treated $htau^{0N3R}$ transgenic larvae with the microtubule stabilising drug NAP. The effect of this treatment upon mitochondria was investigated in *Drosophila* expressing GFP tagged mitochondria (Chapter 5).

Chapter 2: Materials & Methods

2.1 Fly maintenance

All fly stocks used were raised and crossed on standard Bloomington media (see Appendix A). Stocks and crosses were kept at 23°C in temperature controlled rooms with 12/12h light/dark cycle. For all crosses, virgin pupae were picked from the driver line and crossed with 1-4 day old males from transgenic lines.

Table 2-1. *Drosophila* stocks used in experiments

	Fly name (as referred to in text)	**Details**
Transgenic lines	htau^{0N3R}	UAS-htau^{0N3R};+/+ Human 0N3R tau isoform inserted on chromosome 2. Sourced from Bloomington.
	Wld^{S}	+/+;UAS-Wld^{S} Wld^{S} gene inserted on chromosome 3. Generated in Liquin Luo lab, sourced from Torsten Bossing.
	htau^{0N3R};Wld^{S}	UAS-htau^{0N3R};UAS-Wld^{S} Double transgenic line homozygous for human tau and Wld^{S}. Generated by myself.
Driver lines	elav	elav-GAL4/y;+/+;+/+ Pan-neuronal GAL4 expression, (x chromosome). Sourced from Bloomington (C155 line).
	D42	+/+;D42-GAL4 GAL4 expression in motor neurons (chromosome 3). Sourced from Bloomington.
	vGFP.D42	+/+;D42-GAL4, UAS-NPY-GFP Neuropeptide Y fused to GFP recombined onto third chromosome with D42-GAL4. Generated by Cath Cowan (line 28a).
	Or47b-GFP	UAS-mCD8::GFP, Or47b-GAL4/CyO Or47b population of ORNs labelled with membranous GFP. Generated and obtained from Liquin Luo lab.
	D42-mitoGFP	+/+;D42-GAL4, UAS-mito-HA-GFP/TM6B, Tb^{1} Expresses GFP tagged mitochondria in motor neurons. Generated and sourced from Bill Saxton lab.
Other	Oregon R	+/+; +/+; +/+; +/+ Wild type line used as a control in some experiments.
	3703	w^{1118}/Dp(1;Y)y+; CyO/nub^{1} b^{1} sna^{Sco} lt^{1} stw^{3}; MKRS/TM6B, Tb^{1} Chromosomes 2 and 3 doubled balanced plus markers.

2.1.1 Generation of double transgenic line

Two UAS-Wld^S lines were received from Torsten Bossing (line originated from Liquin Luo lab) and the expression level of Wld^S in each line was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (see Figure 7-4 Appendix B), with the higher expresser (pUAST–Wld^S_5) selected, referred to as Wld^S throughout this thesis.

The htau^{0N3R} and Wld^S lines were crossed together (Figure 2-1) to produce a homozygous double transgenic line, htau^{0N3R};Wld^S. Expression of both transgenes was confirmed by SDS-PAGE and western blotting on elav driven lines (Figure 7-5).

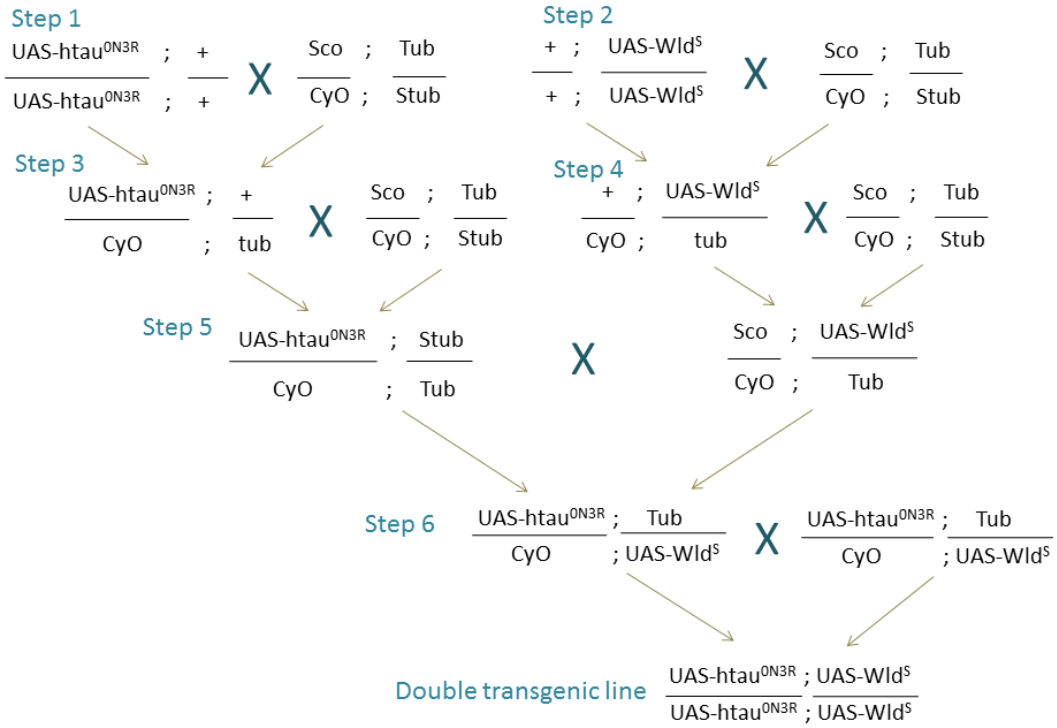


Figure 2-1. Making dual transgenic htau^{0N3R};Wld^S line. UAS-htau^{0N3R} is homozygous on chromosome 2, UAS-Wld^S is homozygous on chromosome 3. The double balancer line w1118/Dp(1;Y)y+; CyO/nub1 b1 snaSco lt1 stw3; MKRS/TM6B, Tb1 was used to track inheritance. On chromosome 2 the balancer chromosomes contain the phenotypic markers curly wings (CyO) and reduced scutellar bristles (Sco). On chromosome 3, the markers on the balancer chromosomes are stubble bristles (MKRS or Stub) and tubby larvae/pupae (Tb or tub). In steps 1 and 2 each of the homozygous lines is crossed onto the balancer line, with resultant progeny crossed back onto balancer line in steps 3 and 4. The balanced single heterozygotes are then crossed with each other in step 5. Resulting double heterozygote progeny are crossed together in step 6 and marker free progeny selected with which to establish the homozygous double transgenic line.

2.2 Western Blot

To confirm the expression of proteins within the nervous system of adult flies, SDS-PAGE of solubilised and homogenised protein extracts from fly heads was performed. Male fly heads from elav driven crosses were homogenised in homogenisation buffer (see Appendix A) before being centrifuged at 3000 x g and the pellet discarded. Supernatants were made up in 2x Laemmli buffer and boiled at 95°C for 5 minutes.

2.2.1 SDS-PAGE

20µl of each sample and 5µl of Amersham Rainbow Molecular Weight Marker (GE Healthcare Life Sciences) were loaded and run on a 5% stacking and 10% resolving gel at 160V for 1 hour. Protein was then transferred onto nitrocellulose membrane (GE Healthcare Life Sciences) at 60V for 1h30.

2.2.2 Protein visualisation & quantification

The nitrocellulose membranes were incubated in 3% bovine serum albumin (BSA) (Sigma-Aldrich) in 0.5% Tween in phosphate-buffered saline (PBST) (Sigma-Aldrich) for 1 hour at room temperature before the application of primary antibodies (Table 2-2) in which membranes were incubated in at 4°C overnight. Following 3 x 5 minute washes in PBST, fluorescently conjugated secondary antibodies were applied (Table 2-2) and incubated with the nitrocellulose membranes for 1 hour at room temperature in the dark. After a final 3 x 5 minute washes in PBST, membranes were viewed on a Li-Cor Odyssey scanner. Bands were quantified using Li-Cor Odyssey software and statistics performed using Graph Pad Prism 6.0 (GraphPad Software, Inc.).

Table 2-2. Antibodies used in western blotting

Antibody raised against (animal raised in):	Supplier	Concentration used
Mouse anti-human tau (monoclonal)	Ab74137, Abcam, Cambridge, UK	1:500-1:1000
Rabbit anti-human tau (polyclonal)	A002401-2, Dako, Cambridge, UK	1:1000
Rabbit anti-Wld^{S} (w18) (polyclonal)	Michael Coleman, Babraham Inst., UK	1:2000 and 1:5000
Goat anti-mouse AF680	Alexa Fluor, Invitrogen, UK	1:20000
Goat anti-rabbit IRDye 800	IRDye, Licor, UK	1:20000

2.3 Behavioural assays

2.3.1 Larval open field assay

Wandering third instar larvae from D42 driven crosses were placed in the centre of 0.3% Alsian Blue (Sigma-Aldrich) 1% agarose (Sigma-Aldrich) plates, 1 per plate with 4 plates recorded at one time. A video was recorded for 2 minutes before the larvae were returned to the centre of the plates and another 2 minute video recorded, to allow the larvae to equilibrate and familiarise themselves with the arena. Larvae were returned once more to the centre of each plate and a final 2 minute video recorded; only the 3rd and final trial was used for analysis. (See Appendix A for recording equipment details). Larval movement was analysed using Ethovision 3.0 (Noldus) tracking software which measured velocity and turning rate; one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison post-hoc analysis were performed using GraphPad Prism 6.0.

2.3.2 Negative geotaxis assay

1-2 day old males from elav driven crosses were selected and placed into vials, 10 flies per vial (constituting 1n). Once weekly, flies were anaesthetised with CO_2 and placed in 50ml measuring cylinders and left to recover and acclimatise for 30 minutes. Cylinders were tapped sharply three times and a video recorded for 20 seconds. The flies were left to recover for 30 seconds before repeating, for a total of three times with only the third and final video used for analysis. This was performed each week for 6 weeks. The height climbed by each fly after 10 seconds was recorded against the markings on the cylinder (arbitrary units) and a mean calculated for the vial. Statistics were performed using GraphPad Prism 6.0; a repeated measures two-way ANOVA and Bonferroni's multiple comparison post-hoc analysis.

2.4 Axonal transport assay

Wandering third instar larvae from vGFP.D42 driven crosses were anaesthetised in diethyl vapour (Thermo Fischer Scientific) for 15 minutes then mounted in 1% agarose (Sigma-Aldrich), ventral side up under a coverslip. Images of vGFP aggregations in the 4 peripheral nerves on each side in segments A3 and A4 (total of 8 axons per larva; Figure 2-2) were taken at x63 on an Axioplan2 MOT upright Epifluorescence microscope (Zeiss) equipped with Micro Max CCD (Princeton Instruments) using MetaMorph acquisition

software (Molecular Devices). Images were thresholded and the area covered by aggregates measured in Metamorph software or ImageJ. A one-way ANOVA and Bonferroni's multiple comparison post-hoc analysis was performed using GraphPad Prism 6.0

2.5 Longevity

1-2 day old males from elav driven crosses were selected and placed into vials, 10 flies per vial, 5 vials per genotype. Control flies were generated by crossing male elav's with female UAS-transgenic lines, from which male progeny (which do not express GAL4) were selected (see Figure 7.3 Appendix A). The number of dead flies was recorded 3 times a week, with flies placed in fresh food vials twice a week. Survival was plotted as a Kaplan Meier curve, with the difference between the curves assessed using a Mantel-Cox test, with the P-value threshold (α) adjusted for multiple comparisons by the Bonferroni method, using GraphPad Prism 6.0.

2.6 Immunohistochemistry

2.6.1 Larval skins – NMJ morphology analysis

Wandering third instar larvae from D42 driven crosses were placed in *Drosophila* saline on a Sylgard dissection plate and pinned through their mouthparts. The posterior tip was cut off and a dorsal midline cut made along the length of the larva. The internal organs were removed with care taken not to damage or remove the ventral nerve cord or segmental nerves. The skin was stretched and pinned out before fixation in 4% formaldehyde for 90 minutes at room temperature.

Dissected and fixed larval skins were washed in 0.1% Triton X in PBS (3 times for 10 minutes each) before blocking in 5% normal goat serum, 3% horse serum and 2% BSA in 0.1% PBS-Tx for 90 minutes at room temperature. Skins were incubated with goat anti-horseradish peroxidase (HRP) antibodies conjugated to fluorescein isothiocyanate (FITC) (1:1000; ICN/Cappel) at 4°C overnight. Skins were washed in 0.1% PBS-Tx (3 times for 10 minutes each) and put through an ascending glycerol series (50, 70, 90 and 100%; 5 minutes in each) before being mounted in Vectashield (Vector Laboratories) and imaged. NMJ's on muscle 4 from segments A3-5 (Figure 2-2) were imaged using a Leica SP2 scanning confocal microscope using the 488nm argon laser. Maximum projections of Z stacks were generated

upon which morphometric analysis was performed using ImageJ. Bouton area was measured using the free draw tool and the interbouton axon width measured between each bouton on the entire NMJ. For each metric, binning into discrete categories was performed to establish the proportion of boutons/axon widths in each bin as a percentage of the total. Statistical analysis was performed using GraphPad Prism 6.0; one-way ANOVA and Bonferroni's multiple comparison post-hoc analysis.

2.6.2 Larval skins – mitoGFP

Wandering third instar larvae from D42-mitoGFP crosses were dissected, washed and blocked as above. Skins were washed in 0.1% PBS-Tx (3 times for 10 minutes each) prior to incubation with goat anti-HRP antibodies conjugated to Rhodamine (1:200; Jackson Immuno Research Laboratories) overnight at 4°C. Skins were washed in 0.1% PBS-Tx (3 times for 10 minutes each) and put through an ascending glycerol series (50, 70, 90 and 100%; 5 minutes in each) before being mounted in Vectashield and imaged. Images of NMJ's on muscle 4 from segments A3-5 (Figure 2-2) were acquired using an Axioplan2 MOT upright Epifluorescence microscope (Zeiss) equipped with a QImaging Retiga 3000 CCD Camera (Photometrics) using Metamorph software (Molecular Devices). A total of 3-6 images were acquired per larva, with 1 larva constituting 1n. Images were thresholded in ImageJ, and the area of the NMJ covered by mitochondria was calculated as a percentage of the total mitochondria area. Differences between the groups were analysed by unpaired Student't t-test, using GraphPad Prism 6.0.

2.6.3 Adult brains

Adults were collected 0-2 days after eclosion from Or47b-GFP driven crosses and aged to the appropriate time point. Flies were anaesthetised with CO_2 and their heads ligated using a blade before being placed in 4% formaldehyde at room temperature prior to dissection. The head was transferred to PBS and, using two pairs of Dumont #5 forceps, the proboscis removed before the eyes and cuticle were peeled away to expose the brain (Figure 6-3 Appendix A). Remaining eye lamina and trachea were carefully removed after which the dissected brain was placed in 4% formaldehyde and fixed at room temperature for 45 minutes.

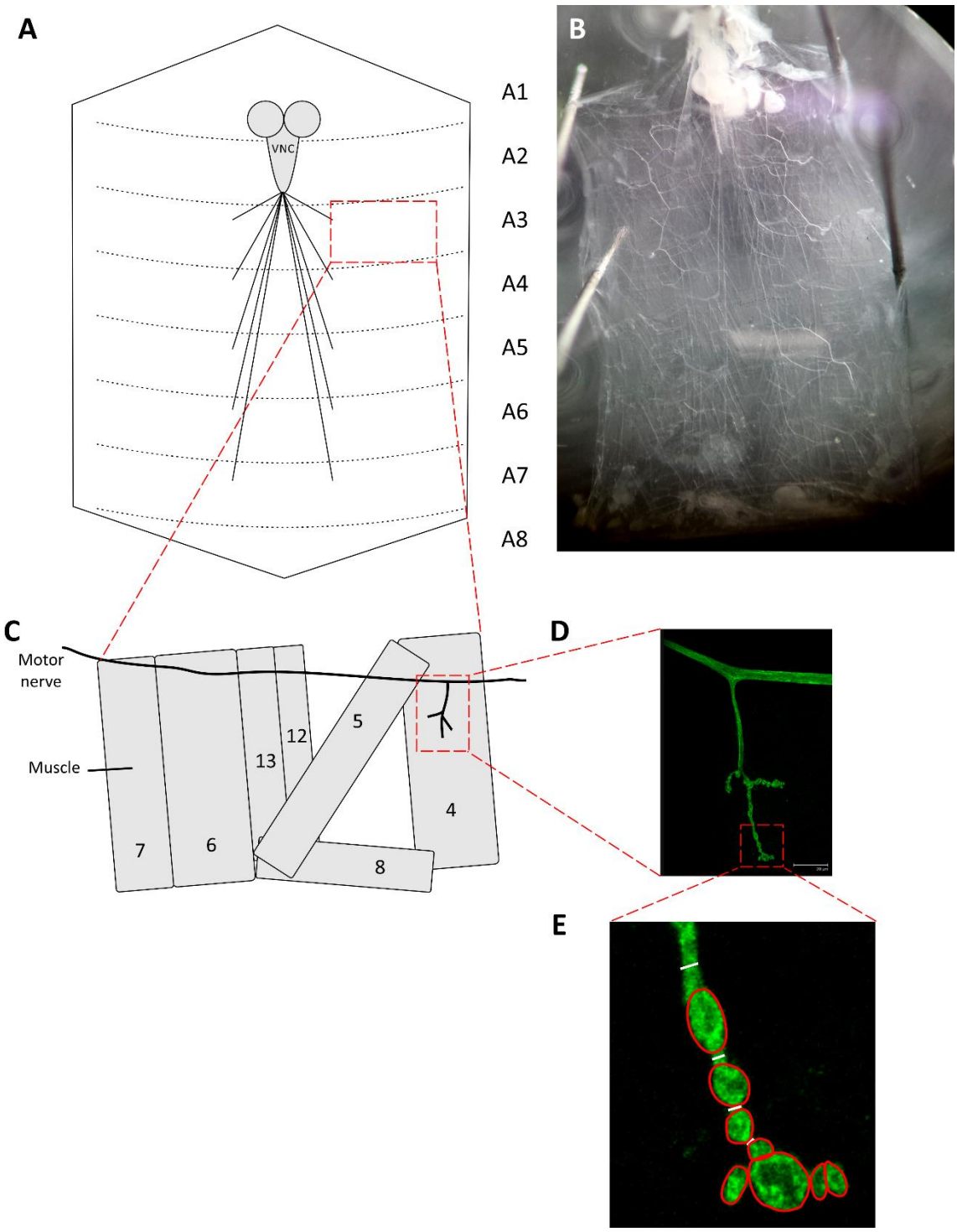


Figure 2-2. Diagram of larval imaging. [A] Diagram and [B] photo of larval dissection showing ventral nerve cord (VNC) and peripheral nerves of the larval nervous system. [C] Identification of muscle 4 in the characteristic muscle layout of each segment. [D] Representative image of muscle 4 NMJ. [E] Bouton area was measured using the freedraw tool (red circles) and the interbouton axon width was measured by drawing a line (white line) the same diameter as the axon inbetween the boutons.

After fixation, brains were washed in 0.1% PBS-Tx (3 times for 10 minutes each) before either mounting in Vectashield or proceeding for staining. Those to be stained were blocked in 5% goat serum, 3% horse serum, 2% BSA in 0.1% PBS-Tx for 1 hour at room temperature. Brains were then stained with rabbit anti-human tau antibodies (1:1000; Dako) for 6 hours at room temperature, washed and incubated in goat anti-rabbit Alexa Fluor 563 (1:1000; Invitrogen) overnight at 4°C. Brains were washed and mounted in Vectashield prior to imaging on an Axioplan2 MOT upright Epifluorescence microscope (Zeiss) equipped with a QImaging Retiga 3000 CCD Camera (Photometrics), images were acquired using Metamorph software (Molecular Devices).

To quantify degeneration, axonal swellings were measured using Image J. Images were coded and all time points and genotypes pooled to ensure the assessor was blind to condition. As swellings exhibited a greater intensity of GFP signal, each image was thresholded and the area of the swellings measured; the glomeruli were not included in the measurement. The mean coverage area for each time point and genotype was calculated and the differences between groups were analysed using GraphPad Prism 6.0; a two-way ANOVA and Bonferroni's multiple comparison post-hoc analysis.

2.7 Injury assay

The third antennal segment was removed from flies, 1 or 3 weeks after eclosion (wae) from Or47b-GFP driven crosses, under CO_2 anaesthesia using Dumont #5 forceps (Figure 2-3). At the appropriate time points, flies were anaesthetised with CO_2 and their heads ligated using a blade before being placed in 4% formaldehyde at room temperature prior to dissection. The head was then placed in PBS and dissected, removing the eyes and cuticle to expose the brain before carefully removing any remaining eye lamina or trachea. The dissected brain was placed in 4% formaldehyde and fixed at room temperature for 45 minutes before being placed in 0.1% PBS Tx and washed. Brains were mounted in Vectashield and imaged on an Axioplan2 MOT upright Epifluorescence microscope (Zeiss) equipped with a QImaging Retiga 3000 CCD Camera (Photometrics), images were acquired using Metamorph software (Molecular Devices).

Degeneration was quantified by previously described methods (Macdonald et al., 2006). Briefly, with the assessor blind to genotype and time point, the presence of the commissural axons was recorded (Y/N). The percentage of brains of each genotype at each time point

with intact axons was calculated and graphed in GraphPad Prism 6.0. The intensity of GFP signal within glomeruli was measured using ImageJ. Using the circle draw tool (70 pixels diameter), the intensity of signal from glomeruli was measured and the background intensity subtracted. The mean was calculated for each genotype and time point, and made relative to the baseline measurement for each genotype. Statistics were performed using GraphPad Prism 6.0; a two-way ANOVA and Bonferroni's multiple comparison post-hoc analysis.

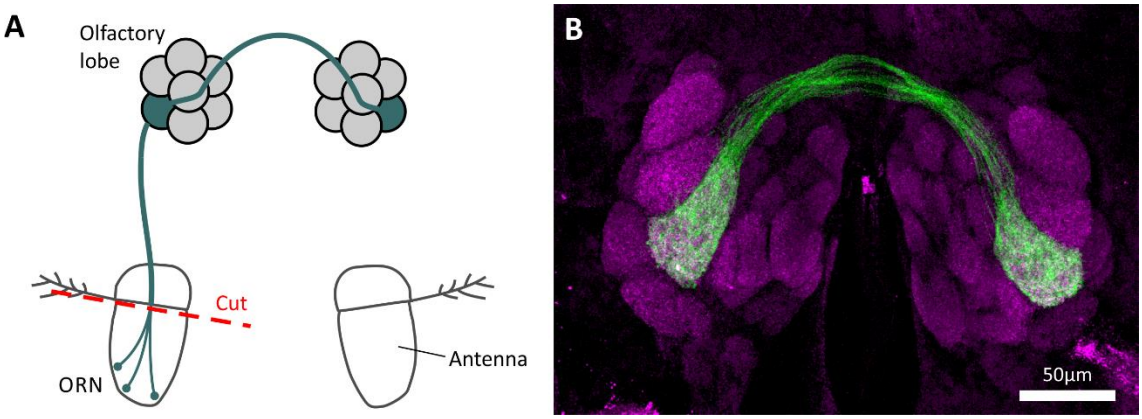


Figure 2-3. Olfactory receptor system in *Drosophila*. [A] Olfactory receptor neurons (ORNs) have their cell bodies in the adults fly's 3rd antennal segment. The ORN projects into the olfactory lobe, sending another projection to the contralateral lobe. Removal of the 3rd antennal segment causes axotomy. [B] Expression of mCD8::GFP in the Or47b population.

2.8 Transmission electron microscopy

Elav driven third instar larvae were pinned out in *Drosophila* saline (Appendix A), a dorsal midline cut made half the length of the larva and the intestines carefully removed. The saline was replaced with 4% formaldehyde, 3% glutaraldehyde, 0.1M PIPES and tissue fixed for 30 minutes at room temperature. The skin was unpinned and placed in fixative overnight at 4°C. Tissue was rinsed in 0.1M PIPES before being post-fixed in 1% osmium tetroxide and rinsed again. Tissue was incubated in 2% uranyl acetate then dehydrated in an ascending alcohol series before being incubated in acetonitrile then 50:50 acetonitrile:Spurr resin overnight. Tissue was transferred to resin for 6 hours, then embedded in fresh resin in flat embedding moulds and polymerised in a 60°C oven for 24 hours. Transverse ultra-thin sections were cut on an ultramicrotome (Leica, UK) and mounted on silver grids before being stained with lead nitrate and imaged on a Hitachi H7000 transmission electron microscope.

2.9 Statistical analysis

Values are presented as the mean ± standard error of the mean. To compare differences between groups, statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.) and unless otherwise indicated, a one way ANOVA was performed, followed by a Bonferroni multiple comparisons test. For adult negative geotaxis behaviour, a two way repeated measures ANOVA followed by Bonferroni multiple comparisons test was used. For longevity data, survival was plotted as a Kaplan-Meier plot and the differences between curves assessed using a Mantel-Cox test, with the P-value threshold (α) adjusted for multiple comparisons using the Bonferroni method. For analysis of degeneration in adult brains (both uninjured and injured), a two way ANOVA followed by Bonferroni multiple comparisons test was used. For the mitoGFP coverage, an unpaired Student's t-test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Chapter 3: Can Wld^S improve tau-mediated axonal dysfunction or delay tau-mediated degeneration?

3.1 Introduction

The axon is emerging as a key site for tau pathology in neurodegenerative disease, with increasing evidence of white matter changes indicative of axonal degeneration in tauopathies such as AD (Huang et al., 2007, Sun et al., 2014). Studies in animal models have demonstrated that axonal dysfunction in tauopathy is typified by disrupted axonal transport (Mudher et al., 2004, Kanaan et al., 2011, Kim et al., 2011), due to tau phosphorylation causing reduced cytoskeletal integrity (Cowan et al., 2010). Evidence of axonal degeneration has also been found in these animal models, such as axonal swellings and loss of white matter integrity in P301L-tau mice (Probst et al., 2000, Lin et al., 2003, Sahara et al., 2014). Understanding the dysfunctional and degenerative mechanisms occurring in tauopathy will aid research into potential therapies for diseases such as Alzheimer's.

Morphological similarities between axonal degeneration in neurodegenerative disease and Wallerian degeneration after injury have led some to suggest that the processes may be related. Axonal swellings are a prominent feature of axonal degeneration in the CNS following injury and in disease, with both situations displaying the following features:

- Accumulation cytoskeletal components (often disorganised)
- Accumulation of autophagosomes
- Interruption of axonal transport (accumulation of vesicles and mitochondria)
- Altered mitochondrial morphology (condensed and rounded)
- Thinning of myelin sheath

The morphological changes associated with axonal degeneration have been described for over 150 years and were thought to be due to the passive wasting of disconnected axons. The more recent discovery of the Wld^S protective mutation, which robustly delays Wallerian degeneration, indicated the existence of a molecular pathway controlling axonal degeneration after injury. Investigation of Wld^S in models of neurodegenerative disease has demonstrated a delay in axonal degeneration in some systems but not in others (Table 1-2). This conflicting evidence has challenged the idea that a single molecular pathway controls axonal degeneration in both injury and disease.

The aim of this chapter is to investigate whether the axonal protection mediated by Wld^S can impact upon tau-mediated axonal dysfunction and degeneration. Considering that cytoskeletal breakdown is a key event in Wallerian degeneration (Schlaepfer and Bunge, 1973, Schlaepfer and Micko, 1978) and is delayed by Wld^S, investigating this protection in

tauopathy is particularly interesting as microtubule destabilisation has been implicated in tau-mediated axonal dysfunction and degeneration (Mudher et al., 2004, Cowan et al., 2010).

The *Drosophila* model of tauopathy is well-established, with characterisation of both larval and adult stages. By co-expressing human tau ($htau^{0N3R}$) and Wld^{S} in the larval nervous system it will be possible to investigate whether Wld^{S} can improve $htau^{0N3R}$-mediated dysfunctional phenotypes, as the larvae represent a dysfunctional system without overt degeneration. Functional readouts of axonal function, such as locomotor behaviour and axonal transport, will indicate whether Wld^{S} can improve axonal dysfunction. In the adult model, both axonal dysfunction and degeneration contribute to phenotypes such as locomotor behaviour and lifespan, therefore changes to these could indicate that Wld^{S} can delay tau-mediated degeneration. In addition, imaging of axons will confirm whether any phenotypic improvements are mediated through a delay of axon degeneration.

3.2 Materials & Methods

3.2.1 Axonal transport assay

Wandering third instar larvae from vGFP.D42 driven crosses were anaesthetised, mounted, and images of vGFP aggregates captured. Whilst blinded to genotype, the coverage of aggregates was measured per animal and the mean per genotype calculated.

3.2.2 Electron microscopy

Wandering third instar larvae from elav driven crosses were partially dissected and fixed in 4% formaldehyde, 3% glutaraldehyde, 0.1M PIPES. Larvae were post-fixed in 1% buffered osmium tetroxide, block stained in 2% aqueous uranyl acetate, dehydrated in an ethanol series and embedded in Spurr resin. Transverse ultrathin sections were mounted on copper grids and peripheral nerves imaged.

3.2.3 Behavioural assays

Larval locomotion

Wandering third instar larvae from D42 driven crosses were placed in the centre of a 10 cm plate, 1 per plate with 4 plates recorded at one time. Videos were recorded and analysed using Ethovision 3.0 tracking software (Noldus). Velocity, angular velocity (turning rate over time) and meander (turning rate over distance) were recorded and calculated per genotype.

Adult negative geotaxis assay

Newly eclosed males from elav driven crosses were selected and placed into vials, 10 flies per vial (constituting 1n). Once weekly, flies were assessed for climbing ability in response to a tapping stimulus, over a total of 6 weeks. The height climbed by each fly after 10 seconds was recorded against the markings on the cylinder (arbitrary units) and a mean calculated for the vial.

3.2.4 Longevity

Newly eclosed males from elav driven crosses were selected and placed into vials, 10 flies per vial, 5 vials per genotype. The number of dead flies was recorded 3 times a week, with flies placed in fresh food vials twice a week.

3.2.5 Immunohistochemistry

Larval skins

Wandering third instar larvae from D42 driven crosses were dissected in *Drosophila* saline and prior to fixation in 4% formaldehyde. Neuronal membranes in larval skins were stained using goat anti-HRP conjugated to FITC (1:1000; ICN/Cappel). Confocal image projections were acquired of neuromuscular junctions, and synaptic morphology analysed.

Adult brains

Newly eclosed males from Or47b-GFP driven crosses were selected and aged to their relevant time points. Brains were dissected in PBS prior to fixation in 4% formaldehyde. Unstained brains were mounted in Vectashield (Vector Labs) and Or47b axons imaged. A subgroup of brains was immunostained for human tau (1:1000 rabbit anti-human tau; Dako) and visualised using a fluorescent secondary (1:1000 goat anti-rabbit Alexa Fluor 568; Invitrogen), before being mounted in Vectashield and imaged.

The coverage of axonal swellings was measured using ImageJ with the assessor blind to genotype and time point.

3.3 Results

3.3.1 Wld^S does not improve tau-mediated dysfunctional phenotypes

Axonal dysfunction

Dysfunctional axonal transport has been reported in many models of tauopathy, both *in vitro* and *in vivo* (Morfini et al., 2002, Kanaan et al., 2011, Mudher et al., 2004). In the *Drosophila* model of tauopathy, disrupted axonal transport is characterised by the accumulation of vesicles into large aggregates, with this phenotype attributed to breakdown of the microtubule cytoskeleton (Mudher et al., 2004, Cowan et al., 2010, Quraishe et al., 2013). To investigate whether Wld^S could improve tau-mediated axonal transport dysfunction, axonal transport was assayed in *Drosophila* larvae. The transparent cuticle of *Drosophila* larvae enables *in vivo* investigation of axonal transport in anathestised animals transgenic for GFP tagged vesicles.

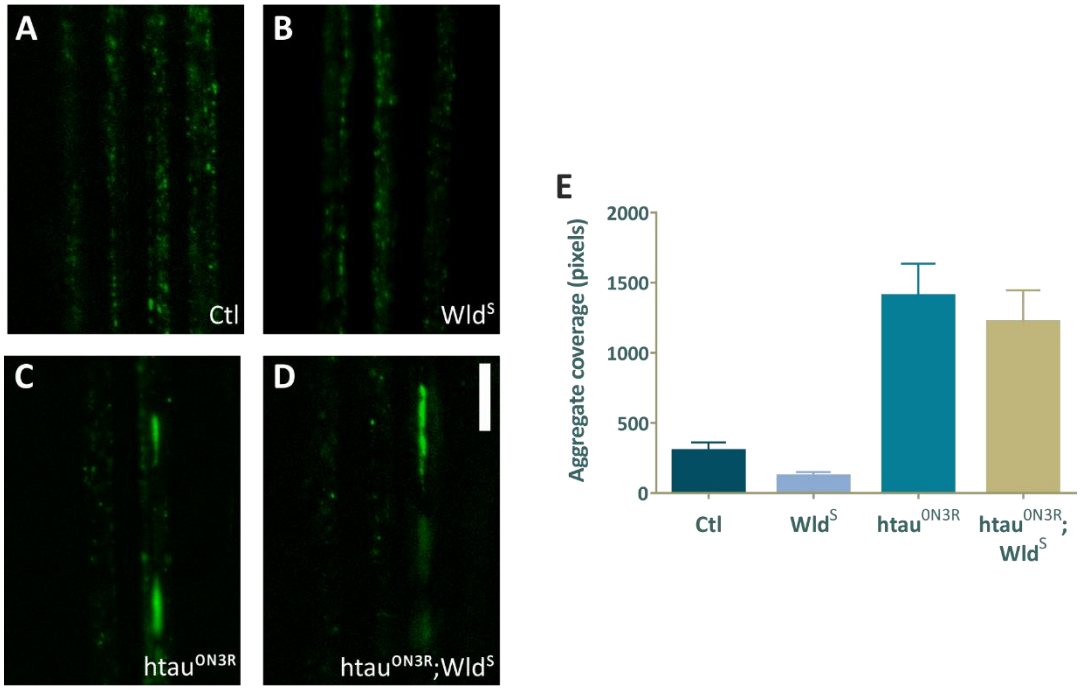


Figure 3-1. Co-expression of Wld^S does not improve vesicular aggregation in larvae. Expression in larvae was driven using D42 Gal4, UAS-NPY::GFP and peripheral segmental nerves imaged. [C] In htau^{0N3R} larvae, aggregates of GFP tagged neuropeptide Y containing vesicles are evident in motorneurons. [D] Co-expression of Wld^S with htau^{0N3R} does not reduce vesicular aggregation. [E] The area covered by vesicular aggregations for each genotype is not significantly altered in htau^{0N3R} larvae by the co-expression of Wld^S. Values are presented as the mean ± SEM. *n*=10 larvae, ANOVA, Bonferroni's multiple comparison.

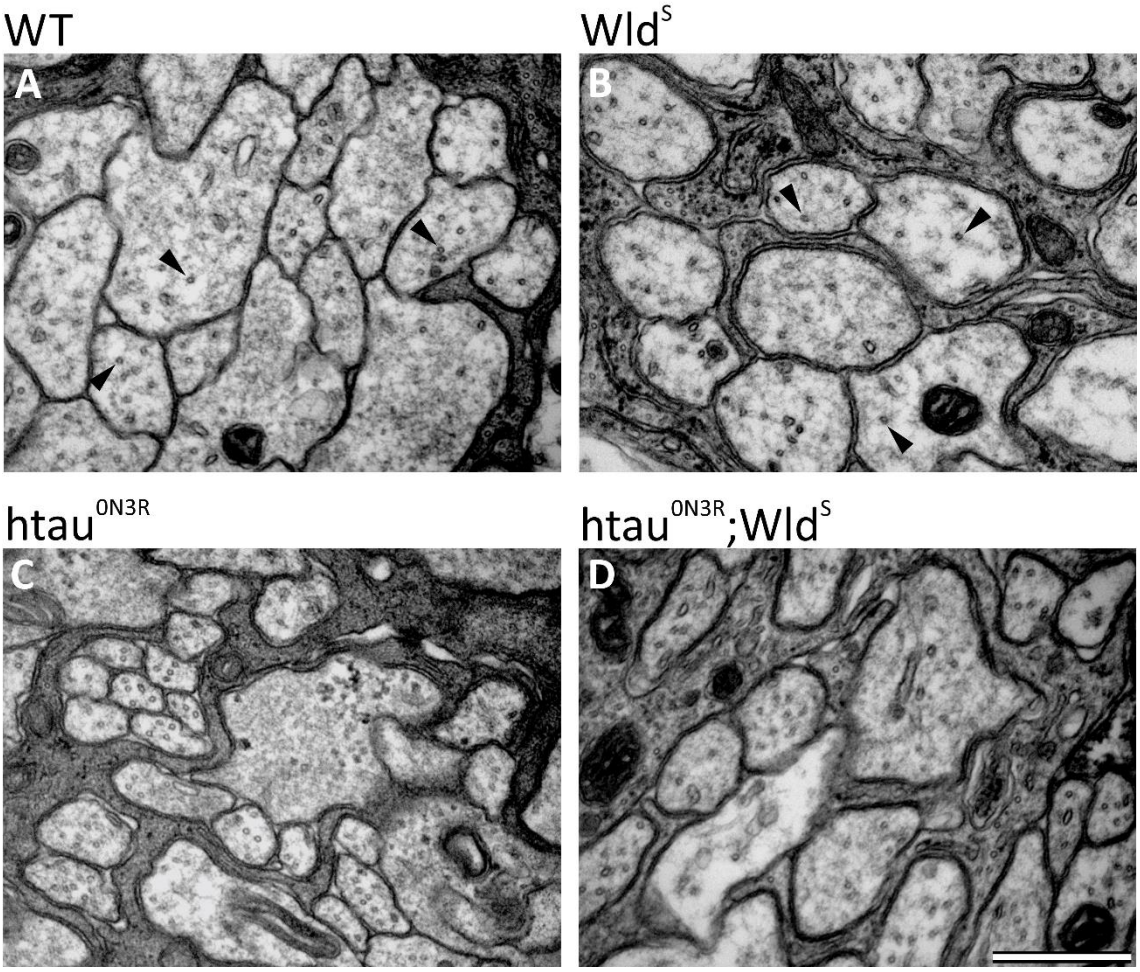


Figure 3-2. Investigation of cytoskeletal integrity in larvae. Transmission electron micrographs indicate that expression of human tau results in fewer microtubule profiles within axons in transverse sections of larval peripheral nerve. **Initial investigations reveal this is not altered by the co-expression of Wld^S with htau^{0N3R}.** Scale bar = 500nm.

Expression of neuropeptide-Y tagged GFP (vGFP) along with htau^{0N3R}, Wld^S and htau^{0N3R};Wld^S was driven in motor neurons using D42-Gal4. In control larvae, a homogeneous distribution of vGFP is observed throughout the motor neurons (Figure 3-1 A). As described previously (Mudher et al., 2004, Quraishe et al., 2013) expression of htau^{0N3R} results in the appearance of large aggregations of vGFP indicating a disruption of axonal transport (Figure 3-1 C). In htau^{0N3R};Wld^S larvae, these vesicular aggregations remain evident, with quantification confirming no significant effect of Wld^S expression on axonal transport dysfunction (Figure 3-1 E). This indicates that htau^{0N3R}-mediated axonal transport dysfunction is unaffected by the co-expression of Wld^S.

The axonal transport dysfunction in this *Drosophila* model is thought to be caused by cytoskeletal breakdown due to the loss of microtubule stabilisation by hyperphosphorylated

tau. This is supported by evidence showing that strategies which rescue the microtubule cytoskeleton also restore axonal transport (Mudher et al., 2004, Quraishe et al., 2013). The integrity of the cytoskeleton can be investigated using transmission electron microscopy (TEM) of transverse sections of larval peripheral nerves. Previous work has demonstrated that expression of htau^{0N3R} results in a disorganisation of microtubules, indicated by a reduction in visible microtubule profiles and the appearance of misaligned microtubules (Cowan et al., 2010).

In electron micrographs of control larval peripheral nerves, regularly spaced microtubules appear as 25nm diameter circles within the axon. However, expression of htau^{0N3R} results in the disruption of the microtubule cytoskeleton, with a reduction in the number of microtubules and an increase in misaligned microtubules (Figure 3-2 C). The reduction in the number of microtubule profiles is most evident in larger calibre axons, with many of these appearing almost devoid of microtubules compared to controls (Figure 3-2 D). This phenotype was did not appear to be improved when Wld^{S} was co-expressed with htau^{0N3R}, however the limited size of this preliminary investigation prevented quantification to confirm this.

This data indicates that Wld^{S} is unable to improve the cytoskeletal alterations occurring in tauopathy which underlie axonal transport disruption. This suggests that the mechanisms of cytoskeletal breakdown in injury differ from those in tauopathy.

Synaptic morphology

Tau induced synaptic dysfunction has been previously report in this model based on both morphological and functional analyses (Chee et al., 2005). There is mixed evidence of Wld^{S}-mediated synaptic protection from both axotomy and disease models (Gillingwater et al., 2002, Vande Velde et al., 2004, Fischer et al., 2005, Wright et al., 2010), so I was interested to see whether Wld^{S} could provide any benefit in our *Drosophila* model of tauopathy.

To gain insight into potential effects of Wld^{S} at the synapse, immunostaining of NMJs was performed using FITC-conjugated anti-horseradish peroxidase, which in invertebrates binds neuronal membrane glycoproteins (Paschinger et al., 2009). In control larvae, the type I boutons of the NMJ on muscle 4 display the characteristic beads on a string structure with arborisation of the synapse over the muscle (Figure 3-3 A). This morphology is severely disrupted in htau^{0N3R} larvae, typified by thinning of the inter-bouton axon and the appearance of smaller 'mini-satellite' boutons (Figure 3-3 C).

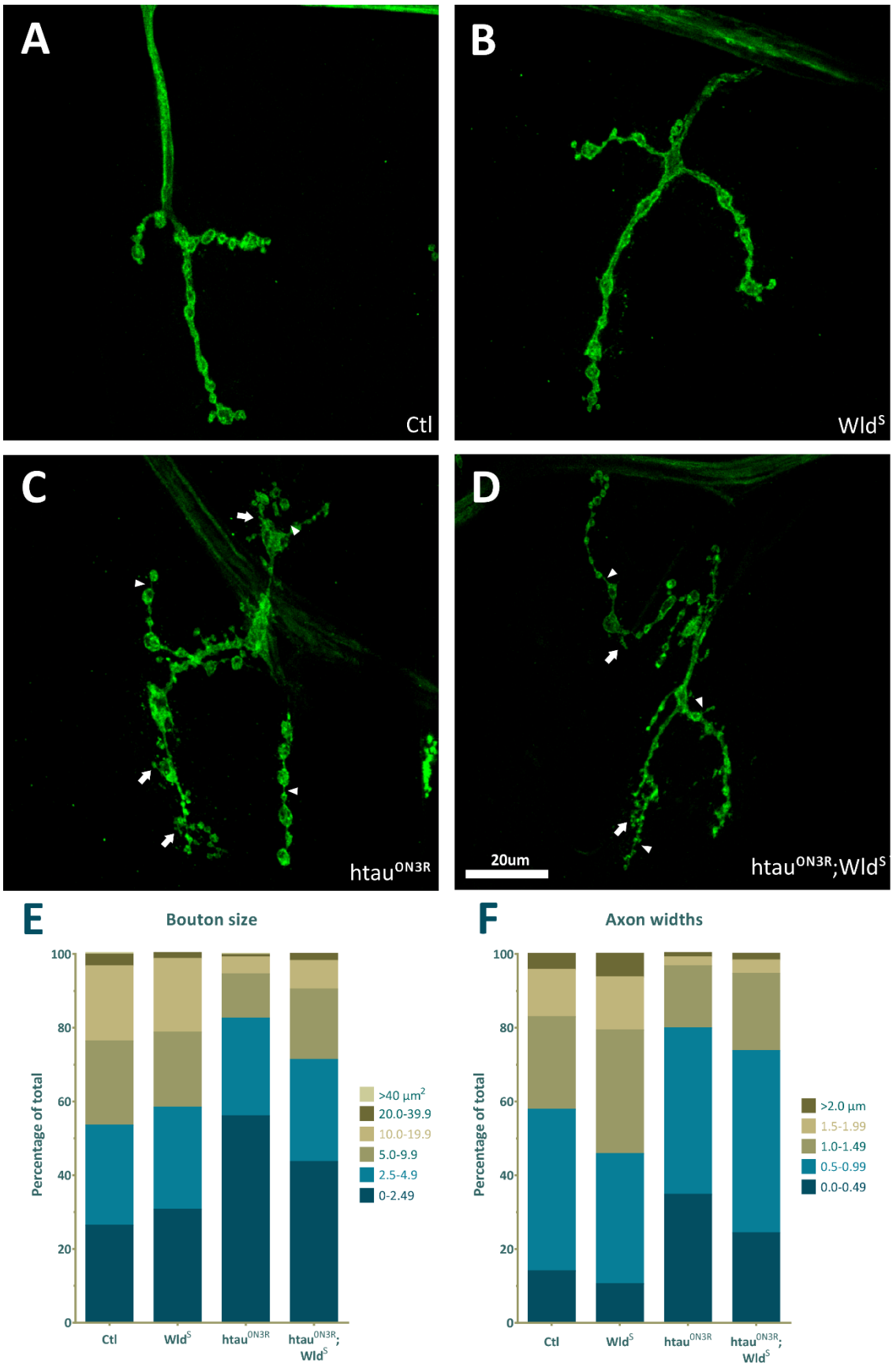

A
Ctl
B
Wld^S
C
htau^0N3R
D
20um
htau^0N3R;Wld^S
E
Bouton size
Percentage of total
100
80
60
40
20
0
Ctl
Wld^S
htau^0N3R
htau^0N3R; Wld^S
>40 µm²
20.0-39.9
10.0-19.9
5.0-9.9
2.5-4.9
0-2.49
F
Axon widths
Percentage of total
100
80
60
40
20
0
Ctl
Wld^S
htau^0N3R
htau^0N3R; Wld^S
>2.0 µm
1.5-1.99
1.0-1.49
0.5-0.99
0.0-0.49

Figure 3-3. (Previous page) Larval NMJ morphology is not rescued by co-expression of Wld^S with htau^{0N3R}. Projections of larval muscle 4 neuromuscular junctions stained with anti-horseradish peroxidase conjugated to FITC (scale bar = 20μm). [A, B] Control larvae display characteristic bead-like distribution of presynaptic boutons. [C] Human tau expression results in aberrant NMJ morphology with thinning of the inter-bouton axon (arrow heads) and the appearance of mini-satellite boutons (arrows). [D] NMJs from htau^{0N3R};Wld^S larvae display the aberrant morphology of htau^{0N3R} NMJs. [E] Quantification of bouton size revealed htau^{0N3R} NMJs displayed an increase in small boutons and a decrease in larger boutons, which was not significantly different when Wld^S was co-expressed. [D] Quantification of axon width showed htau^{0N3R} results in a significant thinning of the inter-bouton axon, and this is not significantly improved upon co-expression of Wld^S . Values are presented as the mean ± SEM. n=4 larvae (3-6 NMJs analysed per larva). ANOVA, Bonferroni's multiple comparison.

When Wld^S is co-expressed with htau^{0N3R}, this aberrant morphology is still evident (Figure 3-3 D), with this reflected in the quantification of bouton size and axonal width (Figure 3-3 E & F). This quantification reveals that there is approximately double the proportion of the smallest boutons (0-2.49 μm^2) in htau^{0N3R} larvae compared to controls, reflecting the appearance of mini-satellite boutons in these animals. There is a concomitant reduction in larger boutons (10.0-19.9 μm^2) in htau^{0N3R} larvae, with this effect not significantly altered in htau^{0N3R};Wld^S larvae (Figure 3-3 E). There is a 3-fold increase in the proportion of axons falling in the smallest width category (0-0.49μm) in htau^{0N3R} larvae compared to controls; whilst there is a reduction in this category in htau^{0N3R};Wld^S larvae the change is not significant (Figure 3-3 F). This data indicates that Wld^S is unable to impact on htau^{0N3R}-mediated synaptic disruption.

Locomotor behaviour

Dysfunctional axonal transport and synaptic disruption manifest in altered locomotor behaviour in htau^{0N3R} expressing larvae, with strategies to improve these also rescuing behavioural defects (Quraishe et al., 2013). As Wld^S was unable to improve tau-mediated axonal transport dysfunction or abberant synaptic morphology, I wanted to confirm that it would be unable to improve tau-mediated behavioural phenotypes.

The use of an open-field assay enabled the assessment of a number of behavioural parameters, shedding light on the impact of Wld^S expression on htau^{0N3R}-mediated deficits, via the use of the image tracking software Ethovision. As shown previously (Quraishe et al., 2013), htau^{0N3R} expressing larvae exhibit restricted movement characterised by a reduced distance travelled with the path taken appearing twisted (Figure 3-4 C).

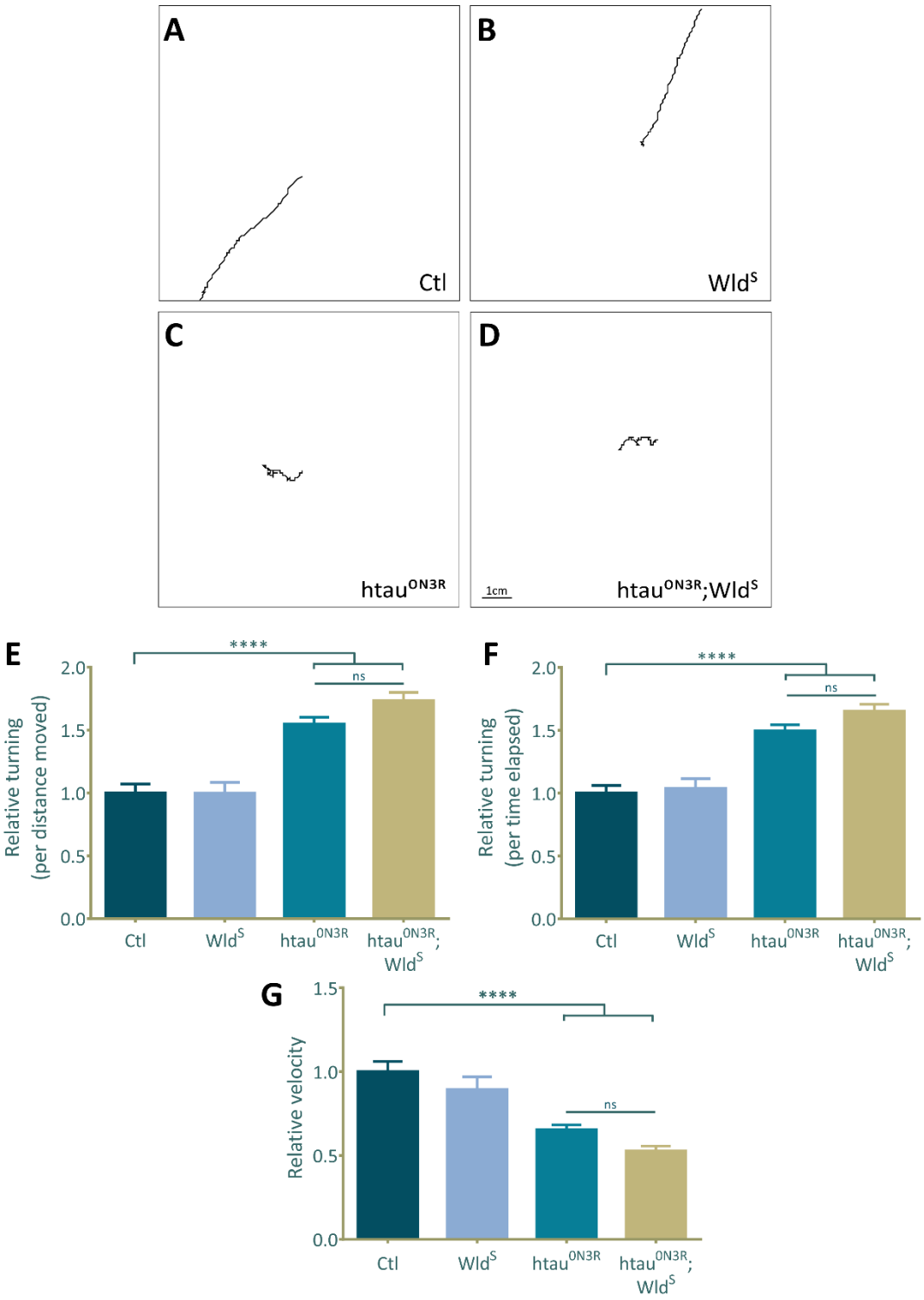


Figure 3-4. Larval locomotor activity is not improved by the co-expression of Wld^S with htau^{0N3R}. Representative traces of the paths taken by [A] control, [B] Wld^S, [C] htau^{0N3R} and [D] htau^{0N3R};Wld^S expressing larvae. Larvae expressing htau^{0N3R} or htau^{0N3R};Wld^S follow a curvilinear path compared to the straight path taken by controls and Wld^S larvae, as indicated by the quantification of [E] meander and [F] angular velocity. [G] htau^{0N3R} and htau^{0N3R};Wld^S larvae also exhibit reduced velocity compared with the control or Wld^S larvae. Values are presented as the mean ± SEM. n=17-20 larvae, ****P<0.0001, ANOVA, Bonferroni's multiple comparison. n.s., not significant.

This behavioural phenotype was not reversed by the co-expression of Wld^S (Figure 3-4 D). Quantification using Ethovision supports these observations, showing a decrease in velocity (Figure 3-4 G) and an increase in turning rate in $htau^{0N3R}$ larvae (Figure 3-4 E & F), which is not improved upon co-expression of Wld^S.

Neither axonal nor neuronal loss have been reported in the *Drosophila* larval model of tauopathy (Mudher et al., 2004, Chee et al., 2005), therefore the phenotypes observed with tau-expression are due to neuronal dysfunction. Considering that Wld^S is reported to provide benefit by delaying axonal degeneration and as degeneration is not present in larvae, I next sought to investigate Wld^S in the adult fly. Both neuronal dysfunction and degeneration contribute to tau-mediated phenotypes in adult *Drosophila*, therefore Wld^S may provide benefits here that it did not in larvae.

3.3.2 Wld^S does not delay tau-mediated axonal degeneration

Investigations in the larval model revealed Wld^S was unable to improve tau-mediated dysfunctional phenotypes, so I next wanted to investigate whether Wld^S could improve phenotypes associated with tau-mediated degeneration. Neuronal loss has not been reported in the larval *Drosophila* model of tauopathy (Mudher et al., 2004), therefore to investigate whether Wld^S can delay tau-mediated degeneration, adult flies were used. Progressive tau-mediated neuronal loss has been observed in the adult *Drosophila* brain, indicated by condensation and fragmentation of neuronal nuclei and vacuolisation (Wittmann et al., 2001, Khurana et al., 2006). Therefore in the adult fly, both neuronal loss and neuronal dysfunction are contributing factors to tau-mediated phenotypes such as altered locomotor behaviour (Mudher et al., 2004) and reduced lifespan (Folwell et al., 2010).

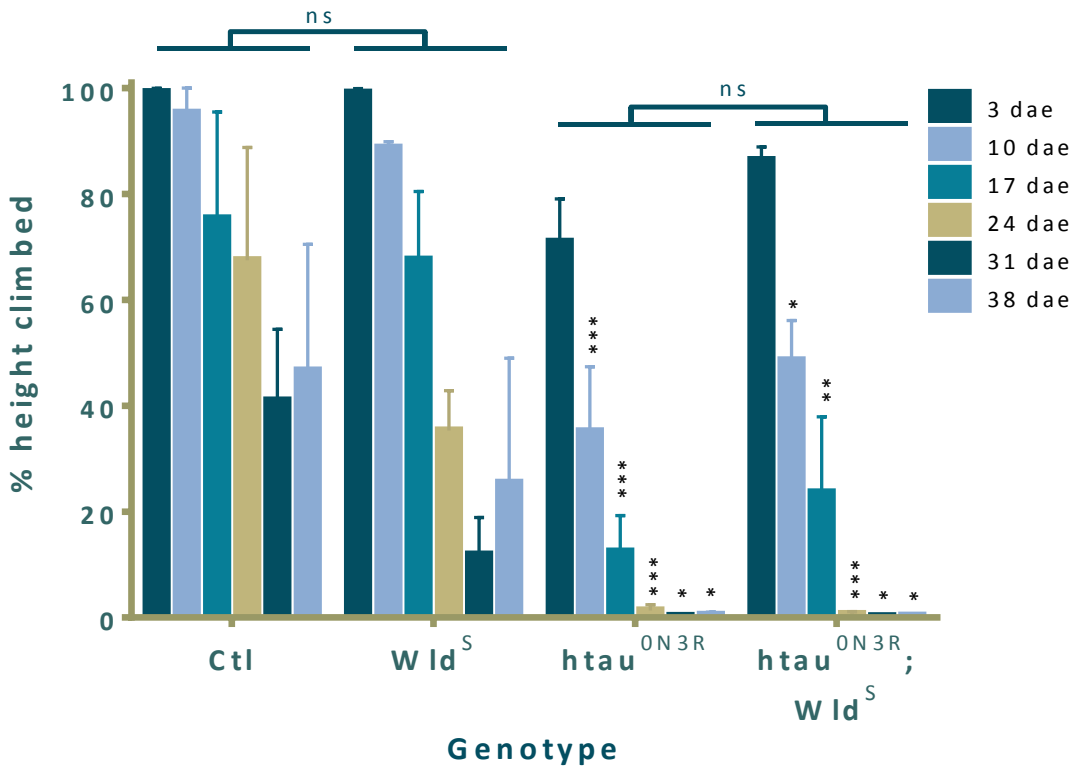


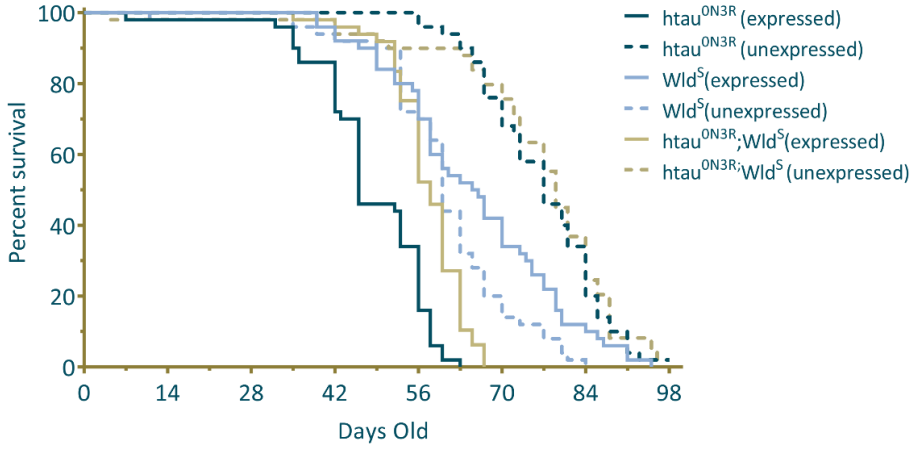
Figure 3-5. htau^{0N3R} causes reduced climbing ability in adult flies and is not improved by co-expression of Wld^S. The decline in climbing ability displayed by htau^{0N3R} flies is significantly worse compared to controls. However there is no significant difference in climbing ability between htau^{0N3R} and htau^{0N3R};Wld^S flies. Values are presented as the mean ± SEM. n=4 (1n=10 flies) *P<0.05, **P<0.01, ***P<0.001 vs. the control group, repeated measures two-way ANOVA, Bonferroni's multiple comparison. n.s., not significant.

Adult *Drosophila* display a negative geotaxis response which can be used as a measure of locomotor function, with the climbing ability of flies declining with age. The expression of htau^{0N3R} results in deficits in this climbing behaviour (Mudher et al., 2004). To test whether Wld^{S} is able to improve locomotor function in adults, htau^{0N3R} was expressed throughout the *Drosophila* nervous system using elav and weekly climbing assays were performed. This resulted in a greatly reduced climbing ability from a few days after eclosion. At 3 days after eclosion (dae), htau^{0N3R} flies have a mean height climbed of 71% compared to controls which reached 100% (Figure 3-5). This deficit is not improved by the co-expression of Wld^{S}, with no significant difference at any time point compared to htau^{0N3R} flies. By 24 dae, both htau^{0N3R} and htau^{0N3R};Wld^{S} expressing flies achieved a mean height climbed of around 1% whilst controls are still climbing well, with a mean height climbed of 67.5% (Figure 3-5). This indicates that Wld^{S} has no effect on the neuronal dysfunction or degeneration that impact on locomotor function when htau^{0N3R} is expressed.

As well as inducing deficits in climbing ability, expression of human tau throughout the *Drosophila* nervous system drastically reduces lifespan (Folwell et al., 2010). To investigate whether Wld^{S} could impact on the reduction of lifespan induced by htau^{0N3R}, a longevity assay was performed. In htau^{0N3R} expressing flies, median lifespan was reduced to 46 days, compared to 77 days in controls (htau^{0N3R} (unexpressed)). Flies co-expressing htau^{0N3R};Wld^{S} also have a reduced lifespan of 58 days compared to 79 days in controls (htau^{0N3R};Wld^{S} (unexpressed)) however this is significantly improved compared to the lifespan in htau^{0N3R} expressing flies (Figure 3-6). This suggests that Wld^{S} is able to ameliorate the detrimental effect of htau^{0N3R}-expression upon lifespan, potentially through delaying axonal degeneration.

Tau-induced degeneration within the *Drosophila* brain has been previously reported using TUNEL staining showing apoptosis and the appearance of vacuoles indicating cell loss (Wittmann et al., 2001, Khurana et al., 2006); however these methods do not provide information on the loss of axons. As Wld^{S} protection is known to be specific for the axonal compartment (Sasaki et al., 2006, Avery et al., 2009, Babetto et al., 2010), a neuronal population was required in which axons are clearly traceable, for this olfactory receptor neurons (ORNs) were selected.

A



B

Genotype	Median survival (days)		P-value
	Unexpressed	Expressed	
htau^{0N3R}	77	46	<0.0001
Wld^{S}	60	65.5	0.0354
htau^{0N3R};Wld^{S}	79	58	<0.0001
Bonferroni corrected P-value threshold			0.008

Figure 3-6. Co-expression of Wld^{S} improves the shortened lifespan of htau^{0N3R} adult flies. [A] Kaplan-Meier curves indicating that the expression of htau^{0N3R} results in reduced lifespan, which is improved by the co-expression of Wld^{S}. [B] Median lifespan of each expressed and unexpressed line. n=50, Mantel-Cox test, Bonferroni corrected P-value threshold 0.008.

ORN cell bodies are housed in the antennae, projecting to the antennal lobe and synapsing on both the ipsilateral and contralateral lobe, with projections to the contralateral side passing through a midline commissure (Figure 2-3). This system has been previously used to track axonal degeneration after axotomy, as following the removal of the antennae containing the cell bodies the axonal commissural projections undergo Wallerian degeneration (Hoopfer et al., 2006, Miller et al., 2009). By driving expression in a population of ORNs (Or47b) it is possible to track axonal degeneration due to the co-expression of membranous GFP.

Flies expressing htau^{0N3R} in the Or47b population of ORNs are indistinguishable from controls at 1 week after eclosion (wae), with smooth axonal processes visible between the two antennal lobes (Figure 3-7 A1 & B1). However as the flies age, progressive morphological aberrations in the axons of htau^{0N3R} transgenic flies are observed, characterised by the appearance of axonal swellings. By 3 wae axonal swellings are beginning to appear over the ORNs, with the number and size of swellings increasing by 6 wae (Figure 3-7 B3).

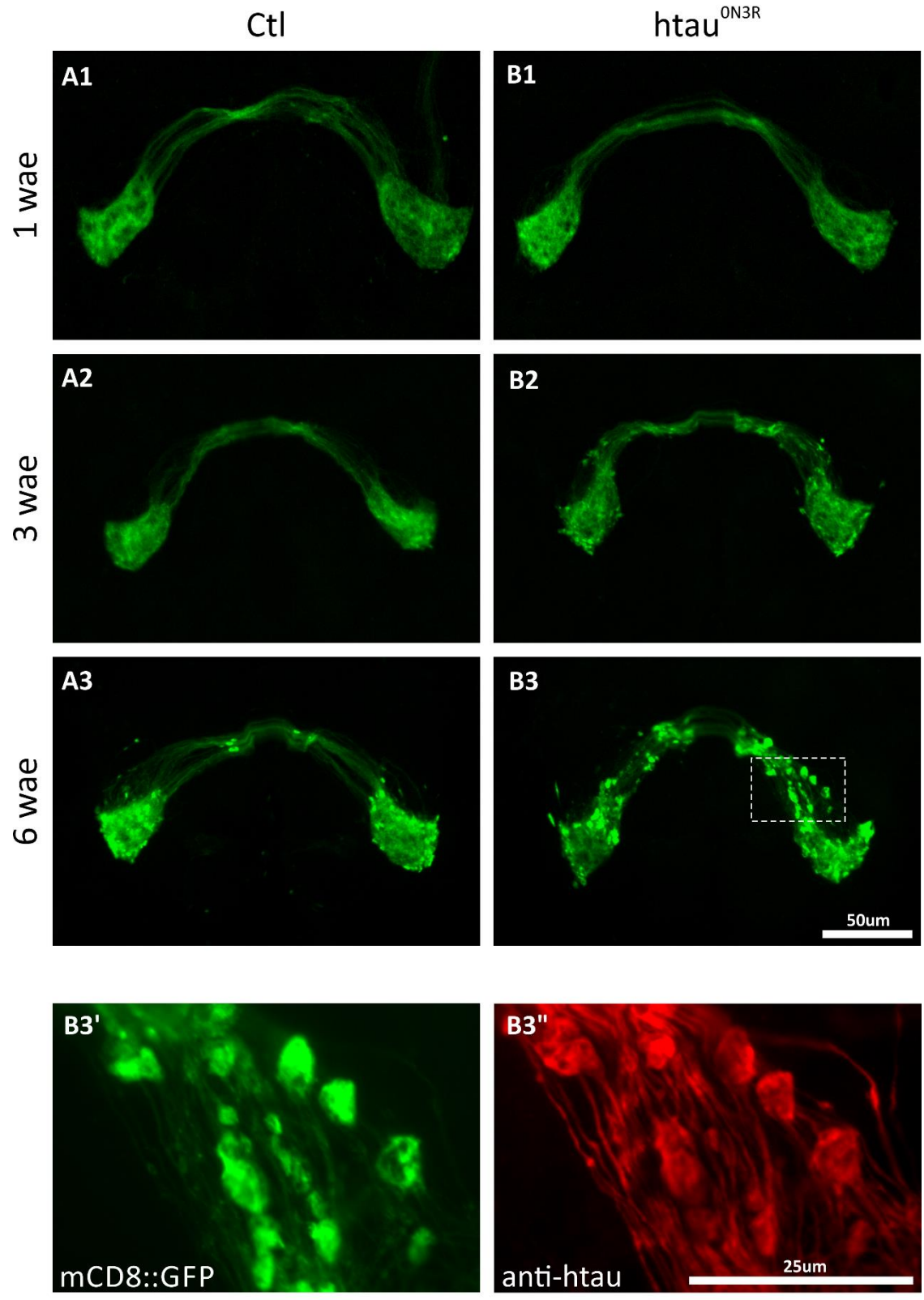
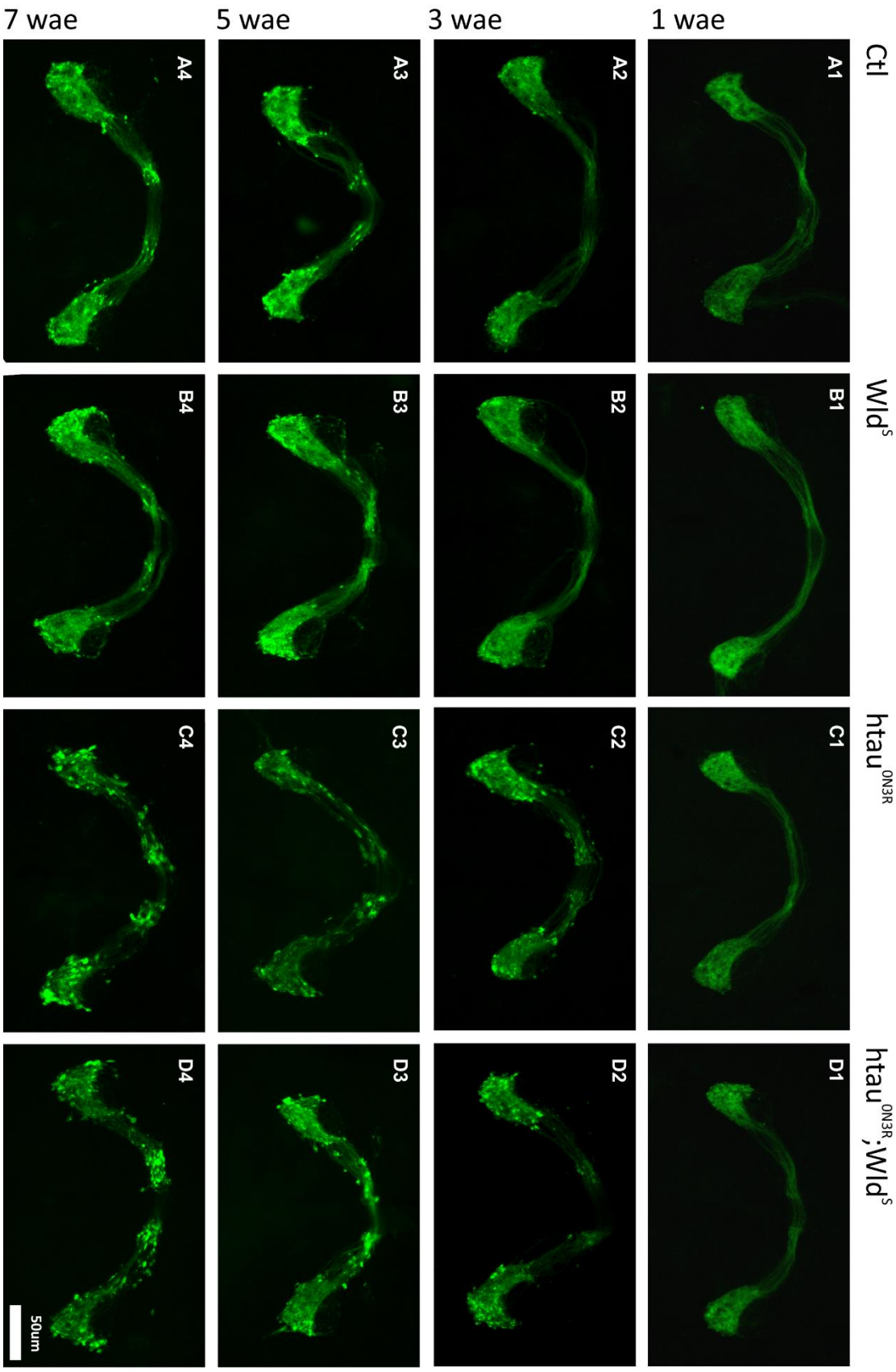


Figure 3-7. Expression of htau^{0N3R} results in axonal degeneration characterised by the appearance of axonal swellings. The axonal swelling begin to appear 3 wae and progressively increase over time in number and size. The swellings are immunoreactive for human tau. wae, weeks after eclosion.

Axonal swellings have been reported in experimental models of AD (Tsai et al., 2004, Stokin et al., 2005, Adalbert et al., 2009) and tauopathy (Probst et al., 2000, Lin et al., 2003, Shemesh et al., 2008, Ludvigson et al., 2011) and are thought to be an early stage of axonal degeneration. In models of tauopathy, swellings have been reported to be filled with cytoskeletal components including tau (Probst et al., 2000, Shemesh et al., 2008). To investigate whether this was the case in this study, immunohistochemical staining for human tau was carried out. This staining revealed that the swellings observed in ORNs expressing htau^{0N3R} were strongly immunoreactive for human tau (Figure 3-7 B3"). High magnification images showed that the swellings appeared to be asymmetrical, an observation also reported by others (Adalbert et al., 2009).

To investigate whether Wld^{S} could protect against this tau-mediated axonal degeneration, htau^{0N3R} and Wld^{S} were co-expressed in the Or47b population. At 1 wae the axons appeared smooth and of normal morphology; however axonal swellings began to appear by 3 wae (Figure 3-8 D2). Similar to htau^{0N3R} expressing axons the number and size of swellings increased over time. Quantification of the axonal area covered by swellings demonstrated that there is no difference in the onset and progression of axonal swellings in htau^{0N3R} and htau^{0N3R};Wld^{S} expressing brains (Figure 3-8 E).

The inability of Wld^{S} to reduce the burden of axonal swellings in htau^{0N3R} expressing axons suggests that the mechanism by which axonal degeneration proceeds in tauopathy differs to that occurring following acute injury.


7 wae
5 wae
3 wae
1 wae
Ctl
Wld^{S}
htau^{0N3R}
htau^{0N3R};Wld^{S}
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
50um

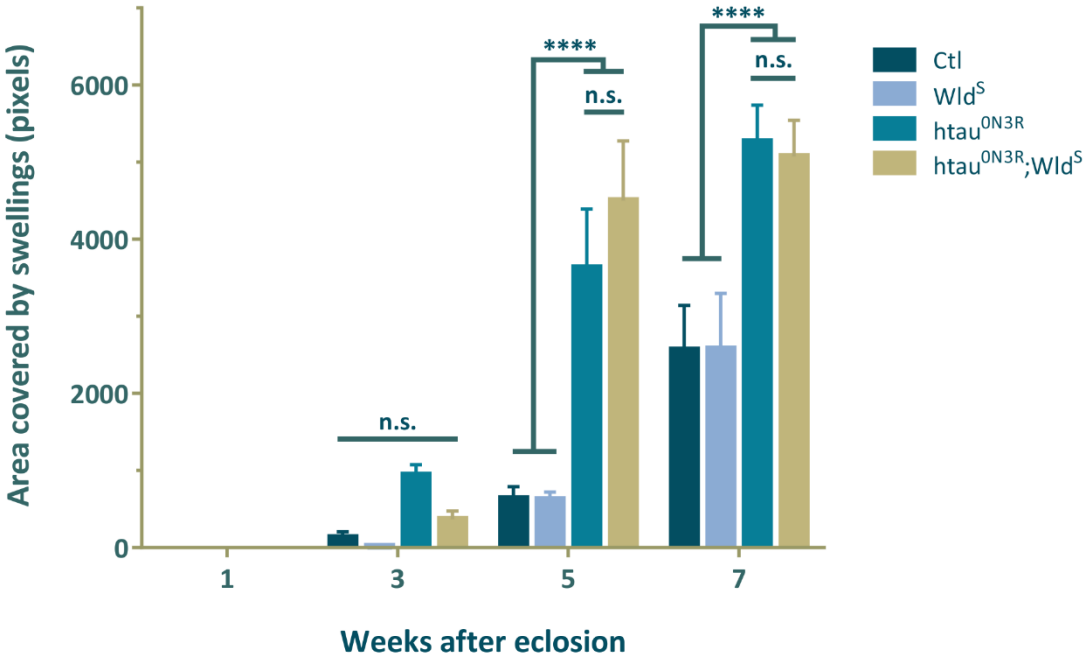


Figure 3-8. Co-expression of Wld^S with htau^{0N3R} does not delay tau-mediated axonal degeneration in adults. Alterations in ORN morphology over time in [A] control, [B] Wld^S, [C] htau^{0N3R} and [D] htau^{0N3R};Wld^S expressing brains. At 1 wae all genotypes display normal ORN morphology. At 3 wae, axonal swellings are apparent in htau^{0N3R} expressing ORNs with the same morphology observed in htau^{0N3R};Wld^S ORNs. [E] Quantification of the swelling coverage confirms that co-expression of Wld^S does not delay the onset nor slow the progression of tau-mediated axonal degeneration. Values are presented as the mean ± SEM. n=6-11 brains; ****P<0.0001, n.s., not significant; ANOVA, Bonferroni's multiple comparison. wae, weeks after eclosion.

3.4 Disussion

The axonal compartment has been recognised as a key site for tau-mediated dysfunction and degeneration and therefore could represent a potential therapeutic target in the treatment of neurodegenerative disease. The work presented here indicates that attempting to delay Wallerian degeneration using Wld^S does not significantly improve tau-mediated dysfunctional phenotypes, including disruption of axonal transport, aberrant synaptic morphology and behavioural deficits. In addition, expression of Wld^S does not appear to delay the onset of axonal degeneration, although improvements in lifespan are observed.

3.4.1 Wld^S does not improve tau-mediated dysfunction

Disruptions in axonal transport underpin neuronal dysfunction in neurodegenerative disease due to the requirement for efficient delivery of organelles and proteins to maintain neuronal function. Dysfunctional axonal transport is well reported in a number of models of tauopathy. In the larval *Drosophila* model of tauopathy, reduced microtubule stabilisation due to tau hyperphosphorylation results in disrupted axonal transport (Mudher et al., 2004, Cowan et al., 2010) and reduced synaptic activity (Chee et al., 2005), leading to impaired locomotor function (Mudher et al., 2004, Quraishe et al., 2013). The work presented here demonstrates that Wld^S is unable to improve tau-mediated dysfunctional phenotypes.

The *Drosophila* larval model is unique in enabling the investigation of axonal dysfunction in the absence of degeneration, and the results from this model system indicate that Wld^S is unable to improve axonal dysfunction. The Wld^S-mediated improvements in axonal function reported in other disease models correlate with protection of axon number and not with improved function of the axon. For example, in the *pmn* mouse model of motor neuron disease an improvement in axonal transport was observed with Wld^S, however this can be explained by a delay in axon loss. Also, despite this apparent improvement in axonal function with Wld^S, there was no improvement in locomotor behaviour observed, indicating that Wld^S was unable to impact on all disease associated phenotypes (Ferri et al., 2003). Similarly, in a model of Charcot-Marie-Tooth disease 1A ($P0^{-/-}$ mice), Wld^S increased grip strength and improved nerve conduction however this was correlated with a delay in axon loss. At a later time point when Wld^S provided no reduction in axon loss, no improvements in functional readouts were observed (Samsam et al., 2003). In addition, the functional benefits provided by Wld^S in reducing axon loss are often very moderate, such as the 2.5 day delay in disease

onset in EAE mice (Kaneko et al., 2006). These studies indicate that improvements in functional readouts reported in other models are due to a delayed onset of axon loss and not through improvements of axonal function *per se*.

If Wld^S can only provide functional benefits through the prevention of axon loss, then it is not surprising that it did not improve axonal transport in the *Drosophila* larval model of tauopathy in which axon loss does not occur. Previous work in this model has indicated that axonal transport disruption occurs due to the loss of microtubule binding by hyperphosphorylated tau, resulting in unstable microtubules (Cowan et al., 2010). By pharmacologically restabilising the microtubule cytoskeleton a rescue of axonal transport occurs, along with improved locomotor behaviour (Quraishe et al., 2013). Contrast this with Wallerian degeneration occurring post-injury; in this situation cytoskeletal breakdown occurs due to a drop in ATP levels causing microtubule depolymerisation (Park et al., 2013b), along with Ca^{2+} influx into the cytoplasm which activates proteases, cleaving MAPs including tau (Sato et al., 1986, Yang and Ksiezak-Reding, 1995). Whilst Wld^S may be able to mitigate the cascade of events which results in reductions of ATP and the Ca^{2+}-mediated events following injury, this does not impact on the cytoskeletal breakdown seen in tauopathy and may explain why no improvement in axonal transport and the associated behavioural phenotypes was observed in this chapter.

3.4.2 Is Wld^S able to delay tau-mediated axonal degeneration?

By using the adult *Drosophila* model of tauopathy it was possible to investigate whether Wld^S could impact on tau-mediated degeneration. Expression of human tau in adult flies has been shown to reduce locomotor function (Mudher et al., 2004) and shorten lifespan (Folwell et al., 2010). Co-expression of Wld^S with $htau^{0N3R}$ resulted in no improvement in locomotor behaviour but significantly increased lifespan compared to $htau^{0N3R}$ expressing flies. However, investigation of axonal degeneration revealed that Wld^S was unable to delay the onset or alter the progression of tau-mediated axonal degeneration. Similar to my data, when Wld^S was expressed in the *SOD1-G93A* mouse an increase in lifespan was observed but no delay in axonal degeneration was seen (Fischer et al., 2005). Therefore Wld^S may somehow influence lifespan without impacting upon axonal degeneration. An important consideration when performing lifespan experiments is the background genetics of the lines being used, as lifespan is affected by multiple quantitative trait loci and so can vary between different strains (Mackay, 2002, Spencer et al., 2003). Those studying lifespan typically backcross their fly lines

to a 'wild type' line, thereby reducing the background genetic variance and enabling any subsequent lifespan differences to be attributed to the transgenes being investigated. However, this lengthy process was not in the scope of this thesis, instead flies were crossed in a way to produce the closest genetic control to the transgenic lines being investigated (see Figure 7-3 Appendix A for details). Therefore, whilst effort was made to eliminate the effect of background genetics on the lifespans recorded, some residual effect may still have occurred and affected the lifespan result.

The overexpression of proteins involved in the *Drosophila* NAD salvage pathway has been previously demonstrated to result in improvements in lifespan. The NAD salvage pathway is different between invertebrates and vertebrates, with nicotinamide converted to nicotinic acid by nicotinamidases, which are not found in vertebrates. In a previous study, when the *Drosophila* nicotinamidase (D-NAAM) was overexpressed pan-neuronally, a significant increase in the flies lifespan was observed. In addition, the expression of D-NAAM in SH-SY5Y cells protected these cells from cell death induced by the application of a ROS generating agent (Balan et al, 2008). Together, this indicates that the overexpression of NAD salvage pathway proteins may provide neuroprotection, therefore the overexpression of Wld^{S} in the brains of $htau^{0N3R}$ expressing flies may have provided neuroprotection by a mechanism distinct from delaying axonal degeneration.

The lack of axonal protection mediated by Wld^{S} in $htau^{0N3R}$ expressing flies indicates that the increase in lifespan did not occur via axonal protection, however protection against neuronal death may still have occurred. Indeed, neuroprotection has been previously demonstrated by the overexpression of Nmnat in both *Drosophila* (Ali et al., 2012) and mouse (Ljungberg et al., 2012) models of tauopathy. Reduced apoptosis and gliosis were observed in mice co-expressing P301L human tau and Nmnat1 or Nmnat2 (Ljungberg et al., 2012). Similarly, the pan-neuronal overexpression of dNmnat with WT (0N4R) or mutant (R406W) human tau was demonstrated to reduce brain vacuolisation in *Drosophila* (Ali et al., 2012). This neuroprotective effect of dNmnat also resulted in a rescue of tau-mediated learning and memory deficits, locomotor behaviour and a reduction in the levels of phospho-tau, tau oligomers and cleaved caspase-3 (Ali et al., 2012). However, this rescue was also observed with the co-expression of enzyme-dead dNmnat, which has been demonstrated to have chaperone activity (Zhai et al 2008). This suggests that the neuroprotection observed was as a result of the chaperone activity of dNmnat on phosphorylated and oligomeric tau species, and not through a direct action of dNmnat upon axonal or neuronal degeneration.

The increase in lifespan in htau^{0N3R};Wld^{S} co-expressing flies reported in this thesis may be associated with a delay in neuronal loss, which could have been investigated by the sectioning of fly brains and the assessment of brain vacuolisation. However, considering the lack of improvement in behavioural measures in htau^{0N3R};Wld^{S} flies, this avenue of investigation was not pursued, with the focus remaining on the axonal aspects of degeneration in tauopathy. The axonal degeneration observed in the *Drosophila* model of tauopathy was characterised by the appearance of axonal swellings, which indicate the early stages of axonal degeneration caused by human tau. In the swAPP^{Prp} mouse model of AD, axonal swellings were observed at early time points with reductions in fibre density at later time points (Stokin et al., 2005), indicating how axonal degeneration manifests and progresses in the CNS in disease. Whilst axonal swellings are not a feature of Wallerian degeneration in the PNS, they have been described to occur following injury in the CNS (Adalbert et al., 2009, Beirowski et al., 2010). Axonal swellings have also been observed in models of neurodegenerative diseases such as AD (Tsai et al., 2004, Stokin et al., 2005, Adalbert et al., 2009) and tauopathy (Probst et al., 2000, Shemesh and Spira, 2010), suggesting that swellings are characteristic of axonal degeneration within the CNS. These morphological similarities have led to the suggestion that Wallerian-like degeneration is occurring in certain neurodegenerative diseases, and the results presented here support that, with the observation of axonal swellings in htau^{0N3R} expressing flies. However, the failure of co-expression of Wld^{S} with htau^{0N3R} in *Drosophila* ORNs was to delay the onset of tau-mediated axonal swellings suggests that the degeneration occurring is not Wld^{S}-sensitive and may proceed via a different axon death pathway.

The data presented in this chapter indicate that the tau-mediated degenerative events which result in the appearance of axonal swellings are not delayed by Wld^{S}. Despite the morphological similarities between injury and disease, this is evidence that axonal degeneration in tauopathy is not Wld^{S}-sensitive, suggesting the existence of a different axon death pathway to the one occurring after acute injury.

3.4.3 Is all axonal degeneration Wallerian-like?

The failure of Wld^{S} to delay tau-mediated axonal degeneration indicates that the degeneration occurring in this model is not Wld^{S}-sensitive, challenging the idea that all axonal degeneration is mediated by a common axon death pathway. Wld^{S} has also failed to impact on axonal degeneration in models of prion disease (Gultner et al., 2009), MND (Vande Velde et al., 2004, Fischer et al., 2005) and hereditary spastic paraplegia (Edgar et al., 2004). These models

all display chronic neurodegeneration and their insensitivity to Wld^S suggests a different mechanism of axonal degeneration occurs in certain chronic conditions compared with acute injury. The majority of instances in which Wld^S has been reported to successfully delay axonal degeneration can be classed as acute models, such as toxic neuropathy (Wang et al., 2002), ischaemic injury (Gillingwater et al., 2004) and MPTP induced-Parkinsonism (Hasbani and O'Malley, 2006, Antenor-Dorsey and O'Malley, 2012).

The differences between acute and chronic neuronal injury are important to consider, however, do not fully explain why Wld^S is protective in certain diseases and not in others. It may be proposed that acute injury and chronic disease initiate distinct signalling cascades which converge on a single effector pathway, which could explain the morphological similarities between axonal degeneration in these different situations. The pathway activated by acute injury is beginning to be revealed, with the identification of key endogenous mediators such as Sarm1 in the response to injury. There is evidence that Wld^S is able to inhibit MAPK signalling downstream of Sarm1 (Yang et al., 2015) and so the failure of Wld^S to impact on certain disease models , such as tauopathy, prion disease (Gultner et al., 2009) and MND (Vande Velde et al., 2004), suggests that this pathway may not be activated in these diseases. However, the investigation of these endogenous mediators of axonal degeneration in these models will be more informative on whether this pathway plays a role.

The failure of Wld^S to protect in tauopathy despite the morphological similarities raises an important consideration: is sensitivity to Wld^S definitive of Wallerian-like degeneration? Or, is Wallerian-like degeneration simply a morphological description for which we currently do not fully understand all the underlying molecular mechanisms? As knowledge of the underlying molecular mechanisms increases, it may become apparent that there are distinct molecular cascades governing axonal degeneration, with tauopathy, for example, occurring via a Wld^S-insensitive pathway. It is important to be clear about what is meant by each of these terms, Wallerian-like degeneration and Wld^S-sensitive degeneration, with the former describing the morphological changes and the later representing the molecular cascade that is currently the most well-known to control axonal degeneration.

To conclude, whilst tauopathy is characterised by axonal dysfunction and Wallerian-like degeneration, the underlying molecular pathway is not Wld^S-sensitive, as indicated by the lack of Wld^S-mediated protection in $htau^{0N3R}$ expressing flies.

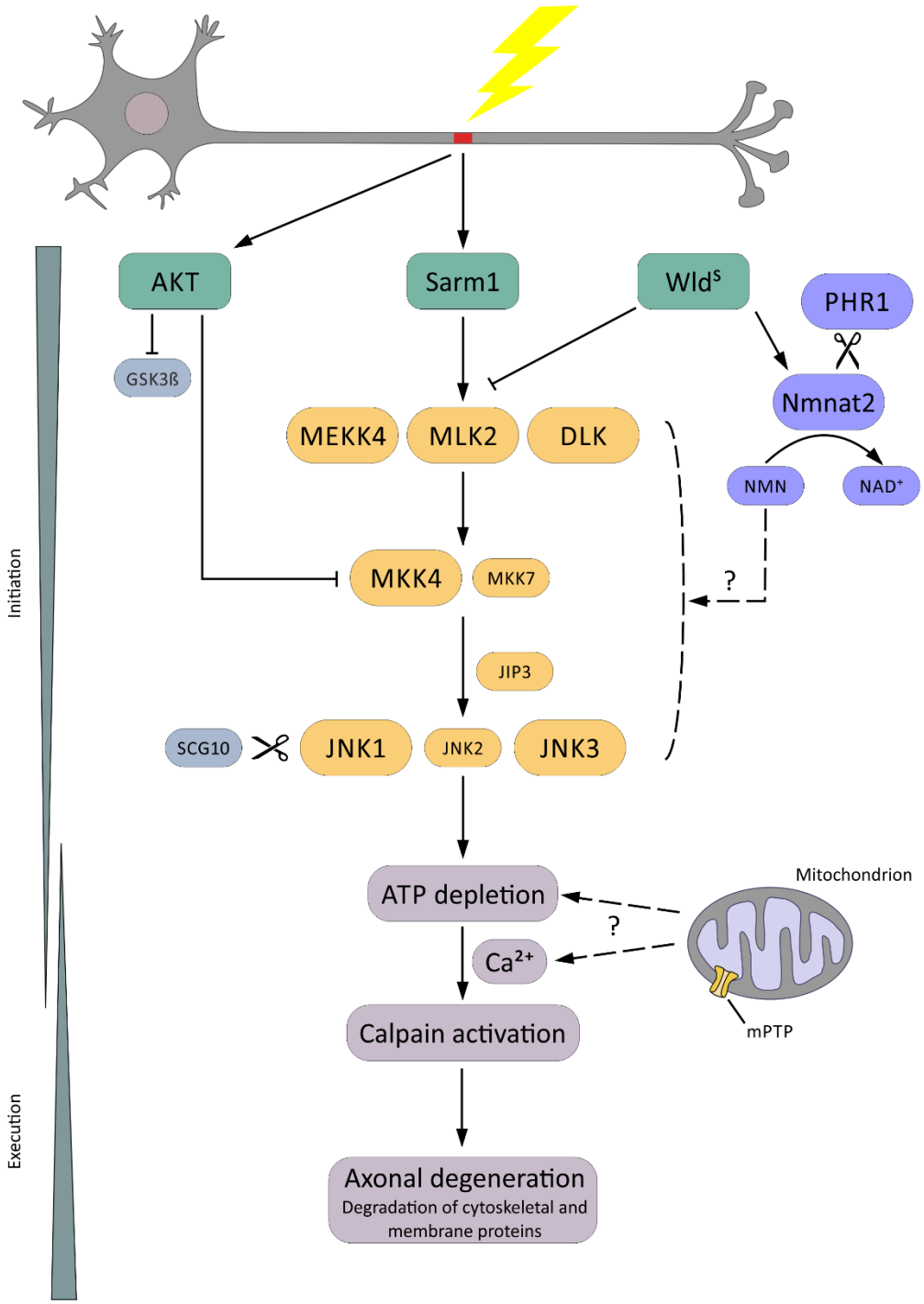


Figure 3-9. The axon death pathway. Acute axonal injury initiates a cascade of events mediated through Sarm1 and the MAPK signalling pathway. This pathway results in ATP depletion and calpain activation through an increase in intracellular Ca^{2+}, leading ultimately to axonal degeneration through degradation of cytoskeletal and membrane proteins. Expression of Wld^S prevents signalling through the MAPK pathway and the downstream consequences such as ATP depletion and calpain activation, resulting in axonal protection.

Whether axonal degeneration in tauopathy proceeds by this pathway is unknown, and the failure of Wld^S in our model suggests degeneration is initiated by an alternate means. There is evidence that calpains are involved in the pathological misprocessing of tau, but whether they are involved in the execution of axonal degeneration is not known.

Chapter 4: Protecting axons against Wallerian degeneration provides bystander protection against tau-mediated degeneration

4.1 Introduction

The previous chapter described how Wld^S was unable to improve axonal dysfunction or delay axonal degeneration mediated by the expression of human tau, indicating that the degeneration occurring in tauopathy was not Wld^S-sensitive. However, there could be a simple explanation for the lack of Wld^S-mediated protection in this model: that the disruption of axonal transport mediated by $htau^{0N3R}$ prevented sufficient transport of Wld^S into the axon, thereby negating its potential protection.

It is now well accepted that for Wld^S to be protective post-axotomy, it must be present within the axon (Avery et al., 2009, Beirowski et al., 2009, Conforti et al., 2009). The level of protection mediated by Wld^S is tightly linked to its concentration within the axon, as demonstrated by the deletion of the nuclear localisation signal from Wld^S, which enhanced the level of protection (Babetto et al., 2010). The disruption of axonal transport in models of tauopathy is well reported; expression of human tau in *Drosophila* larvae causes vesicles to aggregate due to the breakdown of the microtubule cytoskeleton (Mudher et al., 2004, Cowan et al., 2010). Therefore in $htau^{0N3R}$;Wld^S expressing *Drosophila* this disruption of axonal transport may be reducing the level of the Wld^S protein within the axon. This could impact on the protective capacity of Wld^S and may explain why no delay in tau-mediated axonal degeneration was observed. To test this hypothesis, axotomy of $htau^{0N3R}$;Wld^S expressing axons was performed to determine whether Wld^S was still able to delay Wallerian degeneration after injury when co-expressed with $htau^{0N3R}$.

This revealed that whilst Wld^S was able to delay Wallerian degeneration post-axotomy in $htau^{0N3R}$ co-expressing flies, it also appeared to prevent the appearance of tau-mediated axonal swellings. This finding was investigated further using immunohistochemical staining to check for the presence of tau within $htau^{0N3R}$;Wld^S axons post-injury, and by generating the axotomy at a later time point when axonal swellings were established in $htau^{0N3R}$;Wld^S expressing axons.

4.2 Materials & Methods

4.2.1 Antennal injury & dissection

Axotomy was achieved by the removal of the third antennal segments using Dumont #5 forceps whilst flies were under CO_2 anaesthesia, at either 1wae or 3wae.

At the appropriate time points, brains were dissected in PBS and fixed in 4% formaldehyde. Brains were then either mounted in Vectashield or processed for immunofluorescent staining.

For immunostaining brains were incubated with polyclonal rabbit anti-human tau antibodies (1:1000; Dako) then with goat anti-rabbit Alexa Fluor 568 (1:1000; Invitrogen) to enable visualisation. Brains were mounted in Vectashield and imaged using a fluorescence microscope.

4.2.2 Quantification of degeneration

In injured brains, the presence of the commissural axons was scored in the blinded image set and the percentage of brains with intact axons calculated. GFP intensity in the antennal lobe glomeruli was measured using ImageJ, with background levels subtracted.

For uninjured brains, the coverage of axonal swellings was measured using ImageJ with the assessor blind to genotype and time point.

4.3 Results

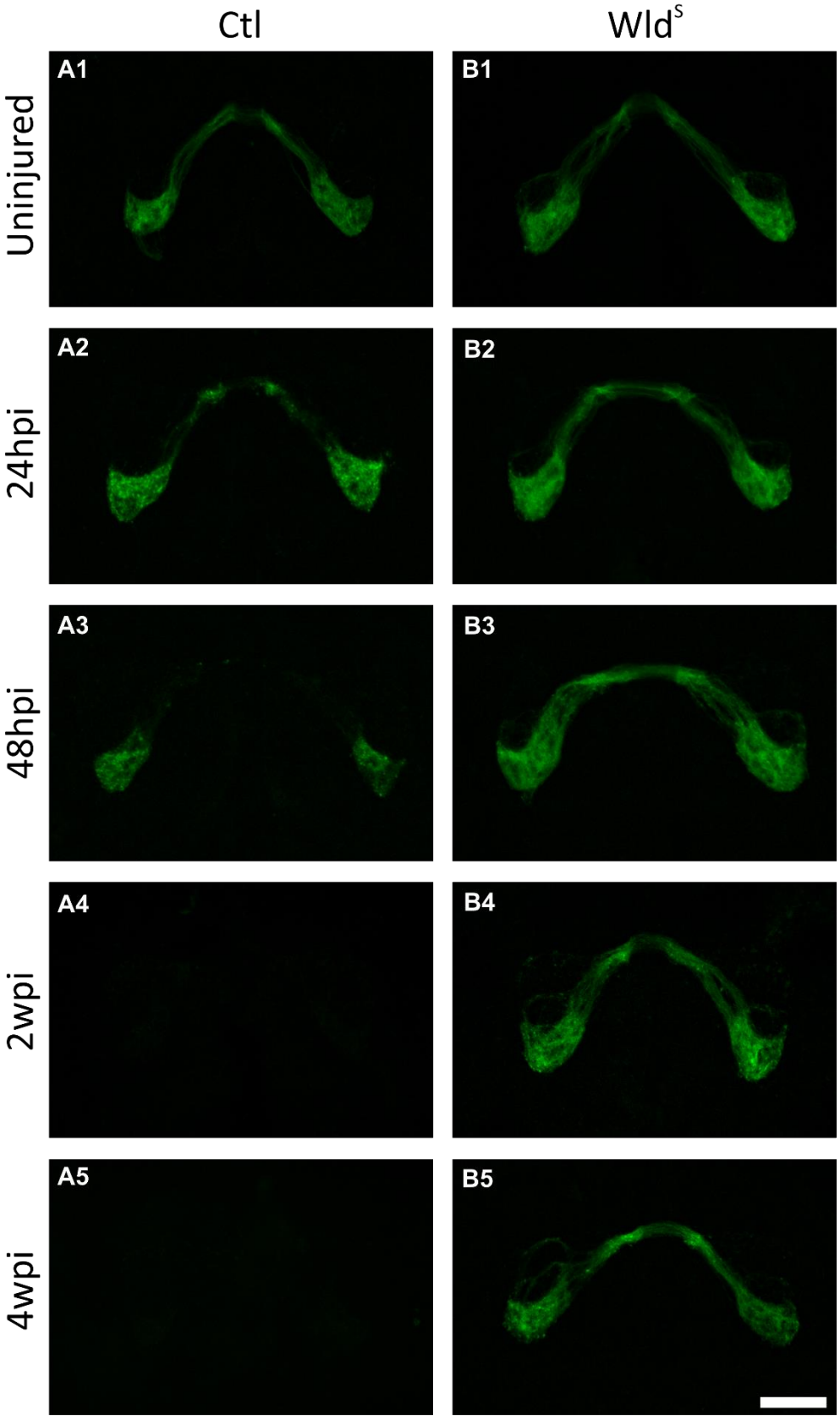
4.3.1 Wld^S protects against axotomy induced Wallerian degeneration

The lack of Wld^S-mediated protection against tau-induced axonal degeneration observed in Chapter 3 could be due to the Wld^S transgene not producing the expected protective phenotype. As it is known that the expression level of the Wld^S protein correlates with the level of protection provided (Mack et al., 2001), the higher expressing of two UAS-Wld^S transgenic lines was selected to make the $htau^{0N3R}$;Wld^S line (See Appendix B Figure 7-2).

Whilst high levels of Wld^S protein are produced by the Wld^S transgenic line, it was also necessary to confirm that this resulted in the expected protective phenotype after injury. A frequently utilised axonal circuit in *Drosophila* for injury experiments are the olfactory receptor neurons (ORNs), as the cell bodies are housed in the third antennal segment and so removal of this segment results in axotomy (Figure 2-3) (Hoopfer et al., 2006, Macdonald et al., 2006).

Expression of Wld^S was driven using the Or47b line, which also expresses mCD8::GFP, allowing for the visualisation of the axons within the antennal lobe. Upon removal of the third antennal segment from Or47b control flies, the commissural axons between the antennal lobes degenerate and are heavily fragmented 24 hours post injury (hpi) (Figure 4-1 A2). This fragmentation increases by 48 hpi, with the mCD8::GFP signal remaining mainly in the antennal lobe glomeruli (Figure 4-1 A3). By contrast, Wld^S expressing axons were protected from degeneration and did not show any evidence of fragmentation at 4 weeks post injury (wpi), the latest time point investigated here (Figure 4-1 B5). This confirms that the Wld^S transgene is producing the expected protective phenotype following axotomy, and so does not explain the lack of protection against tau-induced axonal degeneration.

Figure 4-1. (Next page) Wld^S delays Wallerian degeneration after axotomy. [A] Following axotomy, control axons begin to fragment by 24 hpi, with signal remaining in antennal lobe glomeruli at 48hpi before being cleared completely. [B] Wld^S expressing axons remain intact up to 4 wpi, showing no signs of fragmentation. Scale = 25μm. hpi, hours post injury; wpi, weeks post injury.


Ctl
Wld^S
Uninjured
24hpi
48hpi
2wpi
4wpi
A1
B1
A2
B2
A3
B3
A4
B4
A5
B5

4.3.2 Wld^S protects against axotomy induced Wallerian degeneration in tau-expressing flies

A second explanation for the lack of Wld^S protection against tau-induced axonal degeneration is that disruption of axonal transport mediated by tau could prevent efficient delivery of Wld^S into the axon, thereby limiting its protective capacity. To investigate this, axotomy was performed and the subsequent degeneration in ORNs followed over time.

Following axotomy both control and htau^{0N3R} expressing axons degenerate, with complete loss of mCD8::GFP signal by 2 wae/1 wpi (Figure 4-2 A & C). Both Wld^S and htau^{0N3R};Wld^S axons were protected from axotomy-induced degeneration and maintained a normal smooth morphology up to 5 wae/4 wpi (Figure 4-2 B & D). This indicates a robust protection of htau^{0N3R};Wld^S expressing axons against Wallerian degeneration. However by 7 wae/6 wpi, only 12.5% of htau^{0N3R};Wld^S had intact axons compared to 40% of Wld^S brains (Figure 4-2 F), suggesting that htau^{0N3R};Wld^S axons are not protected for as long as Wld^S expressing axons post-axotomy.

To investigate the difference in axonal protection between htau^{0N3R};Wld^S and Wld^S expressing axons post-axotomy, more frequent time points were carried out between 4 and 6 wpi. This revealed that the latest time point at which all htau^{0N3R};Wld^S expressing axons were intact was 32 dpi, however Wld^S expressing axons remained intact up to 40 dpi (Figure 4-2 H). Whilst htau^{0N3R};Wld^S axons are robustly protected against axotomy-induced degeneration, the length of protection is reduced compared to Wld^S expressing axons.

Western blotting indicated that there was no significant difference in the protein expression levels of Wld^S in htau^{0N3R};Wld^S expressing flies compared with Wld^S expressing flies (Figure 4-3). Therefore, the reduced length of protection in htau^{0N3R};Wld^S axons after injury compared with Wld^S axons cannot be explained by a significant reduction in the expression levels of Wld^S.

Despite the reduction in the length Wld^S protection in htau^{0N3R};Wld^S flies, there was still lengthy protection (<32 dpi) from axonal degeneration after injury. This indicates that Wld^S is able to protect when co-expressed with htau^{0N3R}, and suggests that a disruption of Wld^S protection by htau^{0N3R} is not occurring and cannot explain why htau^{0N3R};Wld^S axons are not protected against tau-mediated axonal degeneration.

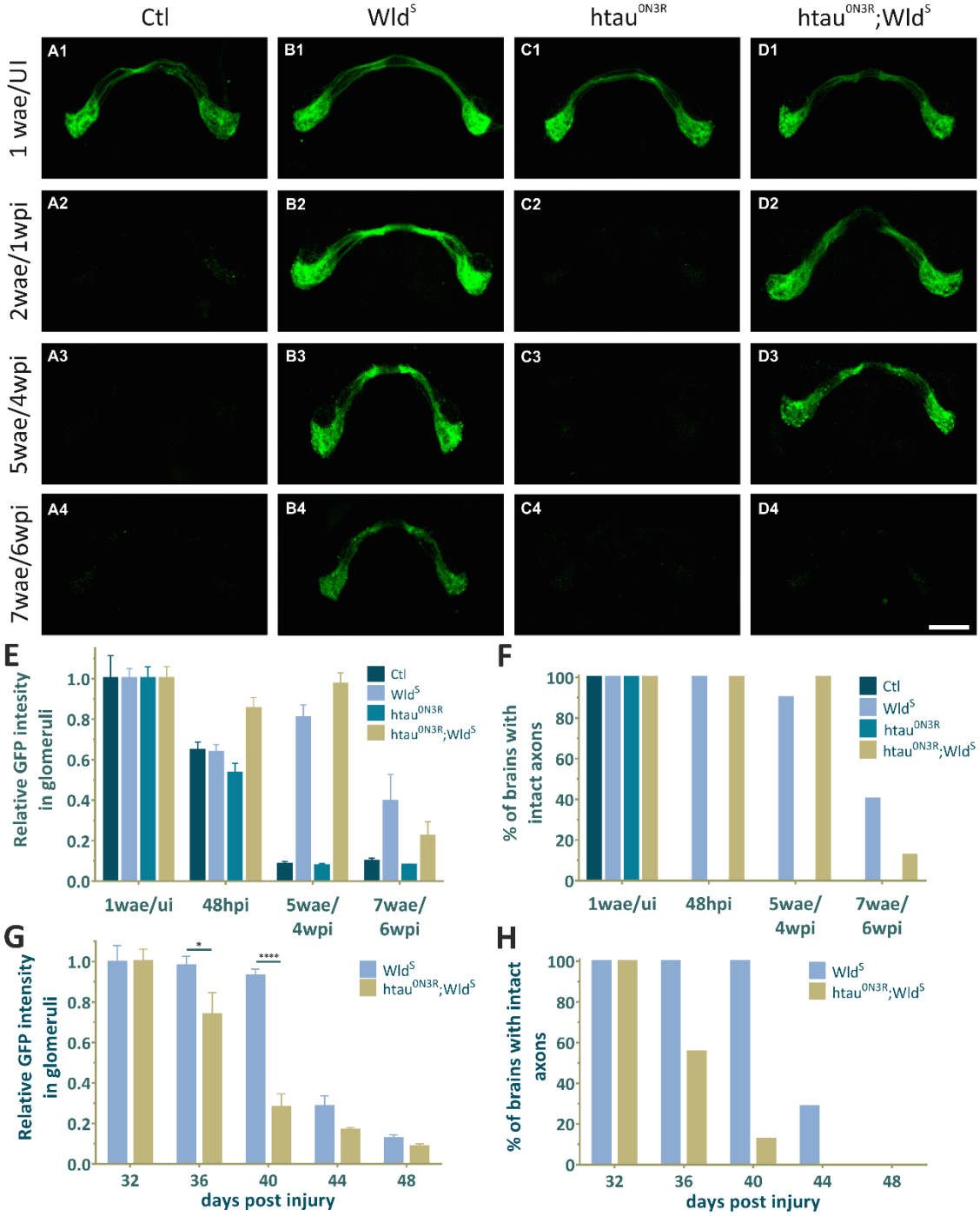


Figure 4-2. Wld^S delays Wallerian degeneration post-axotomy when co-expressed with htau^{0N3R}. Images indicate that lke [B] Wld^S expressing axons, [D] htau^{0N3R};Wld^S expressing axons do not degenerate following axotomy. [E & F] Quantification reveals protection up to 6 wpi, although fewer htau^{0N3R};Wld^S axons remain intact than Wld^S at this time point (n=6-10, ANOVA, Bonferroni's multiple comparison). [G & H] Wld^S axons are protected 8 days longer than htau^{0N3R};Wld^S axons (n=8-14, $^*P<0.05$, $^{****}P<0.0001$, ANOVA, Bonferroni's multiple comparison). Scale = 25µm. wpi, weeks post injury; wae, weeks after eclosion; ui, uninjured; ctl, control.

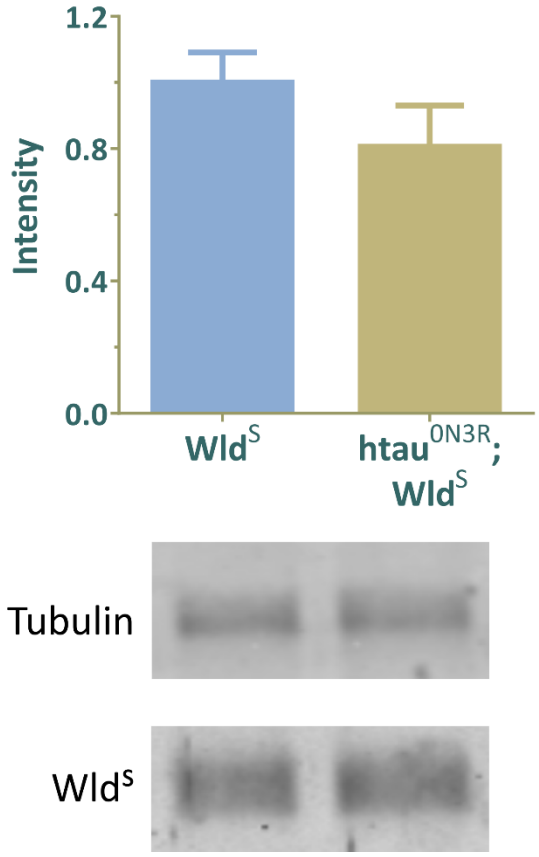


Figure 4-3. Comparison of protein expression in Wld^S and dual transgenic htau^{0N3R};Wld^S line. (n=4, P=0.2556, unpaired t test)

4.3.3 Tau-mediated axonal degeneration does not occur in axotomised htau^{0N3R};Wld^S axons

Axonal swellings are a feature of axonal degeneration post-injury within the CNS (Beirowski et al., 2010, Knoferle et al., 2010, Tang-Schomer et al., 2012), and higher magnification images of axotomised Wld^S and htau^{0N3R};Wld^S expressing ORNs indicate that axonal swellings appear prior to fragmentation (Figure 4-4 A3' & B2'). However, these swellings differ from those observed in uninjured aged htau^{0N3R};Wld^S brains, being much smaller in size and number and only appearing just before the axons fragment. Quantification of the coverage of axonal swellings revealed that injured htau^{0N3R};Wld^S ORNs did not develop axonal swellings like uninjured htau^{0N3R};Wld^S ORNs (Figure 4-4 E). The large axonal swellings observed in 5 wae uninjured htau^{0N3R};Wld^S ORNs (Figure 4-4 C2) were not seen in injured htau^{0N3R};Wld^S ORNs at 5 wae/4 wpi (Figure 4-4 D2).

Due to the degeneration of injured htau^{0N3R};Wld^S ORNs at later time points it was not possible to compare injured versus uninjured brains in older flies (>6 wae). Despite this, the

data from htau^{0N3R};Wld^{S} brains at 5 wae/ui and 5 wae/4 wpi indicates that protection against tau-mediated degeneration has occurred in injured brains that are prevented from undergoing Wallerian degeneration.

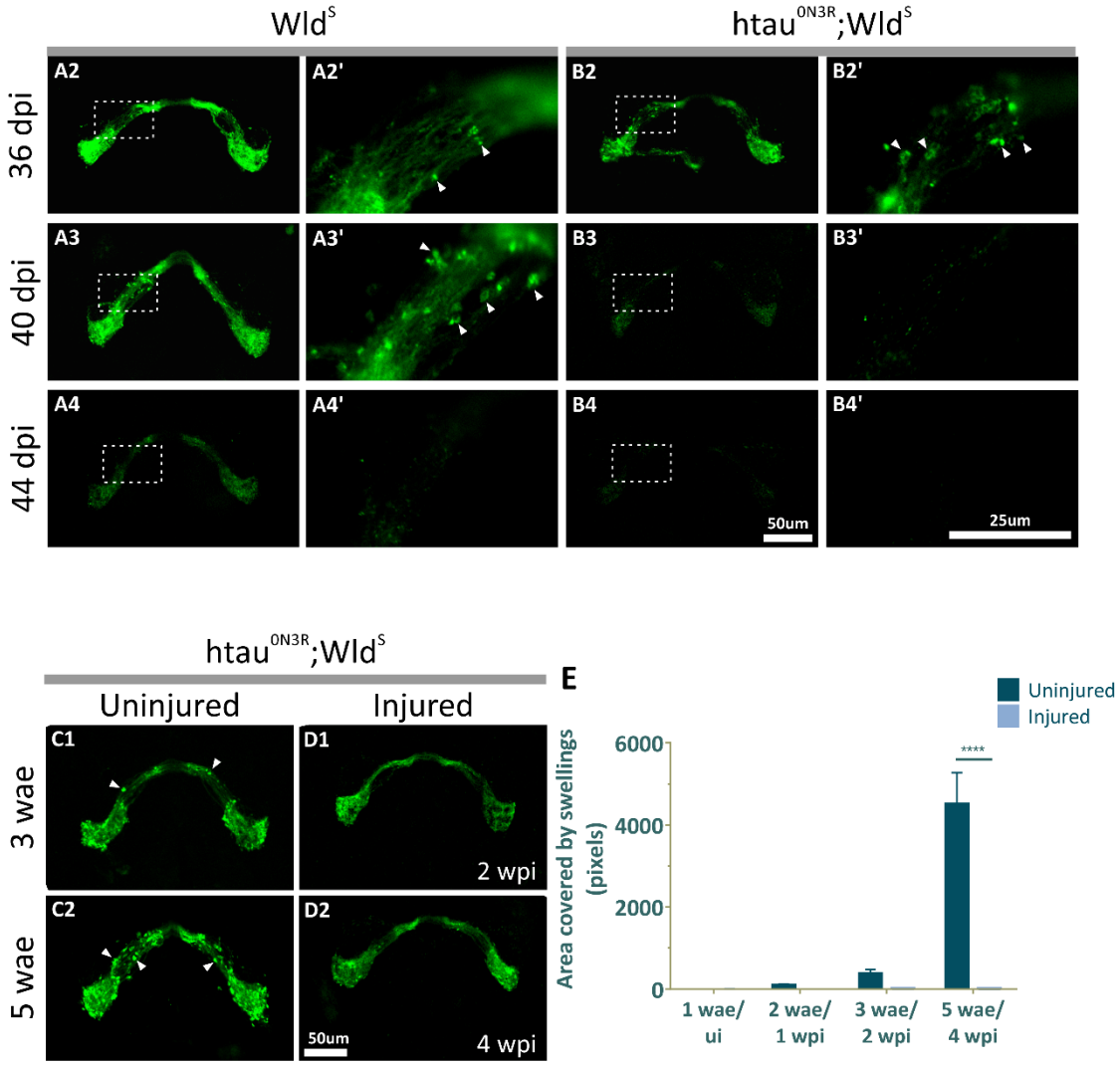


Figure 4-4. **Tau-mediated axonal swellings are not evident in axotomised htau^{0N3R};Wld^{S} axons.** [A & B] Axonal swellings are a feature of axonal degeneration of Wld^{S} expressing axons following axotomy (arrowheads). [C] Axonal swellings in uninjured htau^{0N3R};Wld^{S} axons (arrowheads) do not appear in [D] injured htau^{0N3R};Wld^{S} axons, confirmed by [E] quantification. Values are presented as the mean ± SEM. ****P<0.0001; *n*=6-10; ANOVA, Bonferroni's multiple comparison. Dpi, days post injury, wpi, weeks post injury; wae, weeks after eclosion; ui, uninjured.

4.3.4 Tau levels persist in axotomised htau^{0N3R};Wld^{S} axons

A potential explanation for the protection against tau-mediated axonal swellings in axotomised htau^{0N3R};Wld^{S} brains is that human tau levels drop within the axon following injury. This could occur by a combination of mechanisms including reduced production of human tau due to the loss of the cell body and the degradation of existing human tau within the axon. To investigate this, staining for human tau was performed in injured htau^{0N3R} and htau^{0N3R};Wld^{S} expressing brains.

Following injury to htau^{0N3R} ORNs, human tau immunoreactivity is lost and the mCD8::GFP signal indicates heavy fragmentation of the injured ORNs at 24 hpi (Figure 4-5 A). In injured htau^{0N3R};Wld^{S} axons at 24 hpi, both mCD8::GFP and human tau staining reveal intact continuous axons (Figure 4-5 B). Human tau persisted in the injured htau^{0N3R};Wld^{S} ORNs and was still detected at 6 wae/5 wpi (Figure 4-6 B). This indicates that human tau is not lost from injured htau^{0N3R};Wld^{S} axons, and is present up to the time when the ORNs ultimately begin to fragment.

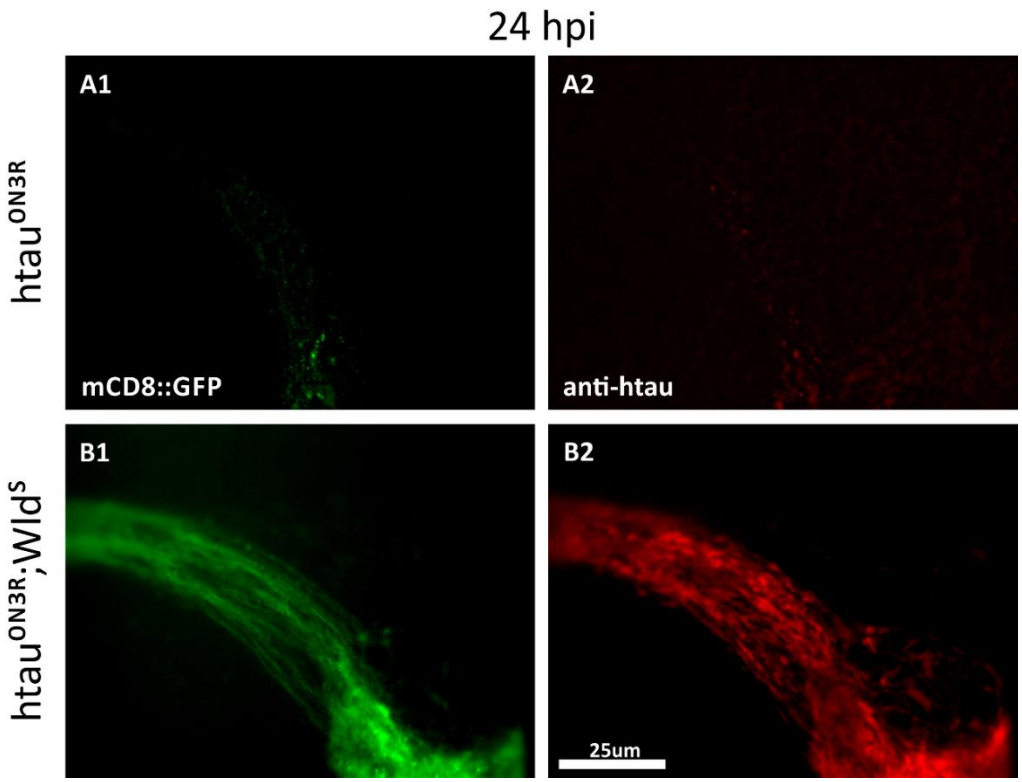


Figure 4-5. Human tau is rapidly lost from injured htau^{0N3R} axons but remains in injured htau^{0N3R};Wld^{S} axons. [A] At 24 hpi the mCD8::GFP signal shows heavy fragmentation along the length of the ORNs, and the human tau signal is similarly reduced. [B] In htau^{0N3R};Wld^{S} ORNs, signal from both mCD8::GFP and human tau staining remains, and the axons appear intact and continuous. Hpi, hours post-injury

Whilst human tau persists in injured htau^{0N3R};Wld^{S} ORNs, the levels may drop and this could be a factor in the apparent lack of tau-mediated axonal swellings after injury. It was not possible to quantify the level of human tau using western blotting as Or47b-GAL4 only drives expression in 50 neurons within the brain, therefore the signal could not be detected above background. However, quantification of the staining intensity in images revealed no difference in human tau immunoreactivity between injured and uninjured htau^{0N3R};Wld^{S} brains, indicating that htau^{0N3R} levels do not drop post-axotomy (Figure 4-6 C). Therefore the lack of tau-mediated axonal degeneration in injured htau^{0N3R};Wld^{S} axons is not due to loss or reduction of human tau within the axon.

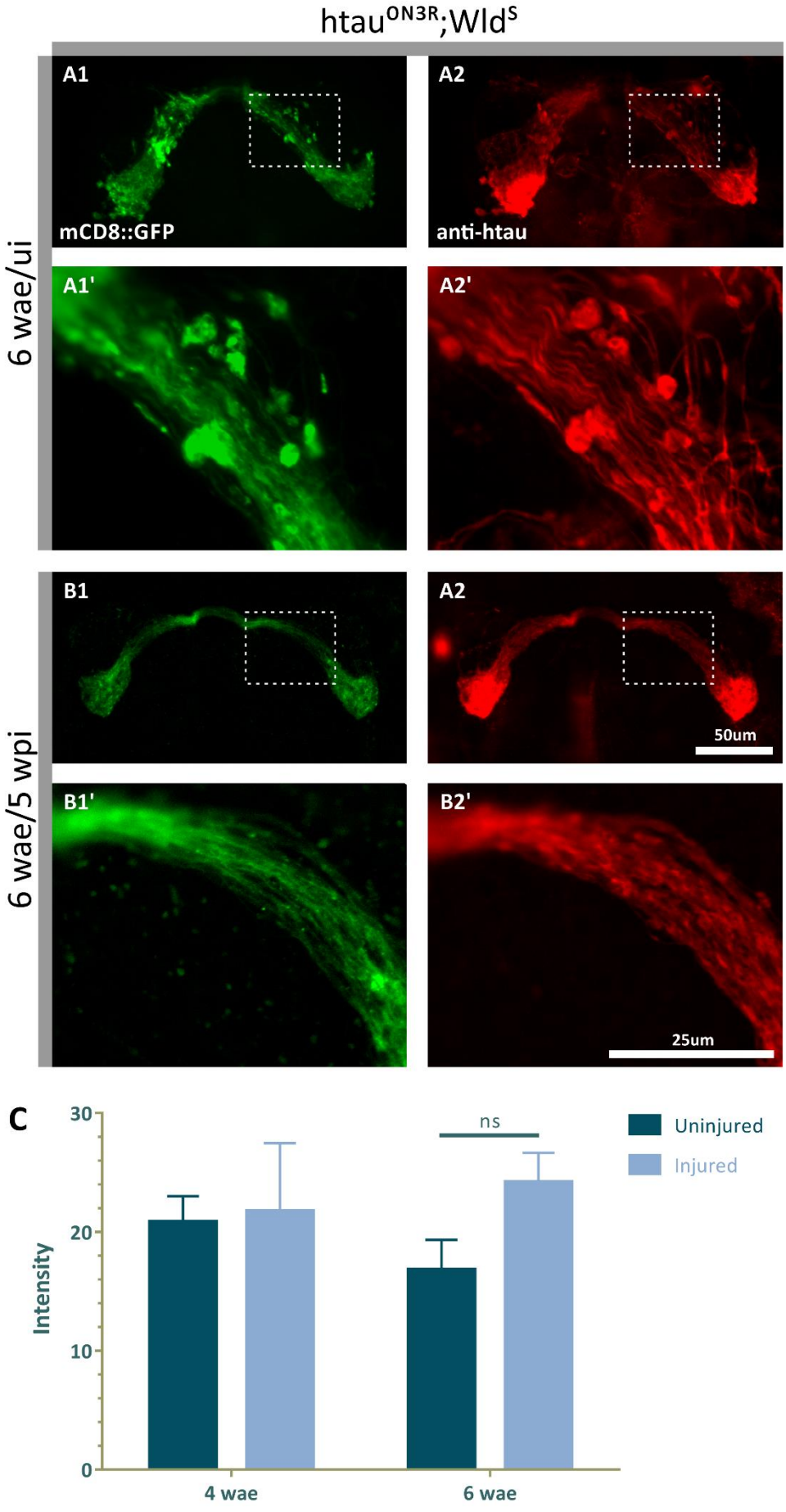

htau^ON3R;Wld^S
A1
mCD8::GFP
A2
anti-htau
A1'
A2'
6 wae/ui
B1
A2
50um
B1'
B2'
25um
6 wae/5 wpi
C
30
20
10
0
Intensity
ns
Uninjured
Injured
4 wae
6 wae

Figure 4-6. (Previous page) Human tau is not lost from axotomised htau^{0N3R};Wld^{S} axons. [A] Human tau staining in uninjured htau^{0N3R};Wld^{S} axons at 6 wae/ui. [B] Human tau staining in injured htau^{0N3R};Wld^{S} axons 6 wae/5 wpi shows tau is not lost from axons. [C] Quantification demonstrates no significant difference between staining intensity between uninjured and injured axons. Values are presented at the mean ± SEM. n=5-13, ANOVA, Bonferroni's multiple comparison.wae, weeks after eclosion; ui, uninjured; wpi, weeks post-injury; n.s., not significant.

4.3.5 Wld^S protection post-axotomy halts tau-mediated axonal degeneration

The results described in this chapter indicate that axotomy of $htau^{0N3R}$;Wld^S expressing ORNs at 1 wae can prevent the appearance of tau-mediated axonal degeneration normally observed in aged uninjured flies. At 1 wae when flies were normally injured, tau-mediated axonal swellings were not observed, so I was interested to investigate the progression of the axonal swelling phenotype when ORNs were injured once tau-mediated axonal swellings have manifested. Tau-mediated axonal swellings appear by 3 wae, therefore I decided to injure flies at this time point and track the progression of axonal swellings in $htau^{0N3R}$;Wld^S ORNs.

In uninjured $htau^{0N3R}$;Wld^S ORNs, the coverage of axonal swellings increases 4.5 fold from 3 wae to 4 wae (Figure 4-7 A) ($P<0.0001$, ANOVA, Bonferroni's multiple comparison). However, when $htau^{0N3R}$;Wld^S ORNs were injured at 3 wae, this increase in axonal swellings was not observed at 4 wae/1 wpi (Figure 4-7 A). This indicates that injury after the appearance of tau-mediated axonal swellings in $htau^{0N3R}$;Wld^S ORNs can halt the progression of the swellings.

The coverage of axonal swellings increases significantly in injured Wld^S ORNs at 1 wpi ($P<0.05$; Figure 4-7 A), however higher magnification images of ORNs indicate that the swellings in injured axons are morphologically different to those appearing in age-matched uninjured $htau^{0N3R}$;Wld^S axons. The swellings observed in injured axons are much smaller, with little difference between the swellings observed in injured Wld^S and $htau^{0N3R}$;Wld^S ORNs (Figure 4-7 D and E), with this supported by the quantification of swelling coverage ($P>0.05$, Wld^S vs. $htau^{0N3R}$;Wld^S at 4 wae/1 wpi and 6 wae/3 wpi). By contrast, the swellings in aged $htau^{0N3R}$;Wld^S axons are very large and cover a significantly greater area compared with age-matched uninjured Wld^S brains ($P<0.0001$, Wld^S vs. $htau^{0N3R}$;Wld^S at 4 wae/ui and 6 wae/ui). This can be observed in the representative images, with larger swellings present in $htau^{0N3R}$;Wld^S brains at 6 wae/ui (Figure 4-7 C) compared with the age-matched uninjured Wld^S brains (Figure 4-7 B) and the injured $htau^{0N3R}$;Wld^S brains (Figure 4-7 E). This indicates that the swellings are morphologically distinct in injury and tauopathy. Additionally, axotomy of $htau^{0N3R}$;Wld^S expressing axons at 3 wae was able to halt the progression of the axonal swellings, with no significant difference observed between 3 wae/ui and 6 wae/3 wpi in injured $htau^{0N3R}Wld^S$ brains. This indicates that the Wld^S-mediated protection post-injury was able to halt the progression of tau-mediated degeneration.

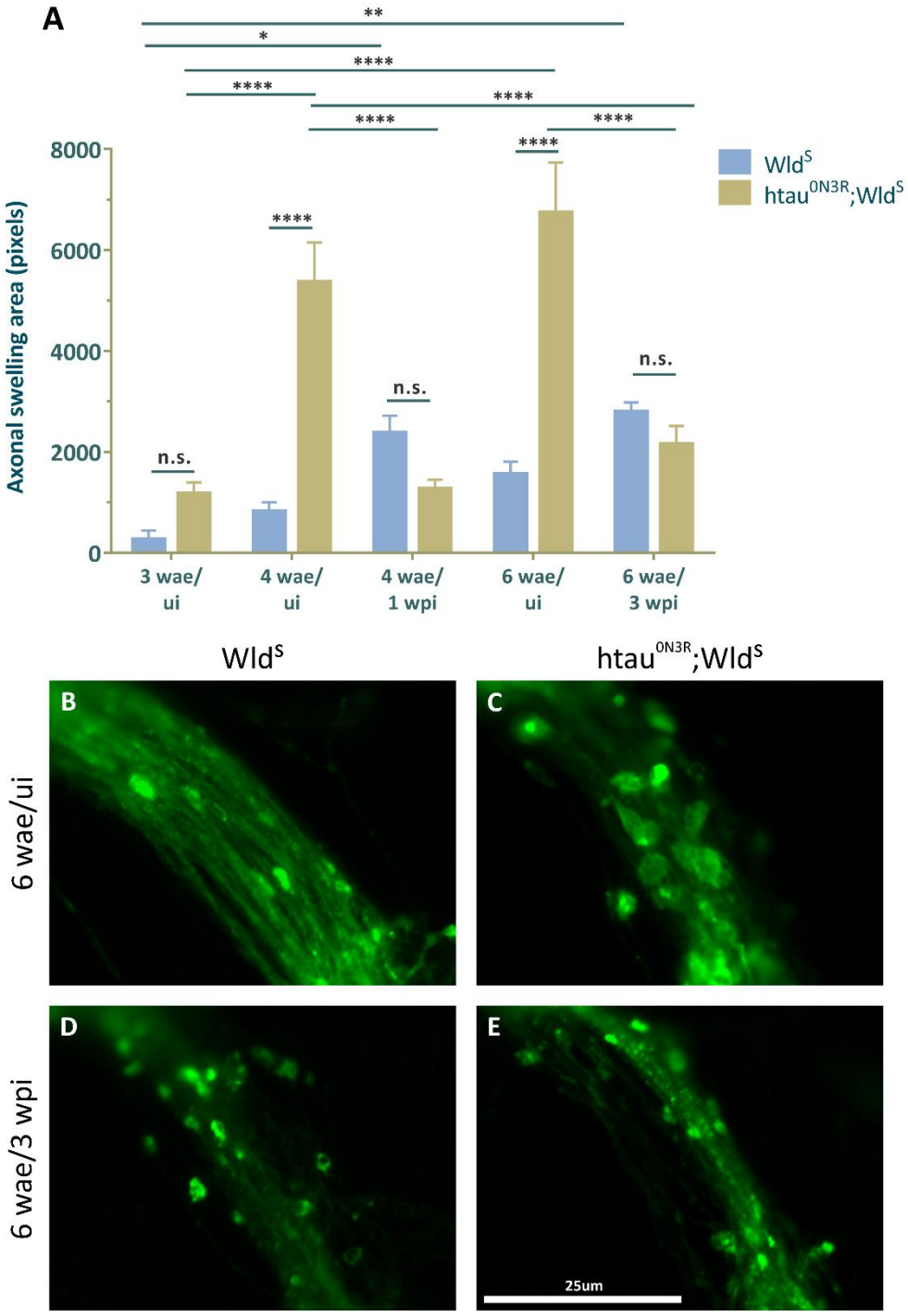


Figure 4-7. Tau-mediated axonal swellings are halted from progressing when axons are injured at 3 wae. [A] Quantification of axonal swellings reveals that in uninjured htau^{0N3R};Wld^{S} axons, the level of swellings increases significantly at 4 wae and 6 wae. In injured htau^{0N3R};Wld^{S} axons the level of swellings does not increase significantly over time. Representative images from uninjured [B] Wld^{S} and [C] htau^{0N3R};Wld^{S} expressing axons. Following axotomy, injury associated swellings appear in [D] Wld^{S} axons, but tau-mediated swellings are prevented in [E] htau^{0N3R};Wld^{S} axons. Scale bar = 25µm. Values are presented as the mean ± SEM. n=>8; *P<0.05, **P<0.01, ****P<0.0001; ANOVA, Bonferroni's multiple comparison. wae, weeks after eclosion; ui, uninjured; wpi, weeks post-injury.

4.4 Discussion

It has been proposed that axonal degeneration occurring after injury and in disease follows the same molecular pathway; however the evidence presented here supports the findings of the previous chapter, that this is not the case. Although Wld^S is unable to delay tau-mediated degeneration, when $htau^{0N3R}$;Wld^S axons are axotomised injury-induced axonal degeneration is delayed. Moreover, the appearance of tau-mediated axonal swellings is also prevented in $htau^{0N3R}$;Wld^S axons following injury. Not only can the swellings be prevented from appearing, but their progression can be halted if $htau^{0N3R}$;Wld^S axons are injured after the swellings have established. This indicates a bystander protection of tau-mediated axonal degeneration may be occurring in injured $htau^{0N3R}$;Wld^S axons, and understanding this phenomenon could yield important information regarding how axons degenerate in neurodegenerative disease.

4.4.1 Wld^S provides axonal protection after injury in $htau^{0N3R}$;Wld^S co-expressing axons

The previous chapter demonstrated that Wld^S was unable to delay tau-mediated axonal degeneration. A potential explanation for this is that Wld^S was unable to be protective due to tau-mediated disruptions of axonal transport. However, the lengthy protection of $htau^{0N3R}$;Wld^S axons occurring post-axotomy does not support this, instead suggesting that a different pathway mediates axonal degeneration in tauopathy than after injury.

This again raises the idea that separate pathways control axonal degeneration after acute injury and in tauopathy. There are previous reports of models of chronic disease in which Wld^S did not provide protection. Wld^S mice inoculated with prion infected brain homogenate displayed no alteration in the progression of disease in either behavioural measures or morphological breakdown of axons (Gultner et al., 2009). Similarly, in SOD1 mutant models of motor neuron disease axon loss was unchanged by Wld^S (Vande Velde et al., 2004, Fischer et al., 2005). Wld^S also showed no protection in a model of spinal muscular atrophy (SMA) (Rose et al., 2008, Kariya et al., 2009), despite providing protection when SMA;Wld^S mice underwent axotomy (Kariya et al., 2009). This mirrors the results presented here, which showed no protection against axonal degeneration in $htau^{0N3R}$;Wld^S axons, but robust axonal protection after axotomy. Together, these cases support the idea that a separate pathway

controls axonal degeneration in certain diseases, and that this pathway is not sensitive to the protective effects of Wld^S.

There is evidence that Wld^S can provide benefit in some models of disease, however the improvements are modest and transient in nature. Wld^S was found to reduce axonal swellings in GAD mice, a model of axonal dystrophy, however this did not coincide with improvements in behaviour (Mi et al., 2005). Whilst Wld^S was found to provide benefit at early time points in both *pmn* mice (Ferri et al., 2003) and P0 null mice (Samsam et al., 2003), this effect was transient and axon loss was found to be unaltered at later time points in both models.

The models in which Wld^S has been reported to have the most benefit all have an acute component to the onset of degeneration. This includes models of physical injury (Beirowski et al., 2008, Wang et al., 2013), toxic neuropathy (Wang et al., 2002), ischemia (Gillingwater et al., 2004), and excitotoxicity (Bull et al., 2012), all of which demonstrated a strong delay in axonal degeneration with Wld^S or cytNmnat1. Although Wld^S has been reported to delay axonal degeneration in models of Parkinson's disease (Sajadi et al., 2004, Hasbani and O'Malley, 2006, Antenor-Dorsey and O'Malley, 2012), it is important to note that these models use the administration of 6-OHDA or MPTP which selectively destroy dopaminergic neurons. These neurotoxic compounds do no recapitulate the actual disease mechanisms occurring in Parkinson's, instead reflecting a more acute injury.

Whilst Wallerian-like degeneration has been observed in numerous disease models, the inability of Wld^S to delay degeneration in a number of models indicates that not all Wallerian-like degeneration is Wld^S-sensitive, and that a separate pathway controls axonal degeneration in diseases such as tauopathy.

4.4.2 Wld^S-mediated protection after injury results in bystander protection of tau-mediated axonal degeneration

The observation that tau-mediated axonal swellings do not manifest in injured $htau^{0N3R}$;Wld^S axons suggests that something associated with the injury and the subsequent Wld^S-mediated protection has prevented $htau^{0N3R}$ from resulting in axonal degeneration. Is this due to activation of Wld^S by injury? Or simply disconnection from the cell body? What other changes are associated with injury and could these impact on human tau?

Is Wld^S protection activated by injury?

The lack of Wld^S protection against tau-mediated degeneration in uninjured axons compared with the protection occurring post-injury could indicate that Wld^S protection requires activation by an acute injury associated signal. The absence of this signal in this model of tauopathy could explain why Wld^S was unable to protect in the absence of acute injury.

The Wld^S protein is not activated by injury, however its protective phenotype is only revealed when an axon receives an acute injury. The biosynthetic activity of Nmnat1 within Wld^S is crucial to its protective phenotype, and in uninjured tissue, Nmnat activity levels are 4-fold higher that in wild-type controls (Mack et al., 2001). The rapid loss of Nmnat2 following acute injury is thought to be compensated for by the presence of Wld^S (and therefore Nmnat1 activity) within the axon, thereby preventing the downstream processes triggered by the loss of Nmnat2 activity post-injury.

Whilst the Wld^S protein itself is not activated by injury, the signalling pathway that it disrupts may be activated by an injury-associated signal not found in tauopathy. Components of MAPK signalling have been found to be activated by acute injury, with evidence showing this pathway sits downstream of Sarm (Yang et al., 2015). Knock out of Sarm1 and expression of cytNmnat1 were both demonstrated to prevent key events in this pathway occurring after injury (Yang et al., 2015). However it remains to be seen whether this pathway is also activated in axons degenerating in disease.

As discussed previously, a separate molecular cascade may initiate axonal degeneration in certain diseases, with this converging on a common execution cascade (Figure 4-8). This would explain why axonal degeneration in injury and disease is morphologically similar. It could also explain why injured htau^{0N3R};Wld^S axons were protected against tau-mediated axonal degeneration. The Wld^S-mediated protection against injury-induced degeneration may over-ride the pro-degenerative signals initiated by human tau (Figure 4-8), and may result in bystander protection against tau-mediated axonal degeneration. These two separate pathways can be distinguished as Wld^S-sensitive and -insensitive pathways. How the activation of the Wld^S-sensitive pathway after injury may override the Wld^S-insensitive pathway initiated by tau is unclear, and further study is required to investigate the potential points of interaction between the two pathways.

Alternatively, changes in normal axon biology as a result of axotomy may halt tau-mediated neurodegeneration, and the presence of Wld^S may simply enable the injured axon to survive and this effect be observed.

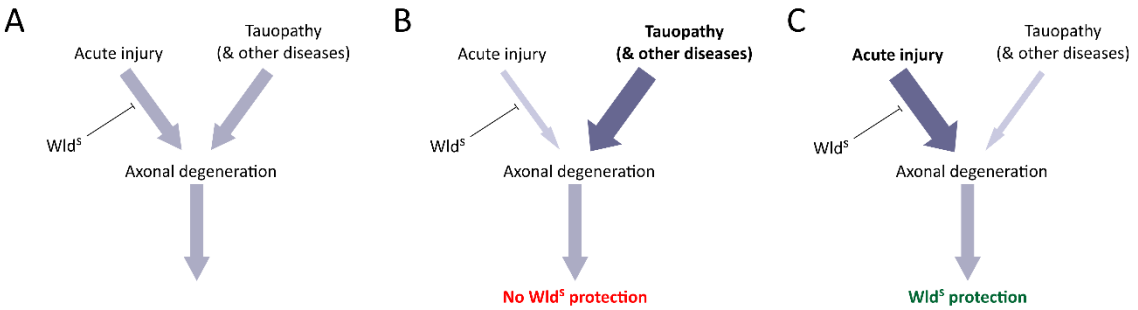


Figure 4-8. Model of Wld^S protection in injury and disease. [A] Acute injury and tauopathy activate different initiator pathways which converge on a common effector pathway in axonal degeneration. Wld^S can only protect against one initiator pathway, which can be said to be 'Wld^S-sensitive'. [B] In uninjured $htau^{0N3R}$;Wld^S axons, human tau drives a different 'Wld^S-insensitive' pathway, therefore no axonal protection is mediated by Wld^S. [C] When $htau^{0N3R}$;Wld^S axons are injured, Wld^S mediated protection acting on the Wld^S-sensitive pathway overrides the effect of human tau, resulting in axonal protection against injury-induced and tau-mediated axonal degeneration.

Could changes in normal axon biology post-injury impact on tau pathology?

How an axon responds to losing connection with its cell body is poorly understood. The discovery of Wld^S has enabled scientists to investigate how protein expression, axonal transport and neuronal excitability are altered after injury and how Wld^S disrupts these changes. These changes combined with the loss of the cell body occurring following injury could all be contributing factors to the bystander protection against tau-mediated degeneration observed in $htau^{0N3R}$;Wld^S axons after injury.

Perhaps unsurprisingly, proteins involved in synaptic and axonal stability were found to be altered after cortical lesion in mice (Wishart et al., 2012). Network analysis on proteomics data found that the 47 hits identified were functionally clustered around synaptic function and neurite development, including vesicular transport and cytoskeletal biogenesis. The authors of this work removed any proteins from their analysis which were also found to be altered in Wld^S mice (19 proteins), as they felt those changes represented a systemic response to injury. As such, they did not report on functional clustering of the 19 excluded proteins, however these could be candidates for the bystander protection in $htau^{0N3R}$;Wld^S injured axons.

How pathological human tau contributes to the formation of axonal swellings is unclear, however the reduction in cytoskeletal integrity and consequent disruption of axonal transport

mediated by tau is thought to be key. If axonal transport were to halt after injury to Wld^S expressing axons, this could explain why tau-mediated axonal degeneration is not observed in injured $htau^{0N3R}$;Wld^S axons.

In *Drosophila*, the transport of mitochondria was shown to immediately halt after axonal injury, but this was not seen in Wld^S expressing axons in which mitochondria maintained motility (Avery et al., 2012, Fang et al., 2012). However a conflicting study in zebrafish has indicated that mitochondrial transport only terminates in close proximity to the injury site, with mitochondria further away being unaffected (O'Donnell et al., 2013). Comparably to *Drosophila,* expression of Wld^S in zebrafish restored mitochondrial motility near the injury site (O'Donnell et al., 2013). Vesicular motility was found to be unaffected by injury and persisted until just prior to fragmentation when both mitochondrial and vesicular transport terminated (O'Donnell et al., 2013). Together these studies indicate that axonal transport of both vesicles and mitochondria continues as normal up until fragmentation in Wld^S expressing axons, suggesting that changes to axonal transport may not contribute to the bystander protection in $htau^{0N3R}$;Wld^S axons.

However, whilst axonal transport has been demonstrated to persist in injured Wld^S axons, these reports have only investigated changes over the first few hours and days after injury. With axons persisting up to 32 dpi in injured $htau^{0N3R}$;Wld^S flies (Figure 4-2), it is not known for how long axonal transport remains functional in these axons. In addition, loss of the cell body means that the demands placed upon the axon for transport are drastically lessened, as there is no new material requiring transport. Therefore in the protected injured $htau^{0N3R}$;Wld^S axon, axonal transport may be reduced and thereby the pathological effect of human tau limited, which could explain the bystander protection after injury.

Neuronal excitability after injury has been investigated in transected axons and found to be lost prior to fragmentation. In Wld^S expressing axons, excitability is maintained for longer however the precise length differs between the studies that have investigated it. In the first, transected axons were able to conduct evoked action potentials for 1-2 weeks (Tsao et al., 1994), however a more recent study found that by 5 days post-injury conductance was lost (Moldovan et al., 2009). Whilst transected axons may have the potential to still conduct, unless stimulated experimentally they would remain electrically silent, due to their disconnection from the cell body. It has been demonstrated that neuronal excitability can affect the rate of Wallerian degeneration; when excitability is reduced degeneration is slowed

and when neurons are hyperexcitable degeneration is accelerated (Mishra et al., 2013). Whether the lack of neuronal excitability in injured htau^{0N3R};Wld^{S} axons has an effect on the pathogenicity of tau is unknown, but it could be a contributor to the bystander protection observed in htau^{0N3R};Wld^{S} axons.

The loss of the cell body in the injured htau^{0N3R};Wld^{S} flies may be key in the prevention of tau-mediated axonal degeneration. Whilst normally tau is predominantly localised in the axon (Binder et al., 1985), numerous studies have demonstrated that it is missorted in tauopathy, with an increased presence in the cell soma. This missorting to the somatodendritic compartment correlates with the loss of synapses (Coleman & Yao, 2003), indicating that is a key event in the progression of tau pathology. Normally, a barrier exists which prevents tau from entering the soma, however, alterations such as increased tau phosphorylation and its detachment from microtubules have been shown to result in the breakdown of the barrier, permitting the entry of tau into the soma (Li et al., 2011). Considering that this missorting of tau is thought to be key in tau toxicity, the loss of the cell body due to axotomy in htau^{0N3R};Wld^{S} is highly likely to affect the progression of tau pathology. This could be a potential explanation for why axonal degeneration was prevent in injured htau^{0N3R};Wld^{S} flies; that the loss of the cell body prevented the initiation of tau toxicity normally mediated through the missorting of tau to the somatodendritic compartment. Together, these functional changes occurring within the injured axon protected from degeneration by Wld^{S} could be contributing to the bystander protection observed in htau^{0N3R};Wld^{S} axons. Understanding how these changes could be altering the pathogenicity of tau could be crucial to the understanding of how pathological changes to tau result in axonal degeneration and neuronal death.

Chapter 5: Restoring the microtubule cytoskeleton does not rescue mitochondrial mislocalisation in tauopathy

5.1 Introduction

Mitochondrial dysfunction is regarded as a significant contributor to neurodegenerative diseases such as tauopathies, since effective mitochondrial function is crucial to the survival of the neuron (Mukherjee and Chakrabarti, 2013). Tauopathy is associated with defects in oxidative phosphorylation (Schulz et al., 2012), increased ROS production (David et al., 2005, Quintanilla et al., 2009, Amadoro et al., 2014) lowered membrane potential (Chee et al., 2005, Quintanilla et al., 2009) and disrupted mitochondrial dynamics (Quintanilla et al., 2009, Duboff et al., 2012, Amadoro et al., 2014). Mitochondrial dynamics, transport, function and degradation are highly inter-dependent processes, with disturbances in one process having effects on all others.

Mitochondrial mislocalisation has been observed in human AD patients (Kopeikina et al., 2011) as well as in animal models of tauopathy (Chee et al., 2005, Kopeikina et al., 2011, Schulz et al., 2012, Amadoro et al., 2014). Alterations in axonal transport are another well reported feature of tauopathy (Ebneth et al., 1998, Shemesh et al., 2008, Ittner et al., 2009, Kanaan et al., 2011, Quraishe et al., 2013); therefore the mislocalisation of mitochondria could be as a result of disrupted axonal transport in tauopathy.

To investigate this, the microtubule stabilising drug NAP was used to investigate whether mitochondrial mislocalisation could be rescued by restoring microtubules and rescuing axonal transport in the *Drosophila* larval model of tauopathy.

5.2 Materials & Methods

5.2.1 Drug treatments

Crosses were set up on normal food and the mating adults allowed to mate and lay eggs for 1-2 days, before being transferred onto drug food. The microtubule stabilising drug, NAP, was present in food at a final concentration of 2.5µg/ml.

5.2.2 Larval dissection & immunofluorescence

Wandering third instar larvae from D42-mitoGFP driven crosses were dissected in *Drosophila* saline and fixed in 4% formaldehyde. Skins were stained with anti-HRP-Rhodamine (1:200; Jackson Immuno Research Laboratories) and mounted in Vectashield. Images of muscle 4 NMJs were taken on a fluorescence microscope. Image analysis was performed in ImageJ, with the area of the NMJ covered by mitochondria calculated as a percentage of the total NMJ area.

5.2.3 Axonal transport

Wandering third instar larvae from vGFP.D42 driven crosses were anaesthetised, mounted, and images of vGFP aggregates captured. Whilst blinded to genotype, the coverage of aggregates was measured per animal and the mean per genotype calculated.

5.3 Results

5.3.1 Mitochondria are mislocalised in larvae expressing human tau

Efficient synaptic function requires a pool of functional mitochondria to be present in close proximity to the synapse, to support the high energy demand associated with vesicle release (Guo et al., 2005). To investigate whether tau-mediated disruption of axonal transport reduces the pool of mitochondria present at the synapse, a line expressing GFP tagged mitochondria within motor neurons was obtained, D42-mitoGFP. Staining with an antibody against HRP conjugated to rhodamine enabled visualisation of the larval NMJ, with mitoGFP indicating the mitochondria contained within it.

In control larvae, the anti-HRP staining revealed the characteristic 'beads on a string' like appearance of the NMJ, with the mitoGFP signal appearing punctate within the boutons (arrowheads Figure 5-1 A). Expression of human tau resulted in a reduced presence of mitochondria at the synapse, with many boutons appearing completely devoid of mitoGFP signal (arrows Figure 5-1 A). To quantify these images, the area occupied by mitochondria as a percentage of the total NMJ area was calculated. Control NMJs contained significantly ($P<0.0001$) more mitochondria ($14.5\pm1.36\%$ $n=10$) than tau NMJs ($1.51\pm0.96\%$ $n=9$), confirming that human tau reduces the presence of mitochondria at the synapse (Figure 5-1 B).

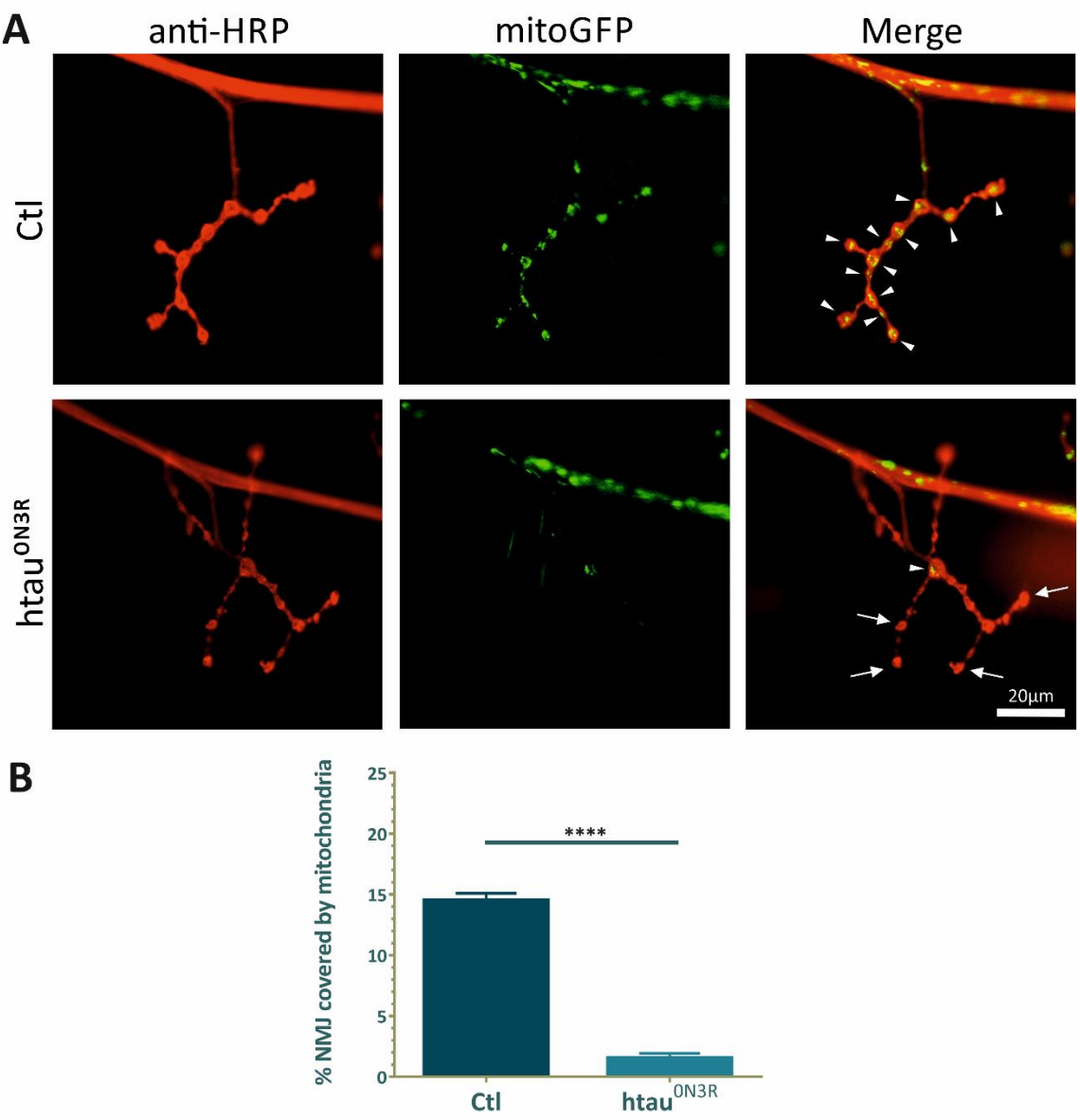


Figure 5-1. Tau expression results in mislocalisation of mitochondria. [A] Images of control and htau^{0N3R} expressing NMJs showing mitochondria within muscle 4 NMJ (arrowheads) and boutons devoid of mitochondria (arrows). [B] Quantification of the area covered by mitochondria indicates a significant reduction in htau^{0N3R} expressing larvae. Values are presented as the mean ± SEM. $^{****}P<0.0001$; n=9-10; Student's t-test.

5.3.2 NAP rescues axonal transport but not mitochondrial mislocalisation in larvae expressing human tau

To investigate whether the reduced presence of mitochondria within the synapse was due to disrupted axonal transport, the microtubule stabilising drug NAP was used. This drug restores the microtubule cytoskeleton and associated tau-mediated phenotypes, without an effect on tau hyperphosphorylation (Quraishe et al., 2013). Thus, its use sheds light on whether mitochondrial mislocalisation in tauopathy is due to the loss of the normal function of tau, microtubule stabilisation.

Images of NAP treated htau^{0N3R} larvae revealed that the NMJs contained fewer mitochondria than controls. Like in the untreated htau^{0N3R} larvae, many boutons were completely devoid of mitochondria (arrows Figure 5-2 A), indicating that NAP did not rescue this phenotype. The percentage of the NMJ occupied by mitochondria was significantly lower ($P<0.0001$) in htau^{0N3R} NMJs (2.47±1.5% n=9) than in controls (18.73±6.39% n=6) (Figure 5-2 B).

To check that the drug was working as expected, a preliminary axonal transport assay was performed using vGFP.D42 larvae. This line enables the visualisation of axonal transport due to GFP tagged neuropeptide Y which is transported in vesicles. As previously described, vesicles are homogenously distributed within peripheral nerves of control larvae (Figure 5-3 A). However in htau^{0N3R} expressing larvae, transport is disrupted resulting in the appearance of large aggregates of vesicles (arrowheads Figure 5-3 B). Treatment with NAP reduced the incidence of aggregates appearing in the larval peripheral nerves (Figure 5-3 C), however, due to the limited nature of this preliminary investigation, it is not possible to determine whether the effect was statistically significant (Figure 5-3 D). This suggests that NAP rescues axonal transport of vesicles but does not restore mitochondrial localisation within synapses.

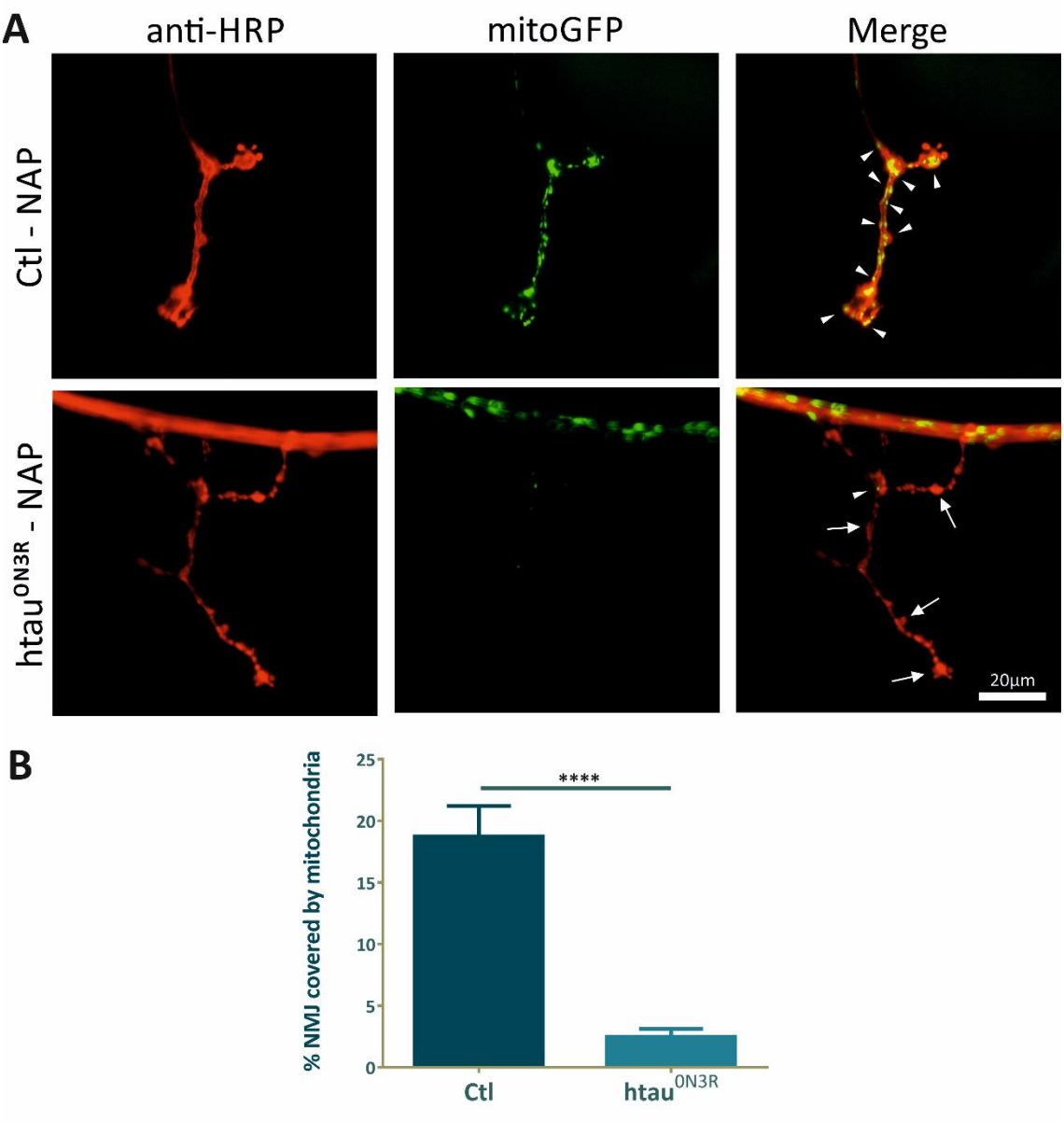


Figure 5-2. NAP does not rescue mitochondrial localisation. [A] Images of NAP treated control] and htau^{0N3R} expressing NMJs showing mitochondria within muscle 4 NMJ (arrowheads) and boutons devoid of mitochondria (arrows). [B] Quantification of the area covered by mitochondria indicates NAP does not increase mitochondria present at the synapse in htau^{0N3R} expressing larvae. Data are presented as the mean ± SEM. $^{****}P<0.0001$; n=6-9; Student's t-test.

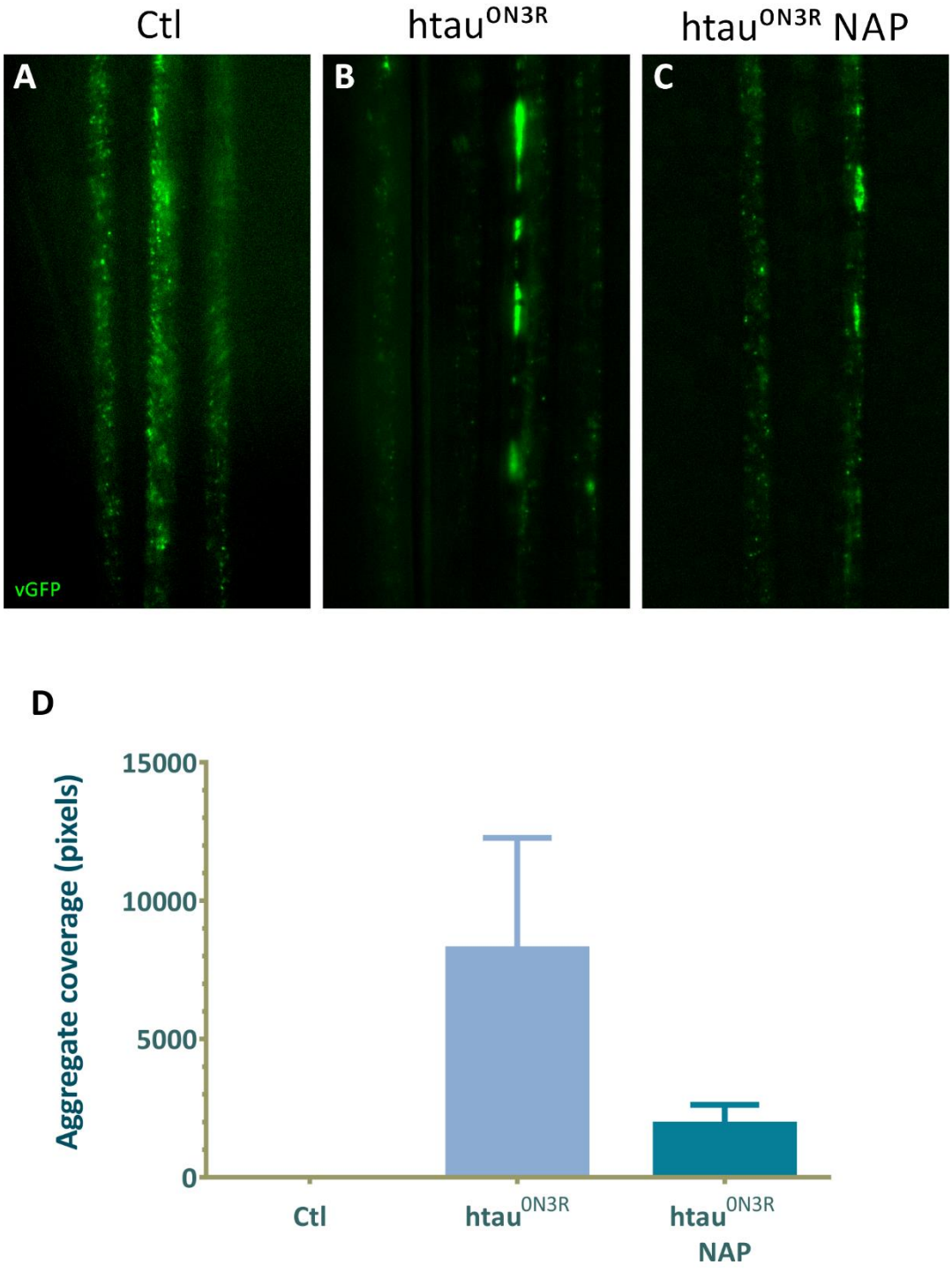


Figure 5-3. Effect of NAP on axonal transport deficits. Images from peripheral axons in [A] control, [B] htau^{0N3R} expressing and [C] NAP treated htau^{0N3R} expressing larvae showing aggregates of vesicles indicative of axonal transport disruption (magnification, x63). [D] Quantification of the coverage of vesicular aggregates. Values are presented as the mean ± SEM. n=3-5; P>0.05, ANOVA; Bonferroni's multiple comparison.

5.4 Discussion

As found previously, expression of human tau results in mislocalisation of neuronal mitochondria, with a depletion from synapses. Whilst restoring the microtubule cytoskeleton using the stabilising drug NAP rescued axonal transport deficits, no improvement in mitochondrial mislocalisation was observed. This suggests that the loss of mitochondria from synapses is not simply due to the breakdown of the microtubule cytoskeleton.

5.4.1 Mitochondrial mislocalisation in tauopathy

The disruption of mitochondrial localisation by tau has been well documented in a number of studies. Previous work in *Drosophila* over expressing WT human tau ($htau^{0N3R}$) also demonstrated a reduction in the number of mitochondria present at larval synapses (Chee et al., 2005). Clustering and accumulation of mitochondria in cell bodies has been seen in cell cultures expressing mutant tau (P301L htau) (Schulz et al., 2012) and in cultures of primary neurons expressing an N-terminal fragment of human tau (NH_2 tau) (Amadoro et al., 2014). Altered mitochondrial localisation has also been observed in mice expressing mutant tau (P301L htau), in which neurons positive for misfolded tau displayed a reduced area of cytoplasm occupied by mitochondria, in both the cell body and neurites (Kopeikina et al., 2011). Furthermore, in human cases of AD both positive and negative staining for misfolded tau was associated with disrupted mitochondrial localisation compared to normal controls (Kopeikina et al., 2011). Together, this indicates that tau-mediated mitochondrial mislocalisation is a key characteristic of tauopathies.

5.4.2 Microtubule destabilisation as a therapeutic target in tauopathy

As microtubule destabilisation is thought to be a key disease associated mechanism in AD and other tauopathies (Cash et al., 2003, Cowan et al., 2010), much effort has gone into finding agents to restabilise the cytoskeleton. NAP is a small peptide which was found to stabilise tubulin *in vitro* (Divinski et al., 2006), and has shown success in *in vivo* models of disease. Treatment of *Drosophila* overexpressing WT human tau ($htau^{0N3R}$) restabilised microtubules, rescued axonal transport deficits and improved behavioural phenotypes. This work indicated that it was possible to compensate for tau loss of function and bypass tau hyperphosphorylation, as NAP was found not to alter tau phosphorylation levels (Quraishe et al., 2013). However these findings conflict with work in NAP treated 3xTg.AD mice, in

which a reduction in tau phosphorylation was observed (Matsuoka et al., 2007). Other microtubule stabilising agents have also shown success in ameliorating tau-mediated neuronal dysfunction. When mutant tau mice (P301S htau) were treated with epothilone D, microtubule restabilisation and improved axonal transport and behaviour were observed, along with reductions in tau phosphorylation (Zhang et al., 2012). The conflicting reports regarding the effects of microtubule stabilising agents upon tau phosphorylation could be explained by the different timescales of the studies. Whilst short term NAP treatment (as in *Drosophila*) impacts upon neuronal dysfunction mediated by the loss microtubule stabilisation by tau, prolonged treatment (as in the mouse studies) and the resultant improved neuronal function may feedback upon the mechanisms involved with tau hyperphosphorylation. Nonetheless, short term NAP treatment in the work described here was unable to improve mitochondrial mislocalisation, indicating that the causal mechanisms go further than disrupted axonal transport due to microtubule destabilisation.

5.4.3 Mitochondrial mislocalisation: more than just a transport deficit?

The failure of NAP to improve mitochondrial localisation indicates that there are additional mechanisms controlling the transport of mitochondria, which remain dysfunctional despite a restabilised microtubule cytoskeleton. Indeed, evidence exists showing that tau affects mitochondria in multiple ways, but due to the interdependent nature of mitochondrial dynamics, biogenesis, transport and mitophagy, disturbances in one process has effects upon all others making it difficult to pin point precisely how tau affects mitochondria.

It has been suggested that disruptions in the axonal transport of mitochondria alters their fission/fusion dynamics resulting in mitochondrial dysfunction. However, some studies have indicated that altered mitochondrial dynamics are not caused by reductions in axonal transport alone. Disrupting mitochondrial transport through the knockdown of adapter proteins Miro and Milton in tau transgenic *Drosophila* results in the accumulation of elongated mitochondria in cell bodies and the exacerbation of tau toxicity (Duboff et al., 2012, Iijima-Ando et al., 2012). Whilst mutations in Miro in *Drosophila* prevent mitochondria from reaching synapses and results in locomotor deficits, the mitochondria themselves show no structural or functional changes, such as reduced membrane potential or elongation (Guo et al., 2005, Duboff et al., 2012). This suggests that tau-mediated disruption in the axonal transport of mitochondria is not responsible for alterations in mitochondrial dynamics or function.

A proposed mechanism for tau-mediated alterations in mitochondrial dynamics and function is via an effect on actin; tau has been demonstrated to cause excess stabilisation of f-actin, which in turn results in an increased associated between f-actin and Drp1 and a concomitant dissociation of Drp1 with mitochondria (Duboff et al., 2012). Drp1 functions in mitochondrial fission, and therefore is an essential component for mitochondrial dynamics. The altered association between Drp1 and mitochondria in tauopathy results in mitochondrial elongation which is associated with neurotoxicity. Increasing mitochondrial fusion through the knockdown of Drp1 and overexpression of Mitofusin causes enhanced mitochondrial elongation and toxicity, whilst increasing fission normalised mitochondrial length and lowered tau toxicity (Duboff et al., 2012). This indicates that altered Drp1 localisation in tauopathy causes mitochondrial dysfunction and contributes to degeneration. Furthermore, Drp1 has been found to directly interact with phosphorylated tau from human AD brains (Manczak and Reddy, 2012), further implicating it in tau-mediated mitochondrial dysfunction.

Altered mitochondrial dynamics is reported in a number of animal models, with different forms of tau altering the balance differentially. Expression of full length (2N4R) WT human tau in immortalised cortical cultures resulted in an increase in mitochondrial length (elongation) whilst expression of Asp421 truncated human tau resulted in decreased mitochondrial length (fragmentation). The fragmented mitochondria were found to have increased ROS production, a lower membrane potential and reduced Ca^{2+} uptake, whilst no adverse effects were seen with the elongated mitochondria (Quintanilla et al., 2009). Mitochondrial fragmentation was also observed in cultures of hippocampal neurons expressing an N-terminal fragment of human tau (NH_2), and was associated with mitochondrial dysfunction, reduced axonal transport of mitochondria as well as a reduced quantity of mitochondria (Amadoro et al., 2014). This study also investigated changes in the mitochondrial proteome associated with the expression of NH_2 tau, revealing changes in protein levels that result in mitochondria with a reduced ability for fusion, such as reduced OPA1 and Mitofusin 1 and 2. They also saw an increase in autophagy, with dying mitochondria associated with autophagosomes (Amadoro et al., 2014).

These reports of tau-mediated mitochondrial fragmentation conflict with the study in *Drosophila* in which expression of mutant tau (R406W htau) resulted in mitochondrial elongation which was associated with toxicity (Duboff et al., 2012). However together these

studies highlight that whilst different tau species may affect mitochondrial dynamics differentially, both lead to mitochondrial dysfunction.

The main purpose of mitochondrial dynamics is quality control, with damaged mitochondria repaired by fusion with healthy mitochondria, and fission enabling the sequestration of irreversibly damaged mitochondria which subsequently can be removed by mitophagy. Therefore the altered mitochondrial dynamics observed in tauopathy (Quintanilla et al., 2009, Duboff et al., 2012, Schulz et al., 2012, Amadoro et al., 2014) impacts upon the quality of the mitochondria present within neurons. Increasing levels of mitochondria that are irreversibly damaged and targeted for mitophagy reduces the pool of healthy mitochondria available for transport. This could be one such mechanism by which mitochondrial localisation is affected in tauopathy.

A further complication in the tau-mitochondrial story is that mitochondrial dysfunction itself can impact upon tau. Loss of a mitochondrial scaffolding protein (Prohibitin1) results in the accumulation of hyperphosphorylated tau (Merkwirth et al., 2012) and mitochondrial poisons cause tau phosphorylation (Hoglinger et al., 2005), phenocopying the effect of tau upon synaptic function (Chee et al., 2005). This results in a chicken and egg situation in which it is difficult to see which is causative of the other; does tau cause mitochondrial dysfunction or does mitochondrial dysfunction cause tau to become toxic?

5.4.4 Future directions

Nonetheless, it is clear that tau impacts mitochondria in a multitude of ways. The failure of NAP to rescue mitochondrial mislocalisation in a *Drosophila* model of tauopathy supports this, as restabilising microtubules and restoring axonal transport did not rescue mitochondrial mislocalisation. As previous work has shown that NAP treatment of htau^{0N3R} expressing larvae does not alter tau phosphorylation (Quraishe et al., 2013), it is likely that the continued presence of phosphorylated tau is impacting upon mitochondria. Due to time constraints it was not possible to investigate whether reducing tau phosphorylation, such as using an inhibitor of GSK3β, could rescue the mitochondrial mislocalisation in this model. However, these experiments would shed more light on tau-mediated mitochondrial dysfunction and the mechanisms involved.

Chapter 6: General Discussion

Ever since Wallerian degeneration was first described over 150 years ago, the idea that neuronal axons damaged by physical injury or disease-associated processes degenerate by a similar mechanism has held much appeal. However, understanding the mechanisms underpinning axonal degeneration has only begun in recent decades, with these investigations shedding light on whether a common mechanism exists. This thesis forms part of these investigations and indicates that there are important differences between how axons degenerate following injury and in certain diseases.

Wld^S robustly delays axonal degeneration after injury, so to investigate whether the same mechanism controls axonal degeneration in disease, I co-expressed human tau and Wld^S. Cytoskeletal breakdown has been implicated in both Wallerian degeneration and tauopathy, and as Wld^S prevents this in Wallerian degeneration I was interested to see whether it could do the same in tauopathy, and rescue the neuronal dysfunction associated with this breakdown. However, these investigations revealed that Wld^S does not impact upon tau-mediated neuronal dysfunction. Co-expression of $htau^{0N3R}$ and Wld^S did not rescue disrupted axonal transport, aberrant synaptic morphology nor improve locomotor behaviour (Chapter 3). The *Drosophila* larval model is unique in providing insight into tau-mediated dysfunction in the absence of degeneration. Previous work in this model has indicated that disrupted axonal transport and altered locomotor behaviour are due to the loss of microtubule stabilisation by hyperphosphorylated tau (Cowan et al., 2010). Whilst Wld^S protection after injury can prevent the breakdown of the microtubule cytoskeleton, the lack of improvement when co-expressed with tau indicates that the mechanism by which it does so after injury is not relevant to tau-mediated microtubule breakdown. In addition, whilst functional improvements have been reported in other investigations of Wld^S in disease models, this correlates with delayed axonal degeneration (Ferri et al., 2003), indicating that Wld^S cannot impact upon the disease mechanisms causing dysfunction.

Investigations into axonal degeneration in $htau^{0N3R}$;Wld^S co-expressing adult *Drosophila* revealed that Wld^S was unable to delay the onset or slow the progression of axonal swellings, which are indicative of axonal degeneration. This correlated with no improvement in adult behaviour, but a rescue of lifespan was observed (Chapter 3). Wld^S has been found to delay axonal degeneration in a number of disease models, such as Charcot-Marie-Tooth disease (Samsam et al., 2003, Meyer Zu Horste et al., 2011), gracile axonal dystrophy (Mi et al., 2005) and Parkinson's disease (Sajadi et al., 2004, Hasbani and O'Malley, 2006). This indicates the degeneration occurring in these diseases is Wld^S-sensitive, and that the mechanisms

associated with these diseases may be related to the pathways projected by Wld^S, such as Ca^{2+} influx and alterations in NAD levels.

For example, reductions in NAD levels have been observed in the EAE mouse model of multiple sclerosis, with this reduction prevented in Wld^S mice (Kaneko et al., 2006). In addition, it has been reported that EAE mice treated with NAD exhibit reduced myelin loss and axonal degeneration, however, it is unclear whether this is due to a direct effect upon axons or via alterations in $CD4^+$ T cells (Tullius et al, 2014). Therefore, the disease-related mechanisms in the EAE model are associated with alterations in NAD, potentially explaining why Wld^S was observed to be protective in this model. This may suggest that the pathway of axonal degeneration occurring in a disease may depend upon the specific pathological mechanisms associated with that disease, and explain why some disease are Wld^S-sensitive and others are not (Table 6-1). This indicates that understanding the underlying disease mechanisms is important in the search for potential therapeutics, and that it is unlikely that targeting a single pathway will be beneficial in all diseases in which Wallerian-like degeneration has been observed.

Supporting the idea of multiple pathways leading to Wallerian-like degeneration, flies co-expressing $htau^{0N3R}$ and Wld^S were robustly protected against injury induced axonal degeneration. In addition, Wld^S protection after injury provided a by-stander protection against tau-mediated axonal degeneration (Chapter 4). This result was not due to loss of $htau^{0N3R}$ from injured $htau^{0N3R}$;Wld^S axons, and was also observed when axons were injured after tau-mediated axonal swellings were established. This interesting result could be explained by an acute injury associated signal. When absent in uninjured $htau^{0N3R}$;Wld^S axons, no protection is observed as the tau-mediated degeneration does not produce this signal, and is therefore Wld^S-insensitive. However injury of $htau^{0N3R}$;Wld^S axons results in the acute-injury signal, which reveals the Wld^S protective phenotype and provides bystander protection against tau-mediated degeneration (Figure 6-1).

Table 6-1. Table of Wld^S-sensitive and insensitive neurodegenerative diseases.

Disease modelled	Insult or mutation	Wld^S-sensitive degeneration?	Reference
Charcot-Marie-Tooth disease 1A	Overexpression of *pmp22*	Yes	(Meyer Zu Horste et al., 2011)
Charcot-Marie-Tooth disease 1B	*P0*-/- mice	Yes	(Samsam et al., 2003)
Excitotoxic injury to retina	NMDA injected	Yes	(Bull et al., 2012)
Glaucoma	Raised IOP	Yes	(Beirowski et al., 2008)
Gracile axonal dystrophy	Loss of *uchl1*	Yes	(Mi et al., 2005)
Hypoxic-ischaemic injury	Carotid artery ligation followed by hypoxia chamber	Yes	(Verghese et al., 2011)
Multiple sclerosis	EAE mice - MOG innoculation	Yes	(Kaneko et al., 2006)
Parkinson's disease	6-ODHA	Yes	(Sajadi et al., 2004)
	MPTP	Yes	(Hasbani and O'Malley, 2006)
Progressive motor neuropathy	*pmn* mice - Tbce mutation	Yes	(Ferri et al., 2003)
Toxic neuropathy	Taxol	Yes	(Wang et al., 2002)
Charcot-Marie-Tooth disease 2D	*Gars* mutation	No	(Stum et al., 2011)
Hereditary spastic paraplegia	*Plp*-/- mice	No	(Edgar et al., 2004).
Motor neuron disease	*SOD1-G93A* mice	No	(Fischer et al., 2005)
	*SOD1-G37*R mice	No	(Vande Velde et al., 2004)
	*SOD1-G85*R mice	No	(Vande Velde et al., 2004)
Prion disease	Scrapie strain 139A intracerebral innoculation	No	(Gultner et al., 2009)
Spinal muscular atrophy	SMN2+/+;SMNΔ7+/+;Smn+/− mice	No	(Kariya et al., 2009)
	SMN2;Smn−/−	No	(Kariya et al., 2009)

Two potential candidates for this acute-injury signal are NMN and Sarm. Application of FK866 to cultured SCGs prevents NMN levels rising after injury and delays axonal degeneration. However, if these cultures are supplemented with NMN then axonal protection is reverted in a dose dependent manner (Di Stefano et al., 2015). Overexpression of Sarm in DRG cultures does not cause spontaneous axonal degeneration, indicating it is activated by injury. Multimerisation via its SAM domain is required to promote axonal degeneration, as supplementation of Sarm1$^{-/-}$ cultures with SarmΔSAM (SAM deletion) did not revert axonal protection after injury as Sarm1 does (Gerdts et al., 2013). Furthermore,

expression of only the SAM and TIR domains (SAM-TIR) results in constitutively active Sarm1, triggering spontaneous degeneration in the absence of injury (Gerdts et al., 2013). Recent work has indicated that Sarm1 sits downstream of the rise in NMN, as NMN levels increase in Sarm1$^{-/-}$ mice as they do in WT mice following injury, despite robust axonal protection. Additionally, Sarm1$^{-/-}$ axons *in vitro* are protected against degeneration mediated by knockdown of Nmnat2 (Gilley et al., 2015), and supplementing cultures of transected Sarm$^{-/-}$ neurons with NMN did not result in axonal degeneration (Loreto et al., 2015). Together, this indicates that the loss of Nmnat2 sits upstream of Sarm and that the rise in NMN could be the acute-injury signal.

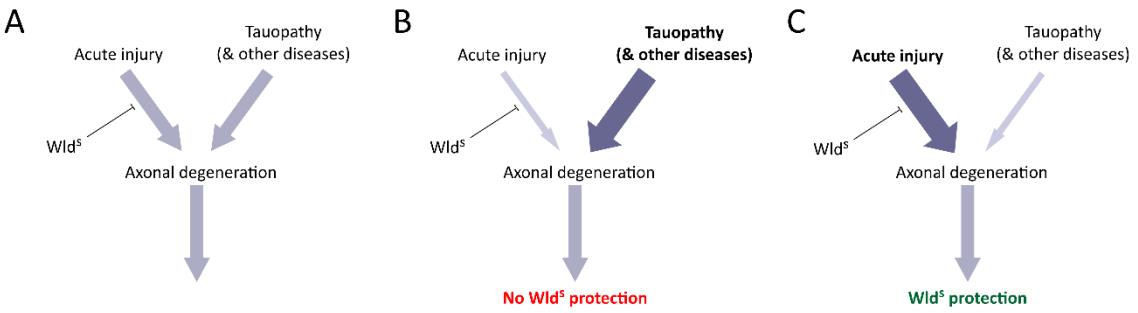


Figure 6-1. Model of Wld^S protection. [A] Acute injury and tauopathy activate different initiator pathways which converge on a common execution pathway of axonal degeneration. [B] In uninjured htau^{0N3R};Wld^S axons, the Wld^S-insensitive pathway is activated, therefore protection is not observed. [C] When htau^{0N3R};Wld^S axons are injured, Wld^S prevents the activation of the Wld^S-sensitive pathway, with this protection overriding the Wld^S-insensitive pathway, thereby providing bystander protection against tau-mediated axonal degeneration.

An alternative explanation for the bystander protection following injury is that injury could have effectively silenced the htau^{0N3R};Wld^S axons, thereby preventing tau-mediated axonal degeneration whilst Wld^S delayed injury-induced degeneration. Whilst axonal transport has been shown to continue after injury to Wld^S axons (Avery et al., 2012), how long this effect continues has not been reported. Furthermore, the loss of the cell body will reduce the pressure placed upon the axonal transport system; therefore this change in demand for efficient axonal transport may reduce the disruption mediated by tau and be observed as a reduction in axonal swellings. Additionally, whilst injured Wld^S axons are capable of conducting evoked potentials, this effect decays over time (Moldovan et al., 2009). Also, in the absence of experimental stimulation and without their cell bodies, the axons are electrically silent, which may affect the ability of tau to cause neurodegeneration. Neuronal activity has been found to enhance the degeneration of transected axons (Mishra et al., 2013, Brown et al., 2015) and of *Drosophila* photoreceptors lacking dNmnat (Zhai et al., 2006). Therefore the lack of neuronal activity in injured htau^{0N3R};Wld^S axons could be a key

difference to uninjured htau^{0N3R};Wld^{S} axons in driving tau-mediated axonal degeneration. Additionally, the loss of the cell body may disrupt key processes associated with tauopathy, such as the somatodendritic sorting of tau.

The mechanism behind the bystander protection of tau-mediated axonal degeneration in injured htau^{0N3R};Wld^{S} axons is an interesting one to explore. This could be done by investigating the changes in axonal function and the impact on tau pathology in Wld^{S} protected injured axons e.g. electrically silencing htau^{0N3R} expressing axons and observing whether this can prevent the development of axonal swellings. It would also be interesting to see whether NMN levels rise in tauopathy and if this triggers signalling through the Sarm/MAPK pathway.

Disruption of the Sarm pathway has currently been described to be protective against acute insults, such as mitochondrial poisons (Summers and Diantonio, 2014, Yang et al., 2015), chemotherapy (Yang et al., 2015), energy deprivation (Kim et al., 2007) and excitotoxcitiy (Massoll et al., 2013). Whether loss of Sarm can protect axonal degeneration in neurodegenerative disease remains to be seen, and would be an important avenue of investigation. However, evidence indicates that Wld^{S} prevents the activation of mediators downstream of Sarm, suggesting that expression of Wld^{S} and loss of Sarm protect via the same pathway. If this is the case then considering the lack of evidence for Wld^{S} protection in certain models of disease, it may be unlikely that this pathway plays a ubiquitous role in axonal degeneration.

In an additional avenue of investigation, this thesis also provided insight into tau-mediated dysfunction, indicating that compensating for tau loss of function was not sufficient to rescue alterations to mitochondria. Restoring microtubule stabilisation using NAP did not rescue mitochondrial mislocalisation in htau^{0N3R} expressing larvae (Chapter 5). Mitochondrial mislocalisation has been observed in a number of models of tauopathy as well as in human cases of AD (Chee et al., 2005, Kopeikina et al., 2011, Schulz et al., 2012, Amadoro et al., 2014) and is one of a number of ways in which mitochondria are altered in tauopathy. Defects in oxidative phosphorylation (Schulz et al., 2012), increased ROS production (David et al., 2005, Quintanilla et al., 2009, Amadoro et al., 2014) lowered membrane potential (Chee et al., 2005, Quintanilla et al., 2009) and disrupted mitochondrial dynamics (Quintanilla et al., 2009, Duboff et al., 2012, Amadoro et al., 2014) are all features in models of tauopathy and are thought to contribute to neurodegeneration. This work indicates that mitochondrial

mislocalisation is not simply due to the loss of microtubule stabilisation by hyperphosphorylated tau. It would be of interest to investigate whether reducing tau phosphorylation, through the use of a GSK3β inhibitor, would restore mitochondrial localisation within synapses and improve mitochondrial function.

Understanding the mechanisms involved with degeneration in disease is important in the search for therapies. This thesis indicates that important differences exist between axonal degeneration after acute injury and in certain neurodegenerative diseases, and that investigation of the mechanisms controlling chronic degeneration requires further investigation. Furthermore, a better understanding of the relationship between tau-mediated dysfunction and degeneration is required to understand whether one leads to the other. Does dysfunction of a number of processes raise the stress level of a neuron until a threshold is reached, triggering degeneration? Or are there key processes which become dysfunctional, initiating a cascade of events resulting in degeneration? Whilst prevention of axonal loss remains an important avenue of investigation, preserving function within protected axons will be crucial to the success of this approach.

Appendices

Appendix A: Recipes, buffers and additional protocols

Standard Bloomington fly media (1L)

dH_2O (L)	1
Agar (g)	6
Yeast (g)	17.5
Soya flour (g)	10
Yellow maize meal (g)	73.1
Light malt extract (g)	46.2
Dry weight sucrose (g)	48
Additional dH_2O (ml)	80
Propionic acid (ml)	5

1. Weigh out agar, yeast, soya flour and yellow maize meal and add 1/5 total water and mix into smooth paste
2. Boil remaining water and add malt and sucrose, stir until dissolved.
3. Add malt/sucrose solution to agar/yeast/soya flour/maize paste, mix and boil, stirring constantly. Boil for a few minutes.
4. Add propionic acid and stir through.
5. Pour into tubes and leave covered to cool (2hrs-overnight) before adding bungs and storing in fridge.

Homogenisation buffer

- 150nM NaCl
- 50mM MES
- 1% Triton-X
- 1% Protease inhibitor
- 1% SDS

Drosophila saline

- 2mM KCL
- 120mM NaCL
- 4mM $MgCl_2(6H_2O)$
- 1.8mM $CaCL_2$ $(2H_2O)$
- 359.7mM sucrose
- 5mM HEPES

Larval video recording equipment & set up

- +R Mediastar DVD discs
- Ikegami digital video camera with a 5mm digital video camera lens
- DVD recorder (Panasonic Diga HDMI DMR-EZ27)
- Monitor (JVC 66W 15" TM1500PS)
- Lamps x2

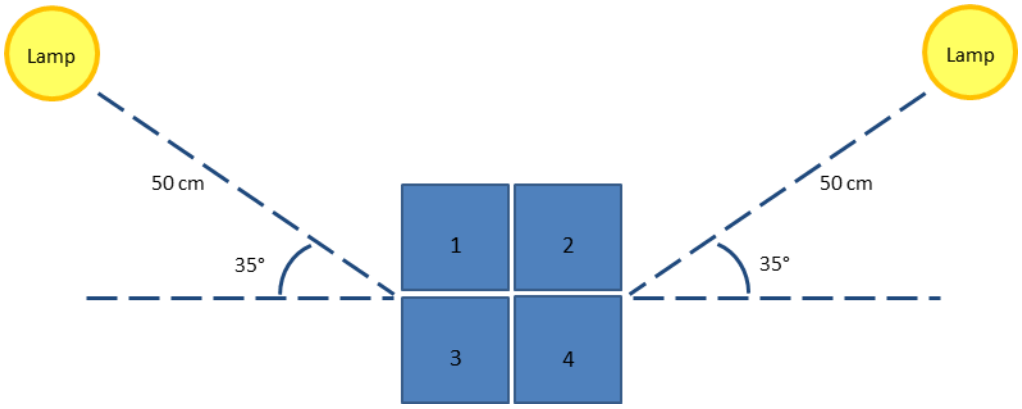


Figure 7-1. Recording equipment set up for recording of larval videos. Video camera mounted 47cm above the plates

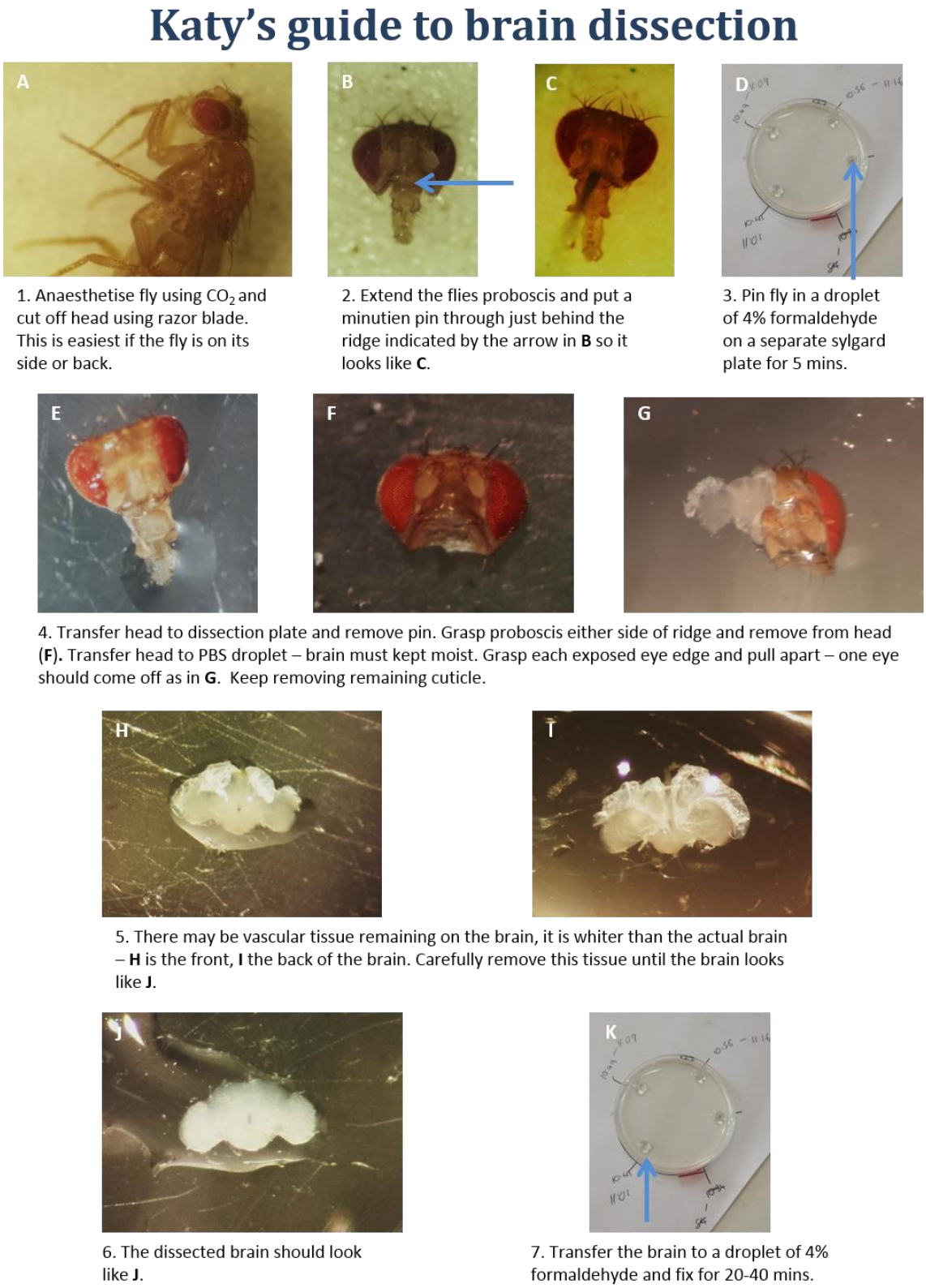


Figure 7-2. Dissection of adult *Drosophila* brains

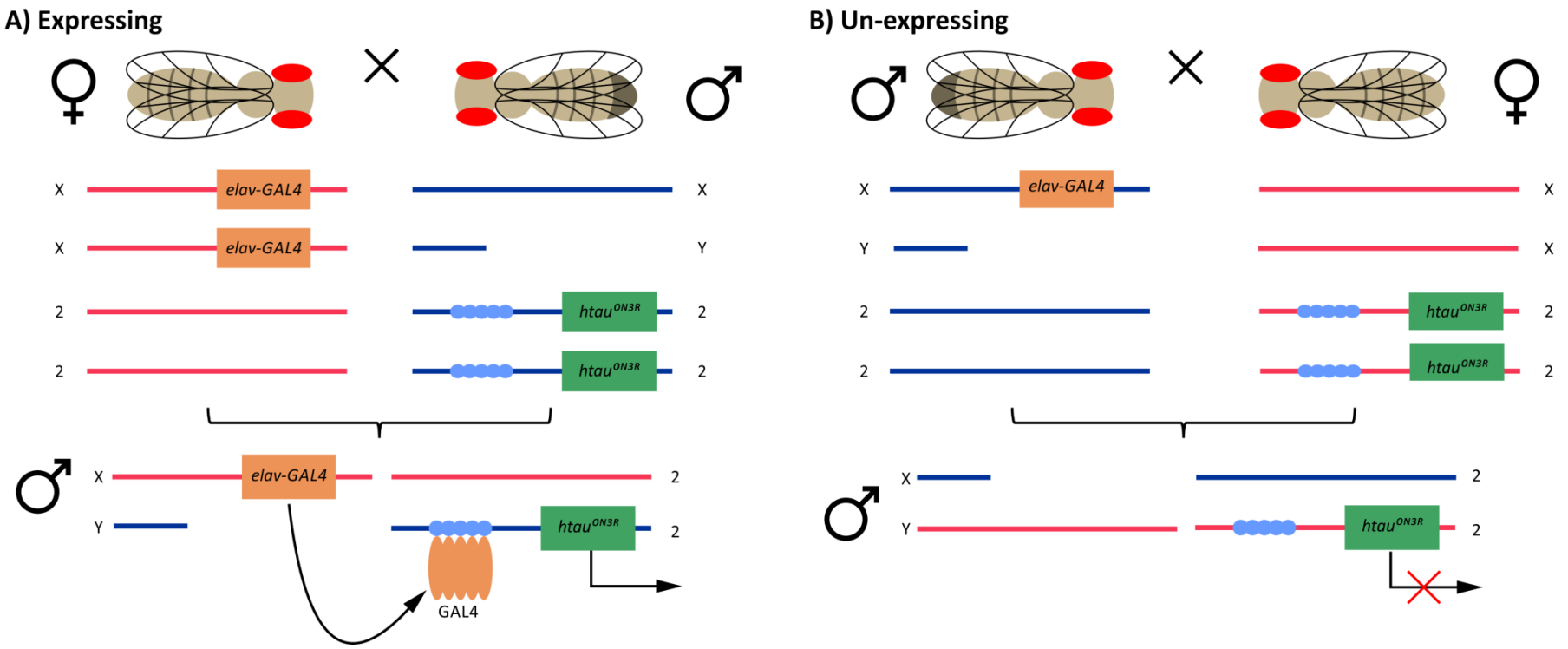


Figure 7-3. Cross scheme for longevity experiments. [A] When a female *elav-GAL4* is crossed with a male *UAS-htau*^{0N3R}, the resulting progeny express htau^{0N3R}. [B] Due to the presence of *elav-GAL4* on the X chromosome, if a male *elav-GAL4* is crossed with a female *UAS-htau*^{0N3R}, resulting male progeny get their X chromosome from the female and so do not carry have the *elav-GAL4* allele.

The resultant male progeny from [B] have very similar genetic background to male progeny from [A], but do not express the transgene, making them the closest control for genetic background in the absence of back-crossing.

Appendix B: Supplementary Data

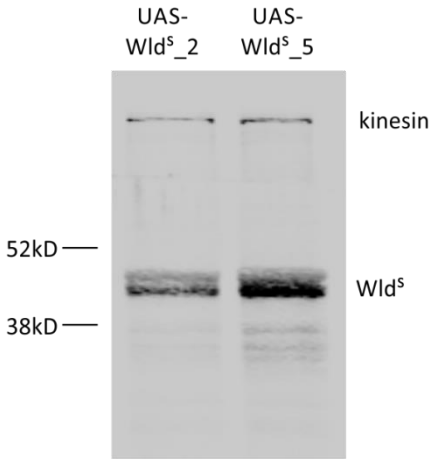


Figure 7-4. Protein expression of the two Wld^S Drosophila lines. Whole fly heads were homogenised and run on SDS-PAGE before blotting with antibodies against Wld^S. Wld^S protein was detected between the 52kD and 38kD markers, with a predicted mass of 43kD. The higher expressing of the two lines was selected to make the htau^{0N3R};Wld^S line and for use in experiments.

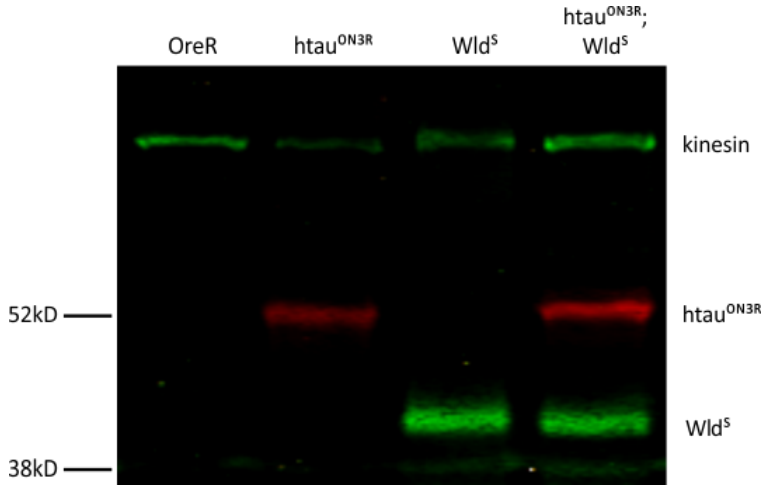


Figure 7-5 Representative blot showing expression of htau^{0N3R} and Wld^S in co-expressing line. Whole fly heads were homogenised and run on SDS-PAGE before blotting with antibodies against human tau and Wld^S.

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