Analysis of SMN function in development and Nedd4, a putative modifier of Parkinson's disease, in *Drosophila melanogaster*

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A thesis submitted for the degree of

DPhil in Physiology, Anatomy and Genetics

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Abstract

Neurological diseases are devastating illnesses that affect over one billion people worldwide. *Drosophila melanogaster* provides a genetically tractable system in which to study gene function and the mechanisms of pathogenesis of neurological diseases. In this study I have investigated the function of survival motor neuron (SMN), the causative gene in the neuromuscular disease spinal muscular atrophy (SMA), in growth and differentiation in *Drosophila*. In addition, I have used the fruit fly to investigate a putative modifer of a previously characterised *Drosophila* model of Parkinson's disease.

Spinal muscular atrophy is an autosomal recessive neurological disease that is characterised by motor neuron loss resulting in muscle weakness. The disease is caused by the deletion or mutation of the survival motor neuron (SMN) gene. In *Drosophila*, SMN was found to be highly expressed in dividing tissues and a reduction in SMN levels resulted in growth defects, stem cell defects and developmental delay. SMN was also shown to regulate chromosome morphology of the endocycling nurse cells of the female germline. Therefore it appears that SMN has a role in growth control and development in *Drosophila*.

Parkinson's disease is a common disorder that results in widespread neurodegeneration with a predilection for dopaminergic neuron loss resulting in movement defects. A defining neuropathological feature of the disease is the presence of α-synuclein containing inclusions. Using a *Drosophila* model of PD, I have shown that specific α-synuclein-induced phenotypes in the fly can be suppressed by the overexpression of the E3 ubiquitin ligase, Nedd4.

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Trinity Term 2012

"Time flies like an arrow; fruit flies like a banana."

Anonymous

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Contents

List of Figures

List of Abbreviations

AED	after egg deposition
ALS	amyotrophic lateral sclerosis
BMP	Bone Morphogenic Protein
CB	central brain
CMA	chaperone-mediated autophagy
CNS	central nervous system
DA	dopamine/dopaminergic
DAE	days after eclosion
ESCRT	endosomal sorting complex required for transport
FGF	Fibroblast Growth Factor
FLP	flipase recombination target site-specific
FRT	flipase recombination target
GFP	green fluorescent protein
GMC	ganglion mother cell
HLB	histone locus body
ILV	intraluminal vesicle
L-DOPA	L-3,4-dihydroxyphenylalanine
mRNA	messenger RNA
MVE	multivesicular endosome
NAC	non-β-amyloid component
NMJ	neuromuscular junction
OL	optic lobe
OPC	outer proliferation centre
P body	processing body
PD	Parkinson's disease
pNB	postembryonic neuroblast
PPN	preproneural
PRMT5	protein arginine methyltransferase 5
RFP	red fluorescent protein
RNAi	RNA interference
RNP	ribonucleoprotein
SMA	spinal muscular atrophy
SMN	survival motor neuron
SN	substantia nigra
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
TH	tyrosine hydroxylase
U body	cytoplasmic U snRNP body
U snRNP	uridine-rich small nuclear ribonucleoprotein
UAS	upstream activating sequence
UNRIP	UNR-interacting protein
UPS	ubiquitin proteasome system
VNC	ventral nerve cord
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Neurological disease

Neurological diseases are disorders of the central and peripheral nervous systems and include common conditions such as Parkinson's disease and motor neuron diseases. The World Health Organisation (WHO) reported that in 2005, neurological disorders attributed for 6.3% of the global burden of disease, compared with a fraction over 5% for both cancers and HIV/AIDS. In developed countries, this proportion rises, with neurological disorders constituting 11.2% of the burden of disease in Europe (1).

As improvements in healthcare have brought about unprecedented increases in quality of life and higher life expectancies, the increasing lifespan of the population has led to a higher incidence of neurological disease in our aging populations. The socio-economic impact of neurological disease is set to rise unless the pathogenesis of neurological disease becomes more fully understood and appropriate therapies developed.

1.2 *Drosophila* in the study of neurological disease

The *Drosophila* model organism has become established as a major player in the study of the pathogenic mechanisms that lead to disease. The advantages of working with *Drosophila* are immediately apparent; their generation time is short, a large number of progeny can be obtained from a single cross and they are inexpensive to maintain.

Fundamental biological processes, such as the cell cycle (2) and cell growth (3) (4) are conserved between *Drosophila* and humans despite their evolutionary distance of approximately 700 million years (5). A small number of complex signalling pathways dictating development are re-used throughout the development of the individual species and are conserved between them (6). Importantly, developmental regulatory genes involved in patterning the basic body plan, including brain and neuromuscular system development are conserved between flies and mammals (7).

Approximately 75% of disease loci identified in humans have orthologues in the fly (8). Therefore, once the causative disease gene has been identified in humans, *Drosophila* models can be employed to investigate disease pathogenesis or disease gene function.

However, arguably the major benefit of using *Drosophila* lies in the organism's amenability to genetic manipulation and the repertoire of genetic techniques available.

In this thesis, these techniques have been employed to study the function of SMN, the causative gene in the neurological disease spinal muscular atrophy, and to investigate whether the ubiquitin ligase Nedd4 is able to modify a *Drosophila* model of Parkinson's disease.

1.3 Genetic techniques

1.3.1 Targeted gene expression using the GAL4/UAS system

The *S.cerevisiae GAL4* gene encodes a transcription regulator that controls gene expression by binding to Upstream Activating Sequences (UAS). In *Drosophila*, GAL4 can similarly induce the expression of genes containing a UAS site within their promoters (9) (Figure 1.1). The *GAL4* gene is inserted into the genome in the vicinity of an endogenous genomic enhancer. *GAL4* expression is consequently dictated by the endogenous enhancer, resulting in cell- or tissue-specific expression characteristic of that enhancer. In turn, transcription of the target gene, being placed downstream of a UAS site, is confined to the expression pattern of the *GAL4* gene. The GAL4 driver and the target gene are maintained as separate parental lines allowing spatial and temporal regulation through the crossing of the target transgene to distinct GAL4 drivers. The GAL4/UAS system can be used to overexpress or knockdown target genes ubiquitously or in any tissue for which a tissue-specific GAL4 driver exists.

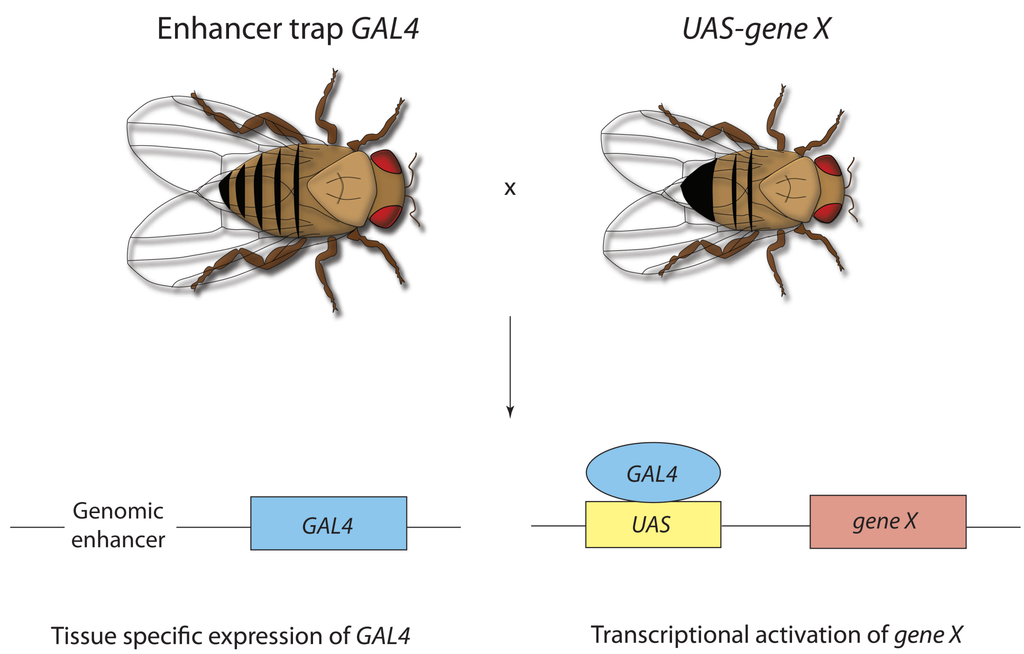

Figure 1.1: Targeted gene expression using the GAL4/UAS system - Targeted gene expression can be achieved by crossing flies carrying a specific enhancer trap *GAL4* to those carrying the gene of interest (gene X), whose promotor contains a UAS site. The resulting progeny have tissue-specific *GAL4* expression which in turn induces tissue-specific target gene expression through GAL4 binding to UAS. Adapted from (9)

GAL4 expression is temperature dependent, with lower temperatures resulting in decreased expression. Therefore expression levels of the *UAS-target* transgene can be modified by altering the temperature at which the cross is maintained (10) (11).

This system can be effectively utilised to model dominant human diseases by driving the ectopic expression of wild-type or mutant human disease genes. Partial loss-of-function of endogenous genes can also be analysed using the GAL4/UAS technique by GAL4 induction of transgenic RNAi lines (10).

This genetic technique is commonly used to efficiently screen for modifiers of neurological and growth phenotypes. The *GMR*-GAL4 driver can be used to induce eye-specific expression of a transgene. In the cases of transgenes whose reduction or over-expression results in neuronal degeneration or misregulated growth, a 'rough eye' phenotype may be observed resulting from disruptions in the regular ommatidial array due to missing or additional cells (10) (12). Changes in levels of putative interactors in the adult eye resulting in suppression or enhancement of the rough eye phenotype can be used to readily identify modifiers (12). The *Drosophila* eye is not required for surivival, therefore is an excellent system in which to manipulate gene expression, bypassing developmental aberrations that may occur if expressed pan-neuronally (13) (12). However, it should be noted that this approach has its limitations; GAL4 itself can cause toxicity, protein function in the eye may differ from other tissues, therefore the observation may be eye-specific, and the rough eye may be the result of the degeneration of non-neuronal cells, such as accessory cells, which should be taken into account when investigating neurodegenerative diseases (12).

1.3.2 Generating mosaics in *Drosophila* using mitotic recombination

An elegant method of analysing loss-of-function in *Drosophila* is the use of site-directed recombination based techniques (14). A desired mutation can be made homozygous in cells of an otherwise heterozygous animal through targeted recombination at specific sites. This technique relies on the yeast flipase recombination target (FLP) site-specific recombinase enzyme which induces recombination at specific flipase recombination target (FRT) sites between homologous chromosomes. The FLP-recombinase gene is under the control of the hsp70 promoter, therefore can be induced by heat-shock, and the placing of an FRT site in relation to a distally located specific mutation allows the generation of homozygous mutant cells in a heterozygous, essentially wild-type background.

Mitotic clonal analysis employs the GFP marker to track wild-type cells. Following mitosis, GFP-positive, homozygous wild-type daughters and GFP-negative, homozygous mutant daughters are produced. Therefore the loss-of-function of a particular gene can be assayed by comparing mutant clones to their wild-type twins (Figure 1.2).

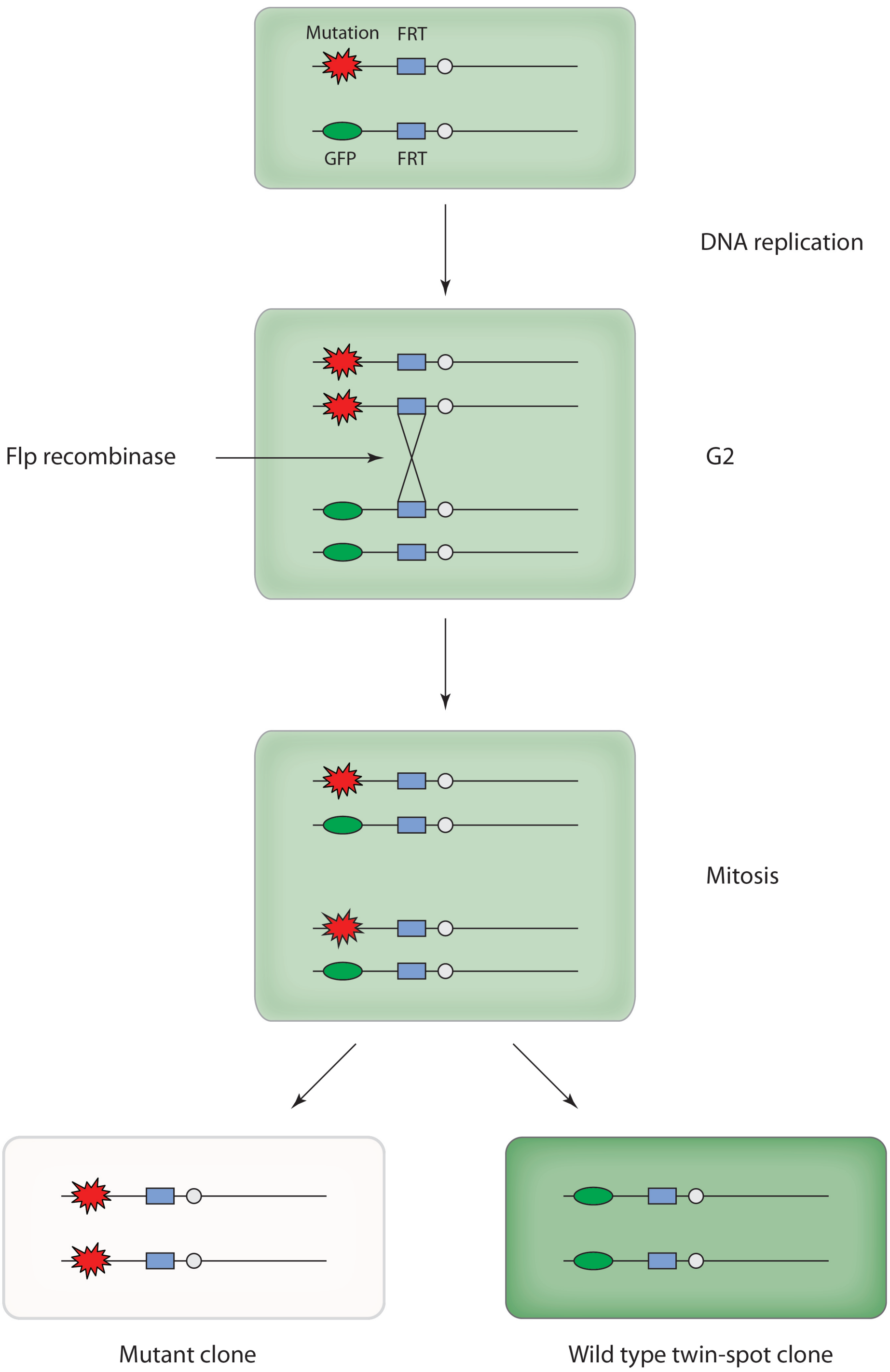

Figure 1.2: Mitotic clonal analysis - Cells that are homozygous for a mutation of interest can be produced in flies in which a GFP marker and the desired mutation are positioned distally to corresponding FRT sites. Following DNA replication, expression of FLP-recombinase induces recombination at the FRT sites resulting in site-specific chromosomal exchange. The consequent segregation of daughter chromosomes during mitosis results in cells being either homozygous for the mutation of interest or for GFP. Figure adapted from (15).

This technique is an excellent option for analysing loss-of-function of mutations that cause lethality, since the animal remains largely wild-type, therefore developmental defects and lethality are averted.

1.3.3 Germline clonal analysis in *Drosophila*

Germline clonal analysis is a modification of the mitotic clonal technique that is unique to the female germline (14). This technique, rather than using GFP for selection of the mutation, utilises the dominant female sterile mutation (DFS) ovo^{D1}. Inheritance of ovo^{D1} results in degeneration of the egg chamber, therefore only egg chambers that are homozygous for the mutation of interest, therefore having removed the ovo^{D1} mutation, survive (Figure 1.3).

Simiarly to mitotic clonal analysis, the generation of germline clones is particularly useful for the analysis of loss-of-function of genes that are mutant homozygous lethal.

1.3.4 Tripartite approach to targeted gene expression: combining mosaic generation with the GAL4/UAS system

The GAL4/UAS system can be combined with site-directed recombination to generate clones in a targeted fashion. In this instance, FRT sites are placed flanking a cassette housing the *GAL80* gene. GAL80 inhibits GAL4-induced transcription of genes therefore, despite the presence of GAL4, transcription of genes under the control of a UAS promoter does not occur. To generate cells lacking GAL80, recombination *in cis* is

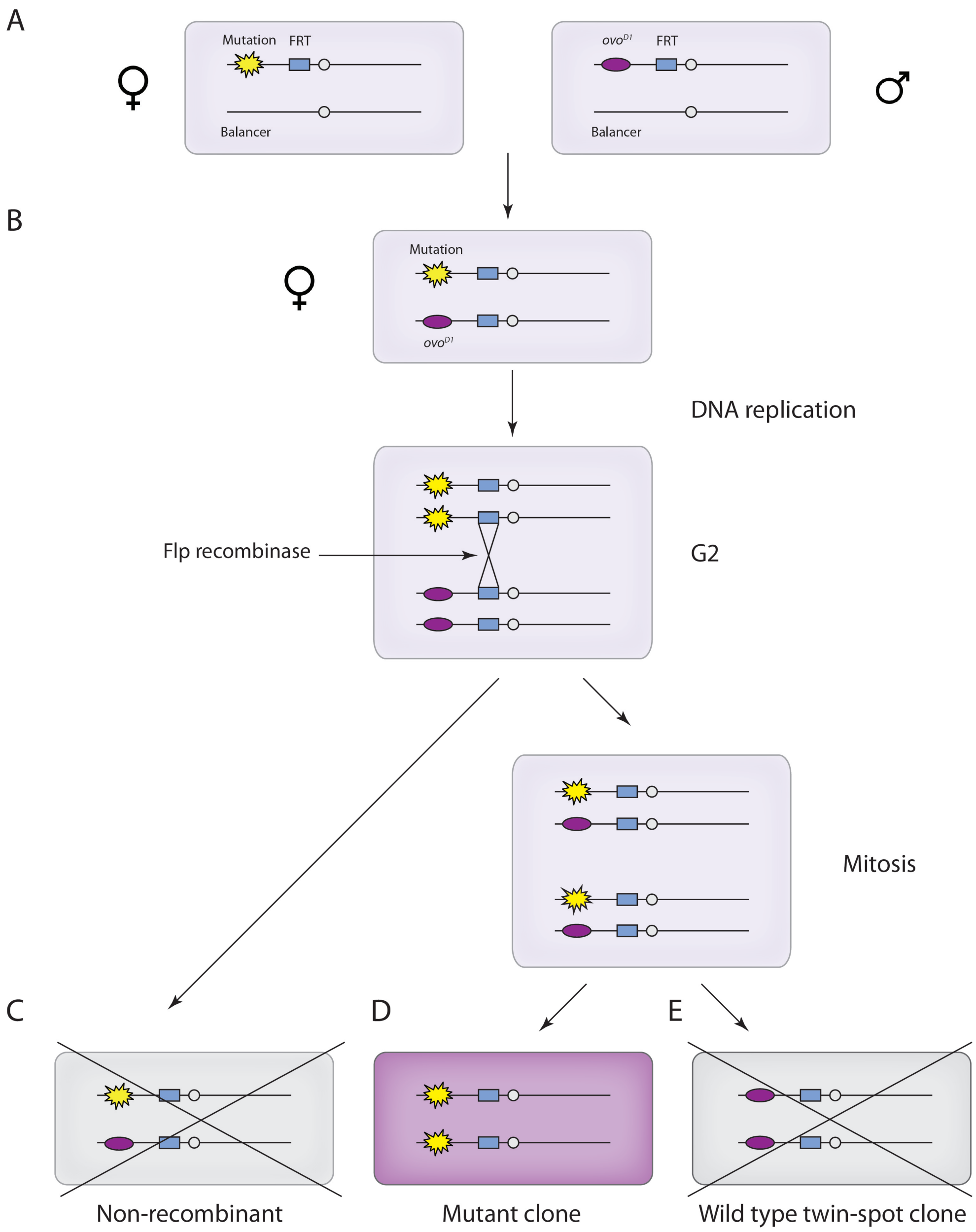

Figure 1.3: Germline clonal analysis - A. Females carrying a desired mutation flanked by an FRT site are crossed to males carrying the DFS mutation, ovo^{D1}. B. Females carrying both mutations are selected and, following DNA replication, FLP-recombinase expression induces site-specific recombination at the FRT sites. C. Non-recombinant daughter cells inherit ovo^{D1} causing degeneration of the egg chambers. D. Germline clones inherit the mutation of interest only, allowing egg chamber survival and analysis of the homozygous mutant germline cells. E. Daughters homozygous for ovo^{D1} degenerate, therefore only mutant clones survive. Figure adapted from (15).

induced by the heat-inducible FLP-recombinase. The GAL80 gene is then 'flipped-out' by FRT-mediated excision. GAL80 inhibition of GAL4 transgene transcription is therefore relieved in the 'flip-out clones' in which GAL80 has been removed. This technique allows targeted production of clones by selecting an appropriate GAL4 driver (Figure 1.4) (11).

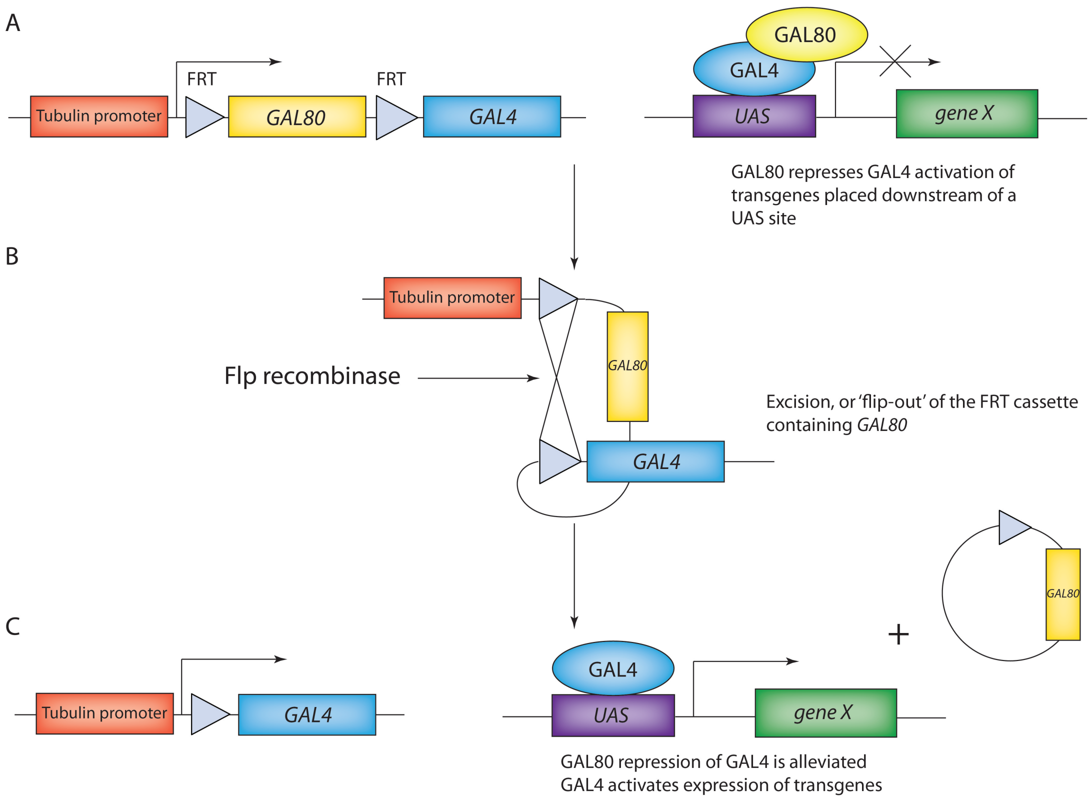

Figure 1.4: Generation of 'flip-out' clones - A. Expression of the *GAL80* gene, housed in an FRT cassette, represses GAL4 induced transcription of genes placed downstream of a UAS site. B. The induction of FLP-recombinase expression causes site-specific recombination at the FRT sites flanking the *GAL80* gene thereby excising *GAL80*. C. Following the 'flip-out' of the FRT cassette containing *GAL80*, *GAL4* alone is expressed rendering it free to activate transcription of transgenes. Figure adapted from (16)

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1.4 Aim of thesis

In this thesis I have used the *Drosophila* model system and the genetic techniques described above to study the function of the causative gene of the neurological disease, spinal muscular atrophy (SMA). SMA is an autosomal recessive disease that results from mutations or deletions in the survival motor neuron (SMN) gene. The disease is characterised by a progressive, selective loss of motor neurons from the anterior horns of the spinal cord resulting in muscle weakness. There is some contention in the SMA field as to whether SMA is a neurodegenerative disease or is the result of aberrant neurodevelopment. In this study I have investigated the function of SMN in development, with particular focus on growth and differentiation of the developing *Drosophila* larva.

Secondly, I have used a previously characterised *Drosophila* model of Parkinson's disease (PD) to investigate a putative modifier, Nedd4. PD results in widespread neurodegeneration with a predilection for degeneration of the dopaminergic neurons of the substantia nigra pars compacta. The defining clinical feature of the disease is the presence of α-synuclein-containing neuronal inclusions termed Lewy bodies and α-synuclein clearance pathways have been recognised as potential avenues for therapy. Recently the E3 ubiquitin ligase, Nedd4 has been implicated in α-synuclein clearance in yeast and human cell lines. In this study I have investigated whether Nedd4 is able to affect α-synuclein-dependent phenotypes in an animal model.

Chapter 2

Materials and Methods

2.1 *Drosophila* stocks and husbandry

Stocks were maintained at 25°C on standard medium (80g/l maize, 18g/l dried yeast, 10g/l soya flour, 80g/l malt extract, 40g/l molasses, 8g/l agar, 6.6ml acid mix). *y, w*, obtained from the Bloomington Stock Centre, was used as the control strain unless otherwise stated. The *smn* alleles, smn^{73A0} and smn^{B}, used in this thesis were previously described by Chan *et al.* 2003 (17). The *P[UAS-FLAG-Smn]*, $[UAS\text{-}Smn\text{-}RNAi]^{C24}$ and $[UAS\text{-}Smn\text{-}RNAi]^{FL26B}$ lines were described in Chang *et al.* 2008 (18). *UAS-dSMN-YFP*, described in Liu *et al.* 2006 (19), was a gift from Joe Gall. The *tub-smn* line used in rescue studies was generated by Sheny Chen.

The $UAS\text{-}\alpha\text{-}synuclein^{WT}$ and $UAS\text{-}\alpha\text{-}synclein^{1-120}$ lines were gifts from Mel Feany (20). The Nedd4 *Drosophila* lines, *UAS-dNedd4* and $UAS\text{-}dNedd4^{C-A}$ have been previously described by Myat *et al.* (21) *UAS-dNedd4-RNAi* was obtained from the Vienna

Drosophila RNAi Center. The *TH*-GAL4 stock was a gift from Stephen Goodwin, *ey*-GAL4, *Elav-C155*-GAL4 and *GMR*-GAL4 were obtained from Bloomington Stock Centre and *HsFlp, UAS-GFP*$_{nls}$*; UAS-Dcr2; tub>GAL80>GAL4/SM5, Cyo-TM6, Tb* was a gift from Joe Gall.

2.2 Immunofluorescence and microscopy

Larval or adult CNS, eye imaginal discs, ovaries, wing discs, larval testes or adult testes were dissected in Grace's Insect Medium, fixed for 10 minutes in 4% paraformaldehyde in PBS and washed in PBT (27ml PBS, 1.5ml 5% Triton X-100, 1.5ml 10% normal horse serum). Samples were incubated sequentially in primary and secondary antibodies overnight. Primary antibodies used were rabbit anti-SMN (J.Zhou, 1:100), rabbit anti-Miranda (C.Doe, 1:1000), mouse anti-Prospero (Developmental Studies Hybridoma Bank, 1:200), rabbit anti-Gemin 5 (C.Thummel, 1:1000), rabbit anti-Gemin 3 (JL. Liu, 1:1000), mouse anti-Hts (Developmental Studies Hybridoma Bank, 1:20), rabbit anti-Lsm11 (J.Gall, 1:1000), mouse anti-Repo (Developmental Studies Hybridoma Bank, 1:100), rat anti-Elav (Developmental Studies Hybridoma Bank, 1:10), rabbit anti-H3K4me3 and rabbit anti-H3K9me2, both from Diagenode, mouse anti-α-synuclein (BD Transduction, 1:500), rabbit anti-Nedd4 (Abcam, 1:500), mouse anti-TH (Chemicon, 1:500). Alexa Fluor® conjugated (Invitrogen) or Cy5 conjugated (Jackson Immunoresearch) secondary antibodies were used at a 1:1000 concentration. Hoechst 33342 (1:250) was used to label DNA. Samples were imaged using a Zeiss LSM 510 META confocal microscope and processed with Adobe® Illustrator® CS5.

2.3 CNS and cell size measurements and post-embryonic neuroblast counting

Flies were placed in vials and an overnight pre-lay was taken. 2hr lays were collected to obtain an appropriate population density. Larvae were grown in vials and age-matched larvae dissected. Cell size measurements were taken from confocal images by manually tracing around the circumference of the cells using the measuring tool in the LSM Image Browser software. pNBs were counted manually followed the processing of Z-stacks into projections. pNBs were identified by their position and relative size within the CNS as well as the presence of the pNB marker Miranda.

2.4 Generating *smn* mitotic clones

smn mitotic clones were generated using Flp-mediated mitotic recombination as described by Chou and Perrimon, 1996 (14). *w HsFlp; ubi-GFP* FRT2A or *w HsFlp; Sp/Cyo;* smn^{73A0} FRT2A/*TM6b* virgin females were crossed to *w;* smn^{73A0} FRT2A/*TM3, Ser*, *w;* smn^{73A0} FRT2A/*TM6b* or *w; ubi-GFP*FRT2A/*TM3, Ser* males for mitotic clonal analysis. Control mitotic clones and rescue clones were generating by crossing *w*

HsFlp; ubi-GFP FRT2A and *w HsFlp; tub-smn; smn*^{73A0} FRT2A/*TM6b* virgin females to *w*; FRT2A and *w; ubi-GFP* FRT2A males respectively.

The resulting progeny were heat shocked for 1hr at 37°C, 48hr after egg deposition for eye disc clones and for 1hr at 37°C on 3 consecutive days, 3, 4 and 5 days after egg deposition for ovary mitotic clones. Non-*TM6b* and non-*Ser* progeny were selected for dissection. Flies selected for ovary dissection were fed on wet yeast every day prior to dissection. The GFP marker was used to identify heterozygous cells and homozygous wild-type cells. The absence of GFP indicated that the clones were homozygous for the mutation.

2.5 Generating *smn* germline clones

Similarly to mitotic clones, *smn* germline clones were generated using mitotic recombination, in the case of germline clones using the FLP-DFS (yeast flippase recombination target-site specfic recombinase-dominant female sterile) system, previously described by Chou and Perrimon, 1996 (14). *w HsFlp; +/Cyo; smn*^{73A0} FRT2A/*TM6b*, *w; smn*^{73A0} FRT2A/*TM3, Ser* or *w HsFlp; Sp/Cyo; smn*^{73A0} FRT2A/*TM6b* virgin females were crossed to *w; ovo*^{D1} FRT2A/*TM3, Ser* or *w HsFlp; ovo*^{D1} FRT2A/*TM3, Ser* males to generate germline clones. *w*; FRT2A virgin females were crossed to *w HsFlp; ovo*^{D1} FRT2A/*TM3, Ser* males for control clones. Rescue clones were generated by crossing *w*

HsFlp; tub-smn/Cyo; smn^{73A0} FRT2A/*TM6b* virgin females to *w; ovo*^{D1} FRT2A/*TM3, Ser* males. The progeny resulting from these crosses were heat-shocked at 37°C for 1hr on 3 consecutive days, 3, 4 and 5 days after egg deposition. Non-*TM6b* and non-*Ser* female progeny were selected for dissection. Flies selected for ovary dissection were fed on wet yeast every day prior to dissection.

2.6 Generating flip-out clones

Flip-out clones were generated using the inducible GAL4 driver, *HsFlp, UAS-GFP*$_{nls}$*; UAS-Dcr2; tub>GAL80>GAL4/SM5, Cyo-TM6, Tb*. The >GAL80> cassette is recombined out following heat-shock, thereby inducing *tub*-GAL4 driven expression of genes under the control of the *UAS* promoter. *HsFlp, UAS-GFP*$_{nls}$*; UAS-Dcr2; tub>GAL80>GAL4/SM5, Cyo-TM6, Tb* virgin females were crossed to the following lines: [*UAS-dSMN-YFP*], [*UAS-FLAG-Smn*], [*UAS-Smn-RNAi*]^{C24}, [*UAS-Smn-RNAi*]^{FL26B}, *UAS-*α*-synclein*^{WT}, *UAS-*α*-synclein*$^{1-120}$, *UAS-dNedd4*, *UAS-dNedd4*$^{C-A}$ and *UAS-dNedd4-RNAi*. The resulting progeny were heat-shocked for 1hr at 37°C at 24hr and 48hr after egg deposition. Larvae were aged in vials and dissected at the 3rd instar larval stage. Flip-out clones were identified using the GFP nuclear localisation signal.

2.7 Scanning electron microscopy of the adult eye

GMR-GAL4 or *eyeless*-GAL4 virgin females were crossed to the following lines: [*UAS-dSMN-YFP*], [*UAS-FLAG-Smn*], $[UAS\text{-}Smn\text{-}RNAi]^{C24}$, $[UAS\text{-}Smn\text{-}RNAi]^{FL26B}$, *UAS-*$\alpha$*-synclein*^{WT}, *UAS-*$\alpha$*-synclein*$^{1-120}$, *UAS-dNedd4*, *UAS-dNedd4*$^{C-A}$ and *UAS-dNedd4-RNAi*, *UAS-*α*-synclein*^{WT};*UAS-dNedd4* and *UAS-*α*-synclein*^{WT};*UAS-dNedd4-RNAi.* Age-matched, male flies were fixed in 70% ethanol. The flies were then dehydrated in 100% ethanol and critically-point dried before being mounted on SEM stubs. The samples were sputter coated with gold and imaged using a JEOL JSM 6390 scanning electron microscope.

2.8 Western blotting

Heads of each genotype were collected and crushed directly using a pestle in 1 × NuPage® LDS Sample Buffer (Invitrogen) and 1 × NuPage® Sample Reducing Agent (Invitrogen) made up to a 100μl total volume with nuclease-free water (Ambion). The samples were heated to 72°C for 10 minutes and centrifuged at full speed for 1 minute. The soluble fractions were loaded into a NuPage® 4-12% gradient Bis-Tris Gel (Invitrogen) and run at 200V for 1hr in 1 × NuPage® MOPS SDS Running Buffer (Invitrogen) with added NuPage® antioxidant (Invitrogen). Following a standard transfer to nitrocellulose (30V for 1hr), blots were blocked for 2hr in 5% Marvel in TBST(10mM Tris pH

7.5, 150mM NaCl, 0.05% Tween-20). Blots were then incubated in primary antibodies in 5% Marvel in TBST at 4°C overnight. Primary antibodies used were mouse anti-α-tubulin (Developmental Studies Hybridoma Bank, 1:1000), mouse anti-α-synuclein (1:5000) and rabbit anti-Nedd4 (1:5000). Blots were washed with TBST three times for 15 minutes before the addition of the secondary antibodies. HRP-conjugated secondary antibodies were diluted in 5% Marvel in TBST and secondary incubations were carried out at room temperature for 45min. The secondary antibodies used were anti-rabbit HRP and anti-mouse HRP (Jackson ImmnuoResearch, 1:5000). Blots were then washed in TBST for 15 minutes three times. The blots were visualised using the ECL Western Blotting Detection Reagent kit (Amersham).

2.9 Survival assays

Age-matched male flies were placed in vials containing standard medium and maintained at 25°C under a 12hr light/dark cycle. Each vial contained 10 flies at the start of the experiment. The flies were transferred onto fresh media every 3 days and their survival recorded. A starting number of between 40 and 140 flies were recorded for each genotype.

2.10 Climbing assay for locomotor function

Locomotor function was assayed using a startle induced negative geotaxis assay and the climbing ability of the flies measured. The climbing assay was performed under red light. Up to 10 male flies were placed in an empty vial and tapped 3 times. The number of flies above 4cm from the bottom of the vial was recorded after 4 sec. 5 trials were performed for each vial and a mean percentage calculated. Alternatively, climbing ability was quantified by measuring the distance travelled by each individual fly and an average of all distances calculated for each vial. All lines were crossed and maintained at 25°C with a 12hr light/dark cycle.

2.11 Analysis of dopaminergic neurons

Adult central brain was dissected from males in Grace's Insect Medium, fixed for 10 minutes in 4% paraformaldehyde in PBS and washed in PBT (27ml PBS, 1.5ml 5% Triton X-100, 1.5ml 10% normal horse serum). Samples were incubated in mouse anti-tyrosine hydroxylase antibody (1:500 dilution) overnight, washed in PBS and incubated overnight in secondary antibody. Alexa Fluor® conjugated (Invitrogen) or Cy5 conjugated (Jackson Immunoresearch) secondary antibodies were used at a 1:1000 concentration. Hoechst 33342 (1:250) was used to label DNA. Dopaminergic neurons were counted manually following the processing of Z-stacks into projections. They were identified by positive staining for tyrosine hydroxylase and their position. Clusters were defined as described by White et al 2010 (22). Samples were imaged using a Zeiss LSM 510 META confocal microscope and processed with Adobe® Illustrator® CS5.

Chapter 3

SMN regulates growth and differentiation in *Drosophila*

In this chapter I will discuss the function of the survival motor neuron (SMN) gene in *Drosophila*. In humans, deletions or mutations in SMN result in the neuromuscular disease, spinal muscular atrophy (SMA). The effect of reduced SMN levels on neuromuscular junction function and muscle development has been extensively studied, however the observation of extra-neuromuscular abnormalities in SMA patients has implicated SMN as a requirement during the development of a number of tissues, as well as neuromuscular development. SMN has also been implicated in growth and differentiation in *Drosophila* and mouse models. In this study I have investigated the effect of modification of SMN levels on the development of a number of tissues with a focus on the developing larval central nervous system, the eye imaginal disc and the female germline.

3.1 Introduction

3.1.1 Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive neurological disease that is characterised by a progressive, selective loss of motor neurons from the anterior horns of the spinal cord resulting in muscle weakness (23). SMA is a prominent disorder with a carrier frequency of approximately 1:50, affecting 1 in 10,000 live births (24) for which there is no known cure. Despite cystic fibrosis being a more prevalent genetic disease, therapeutic advances for this disease has resulted in SMA being the most common genetic cause of infant mortality (25). The elucidation of the molecular basis of SMA pathology is an important step towards the development of new, effective therapies.

The underlying genetic cause of SMA is well characterised, being a monogenic disease resulting from the deletion or mutation of the survival of motor neuron (SMN) gene (23). The SMN gene in humans lies within a large inverted duplication at the chromosome locus 5q13 (23). As a consequence of this duplication event, the human genome contains two SMN genes, the ancestral, telomeric *SMN1* gene and a centromeric *SMN2* gene. Deletions or mutations of SMN1 only result in SMA.

SMN1 and *SMN2* are almost identical; they express their transcripts ubiquitously and have equivalent promoters, their genomic DNA sequences differing by only 5 nucleotides (26). However, a C to T substitution in an exonic splicing region of *SMN2* results in the skipping of exon 7 in approximately 80% of transcripts. These transcripts lacking exon 7 produce a trucated SMN protein, SMNΔ7, that is unable to oligomerise resulting in unstable protein that is rapidly degraded (23) (Figure 3.1).

The ability of *SMN2* to produce approximately 20% full length protein however, results in a low basal level of functional SMN protein even in the absence of *SMN1*. This accounts for the clinical heterogeneity observed in SMA, with the copy number of *SMN2* being the major determinant of disease severity (24).

3.1.2 SMN regulates snRNP assembly

SMN is ubiquitously and constitutively expressed in all tissues and is essential for the assembly of small nuclear ribonucleoprotein (snRNP) complexes required for pre-mRNA splicing (28), (29). SMA is therefore one of a number of neurological diseases, such as amyotrophic lateral sclerosis and Charcot-Marie-Tooth, that is caused by the loss of a protein associated with RNA metabolism. A particularly puzzling aspect of SMA pathology is how the substantial reduction of a 'housekeeping' protein results in a motor neuron specific disease. Although counterintuitive, this phenomenon is not unique to SMA, with many neurological diseases resulting from a subset of neurons being selectively affected, despite the causative gene having a ubiquitous function.

SMN functions within a macromolecular complex called the SMN complex to me-

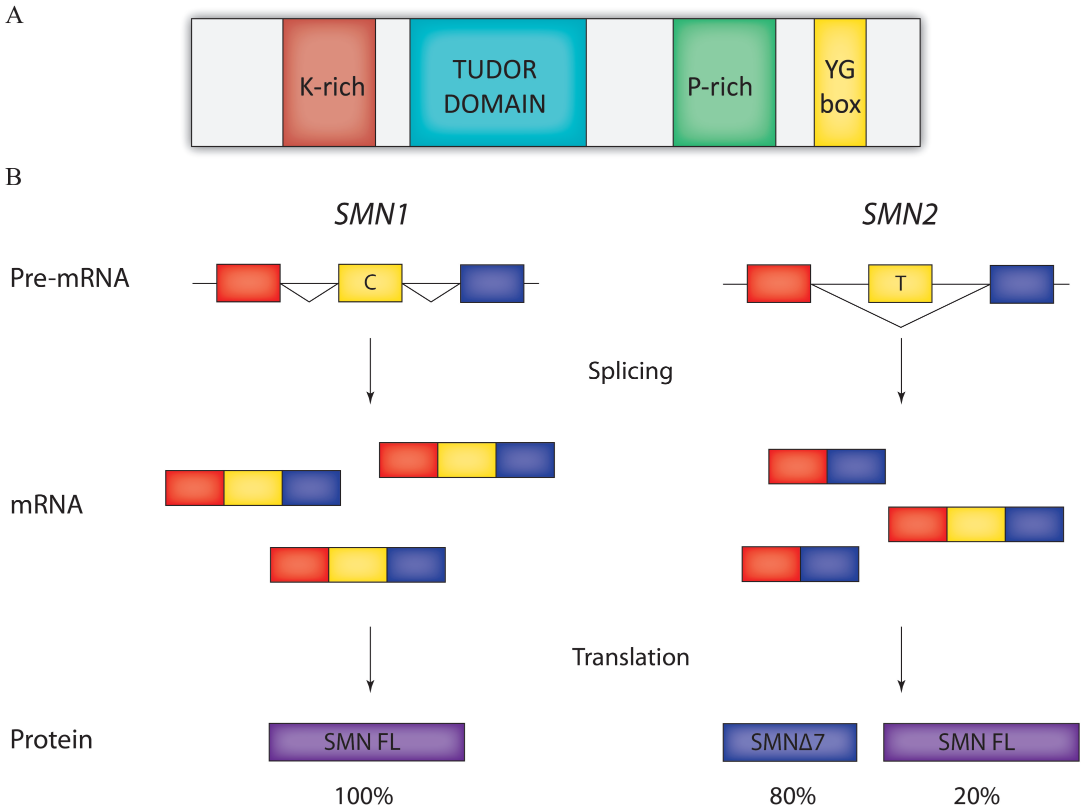

Figure 3.1: Genetics of SMA - A. SMN functional domains: The N-terminal K-rich domain functions in Gemin 2 binding and contributes to SMN self-association. The Tudor domain functions in mediating interactions with Sm proteins as does the Y/G box. This domain is also required for interaction with Gemin 3 and self-oligomerisation. Missense mutations in SMA patients tend to be located around the Y/G box. The P-rich domain has been proposed to be involved in profilin binding. B. *SMN1* and *SMN2* are almost identical however a C to T substitution in exon 7 (yellow) results in a change in splicing causing most *SMN2* transcripts to lack exon 7. SMN protein produced from transcripts lacking exon 7 have reduced ability to oligomerise and therefore are unstable and rapidly degraded. *SMN2* however does produce a proportion of full length, functional SMN protein. Figure adapted from (27).

diate the assembly of snRNPs (29) (Figure 3.2). In vertebrates, this complex consists of SMN, Gemin2-8 and the UNR-interacting protein (UNRIP). *Drosophila* contains only one SMN orthologue which similarly functions as an oligomer in a macromolecular complex however the SMN complex in the fly is comprised of a core SMN and Gemin2 complex. A Gemin3 *Drosophila* homologue is known to interact with SMN and a Gemin5 homologue has also been identified however the fly Gemin5 does not directly interact with SMN (30).

A number of subcellular compartments have been identified as foci of RNA metabolism, including the biogenesis of snRNPs. SMN is expressed diffusely in the cytoplasm of all cells, however SMN is also enriched in punctate subcellular bodies in both the nucleus and cytoplasm proposed to create sites of high concentrations of proteins required for snRNP assembly and processing (31) (32) (33) (34) (Figure 3.2).

3.1.3 SMA - neurodegenerative or neurodevelopmental disease?

A point of contention regarding the pathogenesis of SMA is whether the disease arises from neurodevelopmental abnormalities or neurodegeneration. SMA patients and mouse models display limited motor neuron loss in the pre-symptomatic stages of development (24) indicating that motor neuron loss is a late feature of disease progression. However there is a school of thought that implicates alterations in pre-symptomatic

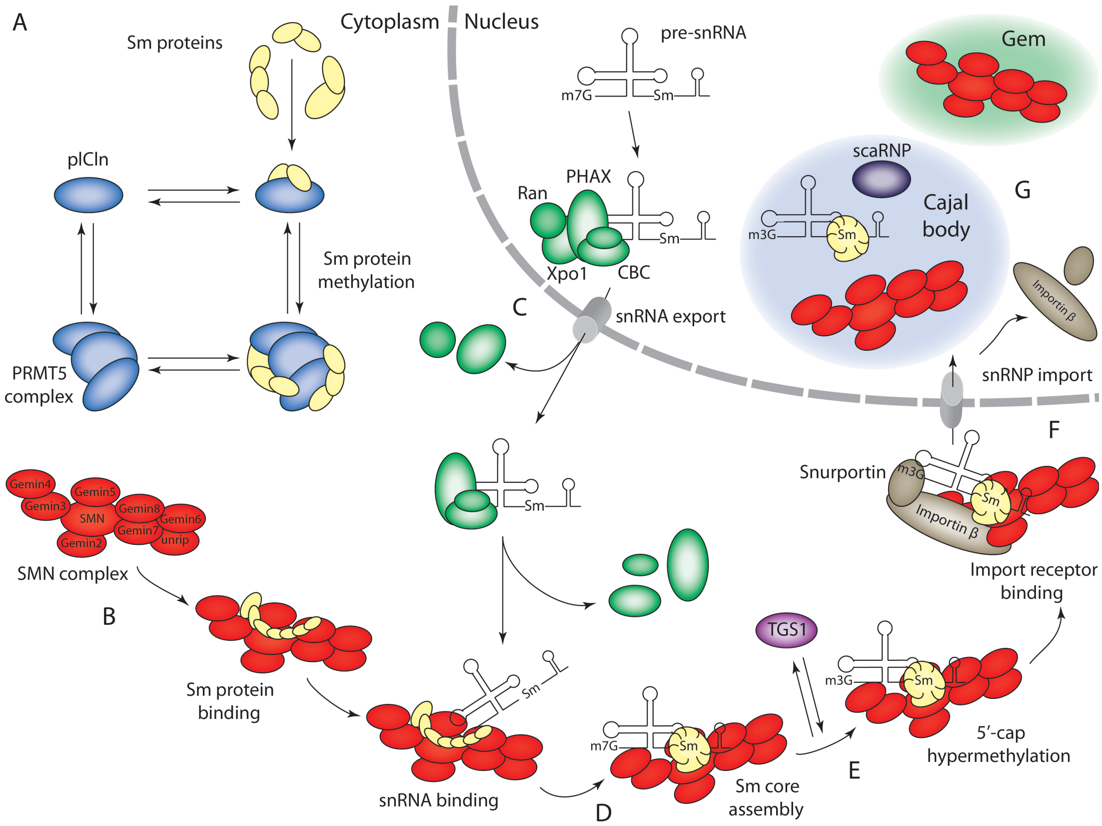

Figure 3.2: SMN function in snRNP assembly - A. In the cytoplasm, Sm proteins associate with the chloride conductance regulatory protein (pICln) in complex with PRMT5. PRMT5 methylates Sm proteins allowing binding to SMN (B). C. snRNA in the nucleus is transported to the cytoplasm by binding the phosphorylated adaptor for RNA export (PHAX), cap-binding complex (CBC), exportin (XPO1) and Ras-related nuclear protein GTP (RAN) mediating its export. D. Once in the cytoplasm, the Sm proteins are then assembled onto the snRNA by the SMN complex. E. The 5' 7-methylguanosine (m7G) cap of the snRNA becomes hypermethylated by trimethylguanosine synthetase 1 (TGS1) to form a trimethylaguanosine (m3G) cap allowing binding to Snurportin and Importin β and consequently nuclear import (F). G. In the nucleus, the SMN complex and snRNPs localise to punctate structures called Cajal bodies. SMN complex members may also be found enriched in Gems. Figure adapted from (27).

neurodevelopmental pathways leading to eventual motor neuron loss as a major factor in pathogenesis.

SMN-dependent snRNP assembly is highest during CNS development and is substantially downregulated during postnatal development in mouse (35) suggesting the need for SMN-mediated snRNP assembly is greater during CNS development. Also, snRNP assembly and synthesis becomes downregulated upon differentiation of neural cell lines (35).

Various studies document specific aberrations in neuromuscular system development, including defects in motor neurons (36) (37), motor axons, neuromuscular junctions (NMJ) (38) (39) (17) (40) (18) and muscle development, (36) (38) (41) (42) in SMA affected individuals or models organisms. Pathfinding defects observed in zebrafish and mouse caused by impaired axonal outgrowth have been suggested as a developmental cause of later motor neuron degeneration (43) (44) (45) (46) since the fidelity of the connection between motor neurons and target muscles is thought to be crucial to motor neuron survival (37) (47).

In *Drosophila* the effect of reduced SMN levels on muscle development and neuromuscular junction structure and function in *Drosophila* has been characterised in a number of *smn* alleles (17) (40) (18). These *smn* alleles all share degrees of muscle defects and in particular, have abnormalities in their larval NMJ organisation. More recently, studies in *Drosophila* and mouse have implicated SMN in the regulation of growth and differentiation (48)(49); *Drosophila* SMN mutant pNBs were found to have a reduced proliferative capacity in the developing larva and similarly, proliferation defects were evident in the brain of SMA mouse models in regions that normally have particularly high levels of SMN in wild-type mice (49). Interestingly, in *Drosophila*, SMN was found to be differentially expressed in distinct cell types within tissues, being most highly expressed in the stem cells (48). Also, modification of SMN levels was found to alter the timing of cellular differentiation in the testis implicating SMN in the regulation of stem cell division and differentiation (48).

Finally, the observation of extra-neuromuscular abnormalities in SMA patients including congenital heart (50) and bone (51) defects has further implicated SMN as a requirement during the development of other tissues. With the exception of muscle development, the consequences of SMN loss on tissue growth and development has been largely overlooked and will be considered in this study using the *smn* alleles described in Figure 3.3 in the light of increasing evidence for SMN function in this role.

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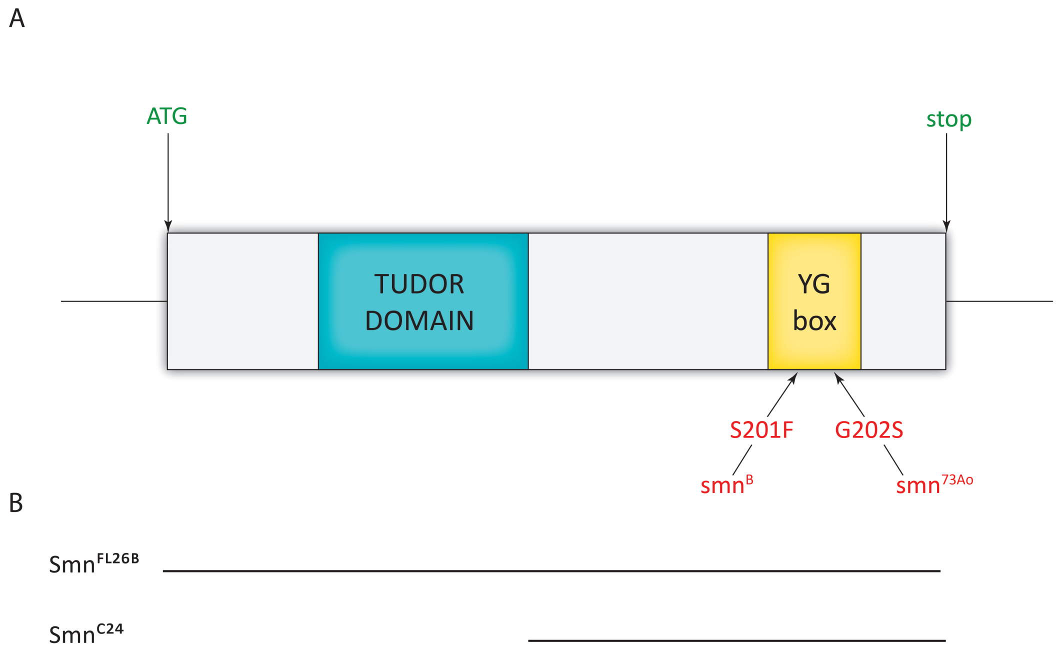


Figure 3.3: ***Drosophila*** **SMN alleles and RNAi contructs** - A. SMN alleles used in this study. The smn^{73A0} and smn^{B} alleles contain point mutations in the conserved C-terminal Y/G box resulting in aberrant self-oligomerisation (17). Both alleles are homozygous late larval lethal with their survival up to this stage being dependent on a maternal contribution of SMN to the embryo. B. SMN RNAi lines used in this study. The RNAi lines described by Chang et al. have varying efficiencies. Ubiquitous expression of the construct targeting the the C-terminus, $[UAS\text{-}Smn\text{-}RNAi]^{C24}$, is lethal at the late pupal stage and the full length-specific, $[UAS\text{-}Smn\text{-}RNAi]^{FL26B}$, is semi-viable (18). Muscle or neuronal specific SMN knockdown using these lines is capable of causing lethality, however, muscle specific SMN loss produces more a severe phenotype (18). Diagram adapted from (18)

3.1.4 Growth pathways modify SMN-induced phenotypes

A number of recent studies have indicated interactions between SMN and growth pathways. It has been shown that SMN mutations in *Drosophila* results in an NMJ phenotype characterised by a reduction in synaptic bouton numbers (17)(18). A screen for genetic modifiers of SMN-dependent reduced viability identified bone morphogenetic protein (BMP) signalling components as modifiers. BMP components are members of the TGF-β family of signalling proteins, one of the unifying developmental regulatory pathways discussed above, also known to function in synaptic growth in *Drosophila* (52)(53). BMP members, namely *wishful thinking* (*wit*), *mad* and *dad*, were tested for their ability to modify the SMN-induced NMJ phenotype and were confirmed as genuine modifiers (18)(54).

Another modifier identified by this screen was the FGF receptor *breathless* (18)(54). The FGF pathway in known to regulate a number of downstream events in *Drosophila* including proliferation, migration, differentiation and apoptosis (54) (55) (56) (57). Further investigation revealed that SMN regulates the expression of FGF signalling components and similarly to the effect observed with BMP signalling, alterations in FGF signalling modified the SMN-dependent NMJ phenotype in *Drosophila* (54).

The mTOR pathway regulator, PTEN, has also been shown to genetically interact with SMN. PTEN is a negative regulator of protein synthesis and cell growth whose reduction promotes motor neuron survival and axonal growth in healthy purified motor neurons. In SMN-depleted motor neurons, defects in axonal growth and survival are observed. Reducing PTEN levels activates Akt and rescues motor neuron survival and axonal growth (58).

3.1.5 Developmental regulation of growth in *Drosophila*

The developing *Drosophila* larva is an ideal system in which to study the function of a gene during development. The larval phase of *Drosophila* development is a period of rapid growth and in mitotic tissues, differentiation. Therefore, the effect of SMN reduction on growth and differentiation and the downstream consequences of perturbations in very early developmental decisions can be effectively modelled in this system. The larval stage involves two major developmental acts; increasing larval size and the differentiation of adult tissue progenitors (59).

Growth by endoreplication: Larval tissues differentiate early in development, therefore the majority of larval cells have reached their most differentiated state, with the exception of the imaginal cells, the precursors of pupal and adult tissues. During larval development, the differentiated body tissues grow rapidly. Increasing DNA content is the most efficient mechanism of increasing cell size and therefore tissue growth, therefore, endoreplication is adopted to achieve overall growth during the larval stage (60). Endoreplication by endocycling is characterised by a loss of a cell cycle mitotic phase, instead oscillating between a G- and S-phase without cell division. This modified cell cycle results in polyploidy thereby dramatically increasing cell size and tissue growth (59)(Figure 3.4). Endoreplication is also utilised in the nurse cells of the adult female germline to achieve polyploidy to meet the high metabolic activity required for embryogenesis (33).

Growth by mitosis: As the larva rapidly increases in size by endoreplication, the undifferentiated adult progenitors grow via cell proliferation; cell growth through division. The developing central nervous system and the imaginal discs constitute the larval mitotically dividing tissues (Figure 3.4). To study the role of SMN function in growth and differentiation, the eye imaginal disc and larval central nervous system will be used in this study.

The developing *Drosophila* eye: The eye imaginal disc is a two-dimensional mitotically dividing epithelial structure in the developing larva that differentiates into the *Drosophila* adult eye. Its growth and differentiation occurs in a defined pattern during the 3^{rd} instar larval stage, sweeping across the disc from the posterior to the anterior end generating generating differentiated cell types, including neurons and glia (56).

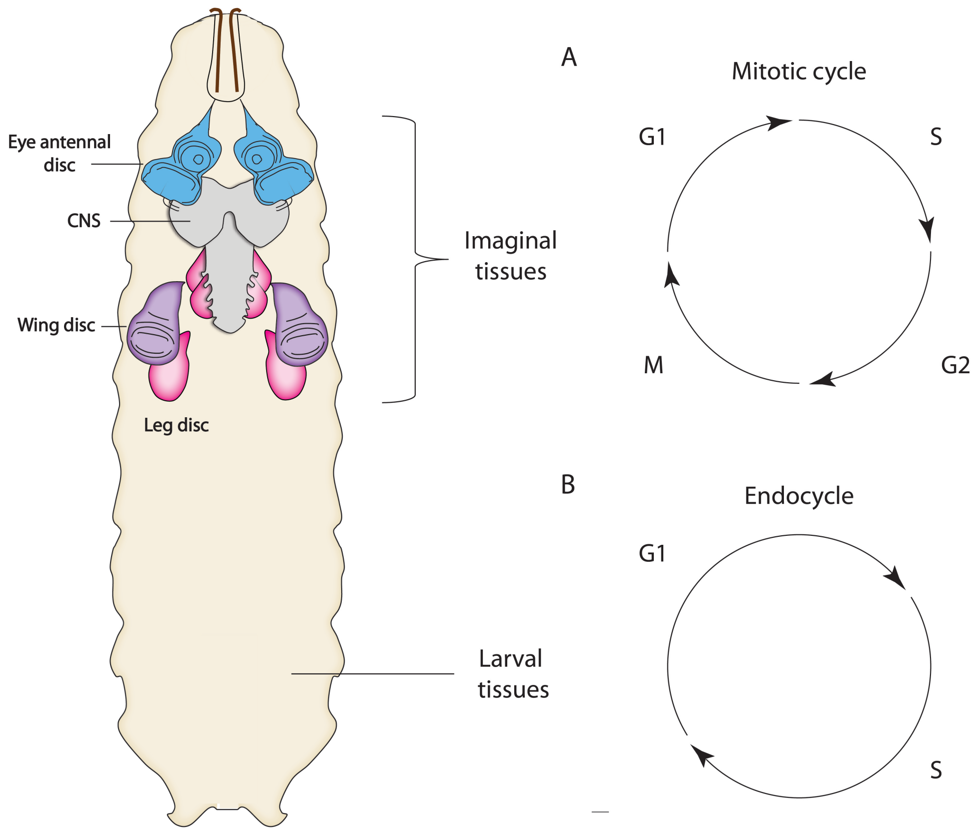


Figure 3.4: Growth by mitosis and endoreplication in *Drosophila* larvae - During the larval stage of development, rapid growth occurs. A. The imaginal tissues, the undifferentiated adult tissue precursors, and the central nervous system grow via cell proliferation; cell growth by mitotic division. B. In the differentiated larval tissues, endocycling is adopted to more efficiently increase growth rates. Endocycling utilises a modified cell cycle that lacks the mitotic phase, oscillating between G- and S-phase without division. This type of cell cycle results in polyploidy and dramatically increases cell size and tissue growth. Diagram adapted from (61).

Importantly, all stages of differentiation can be observed in each eye disc at a single time. The mitotic divisions progress in a 'mitotic wave', appearing as in indentation in the eye disc called the morphogenetic furrow, from the anterior to the posterior of the disc. The result is the generation of rows of ommatidia, the precursors to the adult eye ommatidia. (Figure 3.5).

The developing larval central nervous system: The larval CNS consists of 3 major regions, the central brain (CB), the optic lobes (OL) and the ventral nerve cord (VNC) (Figure 3.6). The larval stage of development provides the major phase of neurogenesis, with approximately 90% of adult neurons generated during this period (63). Neural stem cells in the CB and VNC, termed postembryonic neuroblasts (pNBs), generate cellular diversity and expansion in the CNS by asymmetric division, generating one large stem cell daughter and one smaller daughter, the ganglion mother cell (GMC). Asymmetric division results in the sequestering of cell fate determinants into the GMC that dictate the fate of the GMC daughter cell, repressing stem cell-like genes and activating differentiative genes (63) (Figure 3.7A) allowing the GMC to undergo terminal differentiation producing two neurons or glia (64).

The optic lobe neuroepithelium is determined during embryogenesis, which subsequently forms the inner proliferative centre (IPC) and outer proliferation centre (OPC)

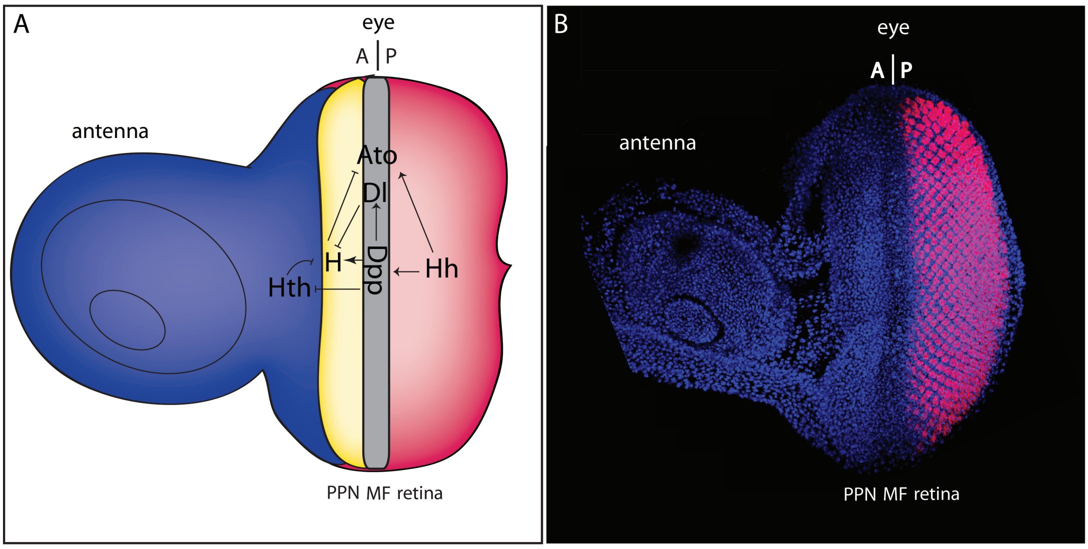


Figure 3.5: Proliferation in the eye disc - A. During the 3^{rd} instar larval stage, cell differentiation occurs in a defined pattern from the posterior to the anterior of the eye disc in a 'mitotic wave' led by the morphogenetic furrow. Hh expression activates Ato in cells of the morphogenetic furrow resulting in the specification of R8 and consequently the remaining cells of the ommatidia. These cells are made responsive to Hh signalling via Hh induction of Dpp expression at the MF which in turn induces H and suppresses Hth, thereby setting up a preproneural state in which those cells are able respond to differentiation cues. H suppresses Ato in cells directly anterior to the MF to prevent precocious neuronal differentiation while within the MF, Dpp induces Dl expression that turns off H and induces Ato expression resulting in photoreceptor differentiation. Finally, the differentiated photoreceptors secrete Hh displacing the MF further into the anterior of the disc, inducing the next wave of photoreceptor differentiation. H, hairy; Dpp, decapentaplegic; Hh, hedgehog; Ato, atonal; Hth, homothorax; PPN, preproneural state; MF, morphogenetic furrow. Diagram adapted from (62). B. 3^{rd} instar larval eye disc. The differentiated neuronal photoreceptors in the posterior side of the disc are labelled with Elav (magenta).

of the optic lobes. During the early larval stages, this neuroepithelium proliferates via symmetic divisions before switching to asymmetric divisions in the late larval stages (63).

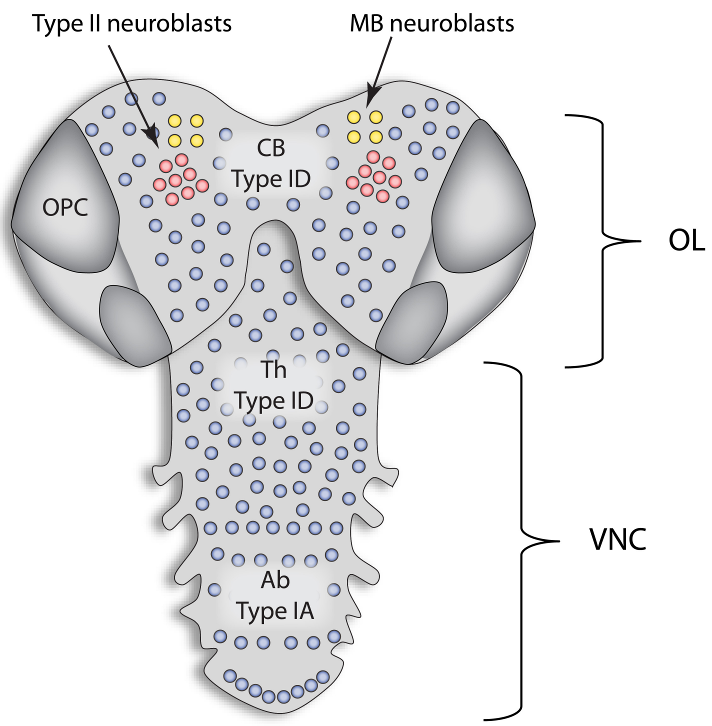


Figure 3.6: Larval developing CNS - The larval CNS consists of 3 major regions, the central brain (CB), the optic lobes (OL) and the ventral nerve cord (VNC). The VNC is comprised of the thoracic (Th) and abdominal (Ab) ganglions. Cellular expansion and differentiation in the CNS is generated the pNBs. Each region of the larval CNS contains distinct types of pNB. A small number of Type II and mushroom body (MB) neuroblasts are present in the CB. Type ID neuroblasts are contained in the CB and the Th ganglion of the VNC. Finally, the outer proliferation centre (OPC) of the optic lobes contains symmetrically dividing neuroblasts. Figure adapted from (63)

The developing larval CNS is therefore a highly proliferative, well characterised tissue in which to study the effect of SMN loss on growth and differentiation.

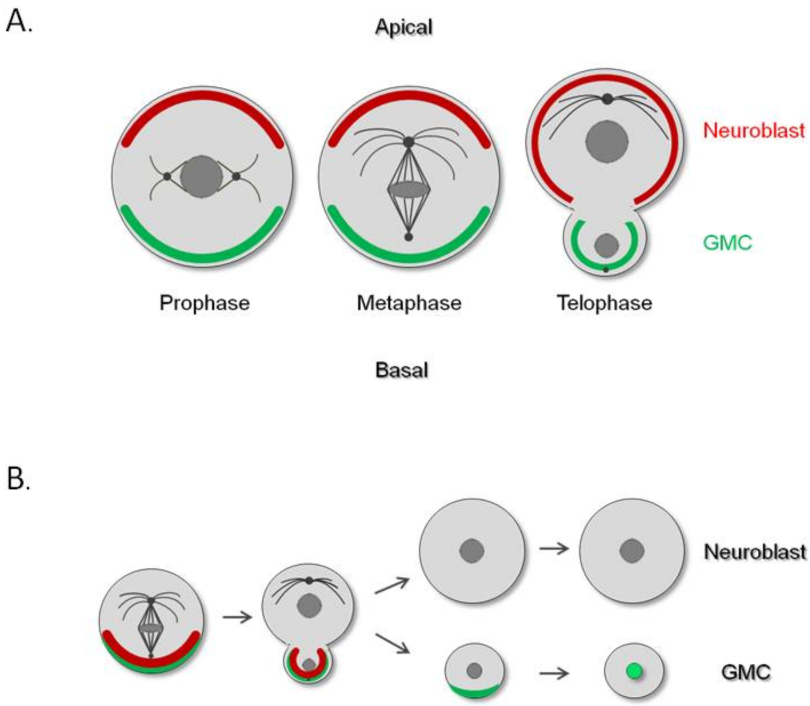


Figure 3.7: Neuroblast asymmetric division and cell fate - A. Neuroblasts asymmetrically divide producing a larger daughter cell that is capable of self-renewal (Neuroblasts) and a ganglion mother cell (GMC) that can divide once to produce two post-mitotic neurons or glia. The basal (Numb, Pon, Prospero, Miranda) and apical (Par, Inscuteable, Pins, Gαi) complexes are shown in green and red, respectively. These complexes function in spindle orientation and localisation of the cell-fate determinants Numb and Prospero. Upon cytokinesis a larger daughter containing the apical proteins and a smaller daughter containing the cell-fate determinants Numb and Prospero are generated. B. Role of Prospero in determining cell fate. During asymmetric division *prospero* mRNA (green) becomes localised to the basal side of the dividing neuroblast by its adaptor protein Miranda (red). Upon cytokinesis Miranda and Prospero are segregated to the ganglion mother cell (GMC) where Miranda is degraded and Prospero released and translocated to the nucleus where it functions as a transcription factor. Diagram adapted from (65)

3.1.6 Aim of chapter

In this chapter, I aim to evaluate the function of SMN in tissue development, with a focus on growth and differentiation in the eye and larval central nervous system, and the role of SMN in nurse cell chromosome morphology in the female germline. *smn* mutants were used to analyse defects in larval growth and development and the clonal analyses described in this thesis were employed to investigate defects in proliferation and differentiation in the CNS and eye imaginal disc. Clonal analyses were also utilised in the female germline in the study of the function of SMN in chromosome morphology. A number of *Drosophila* tissues have been extensively studied and the development of the systems used in this thesis are well characterised, strengthening their use in the investigation of SMN in growth and development.

3.2 Results

3.2.1 SMN reduction results in larval growth defects

The *Drosophila* smn^B allele contains a point mutation in the Y/G box required for SMN oligomerisation, therefore is proposed to produce SMN defective in self-binding, resulting in unstable protein that is rapidly degraded. Western blotting from 3^{rd} instar larval lysates have shown that smn^B is essentially a protein null at this stage (40), however to reach the late larval stages, maternally derived protein is required. Therefore *smn*^{B} larvae provide a system in which SMN levels are reduced, but not completely removed. The *smn*^{B} mutant larvae display progessive loss of motor function until death suggesting that the decreasing levels of maternally supplied SMN cannot support muscle or neuronal function at later larval stages (17).

smn^B larvae display growth defects, being smaller than their control counterparts at the 3^{rd} instar larval stage (Figure 3.8). Dyed food can be observed through the semi-transparent cuticle at this stage therefore feeding appears to be normal in the larvae.

The *smn*^{B} allele is late larval lethal. At this stage *smn*^{B} mutants cannot proceed through to pupation however a number of different states are reached. At 8 days AED, control larvae have pupated, however, at this time point *smn*^{B} animals remain as larvae. A proportion of mutants die or form a variety of 'pseudo' pupae (Figure 3.9). The maternal SMN supplied to the developing *smn*^{B} larvae does not appear to be absolute and western blotting of 3^{rd} instar larvae show variations in SMN levels at this stage (40). Therefore it is probable that depending on SMN levels in the larvae, various stages may be reached. Those animals having higher levels may survive for longer as wandering larvae. Ultimately the depletion of maternally contributed protein results in death before pupation can fully proceed.

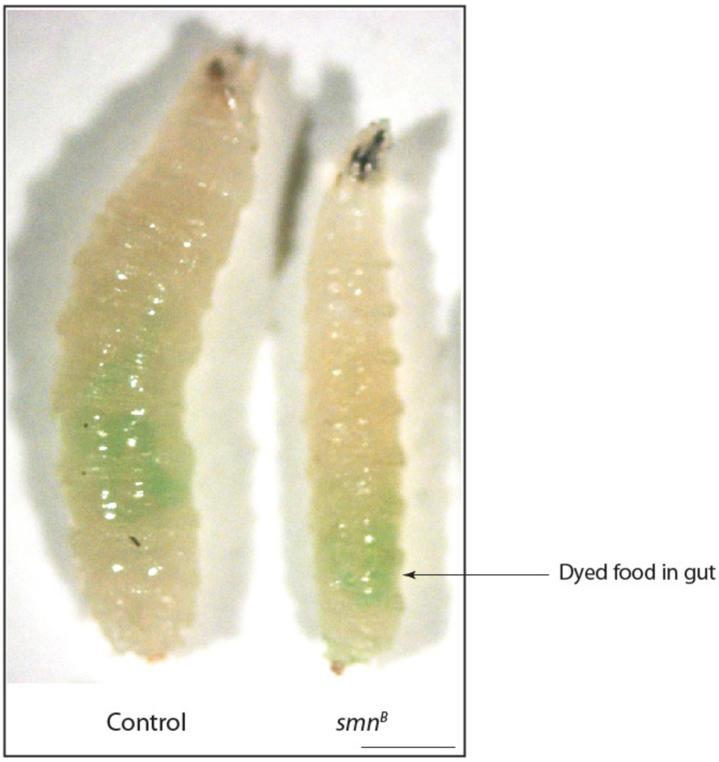


Figure 3.8: *smn*^{B} mutant larvae are reduced in size - At 5 days after egg deposition (AED) *smn*^{B} mutant larvae are shorter and markedly thinner than age-matched *y,w* control larvae. The presence of dyed food in the gut of mutant larvae, observed through the cuticle, suggests food uptake in these mutants is normal, therefore does not contribute to the growth defects observed. Scale bar: 0.5mm.

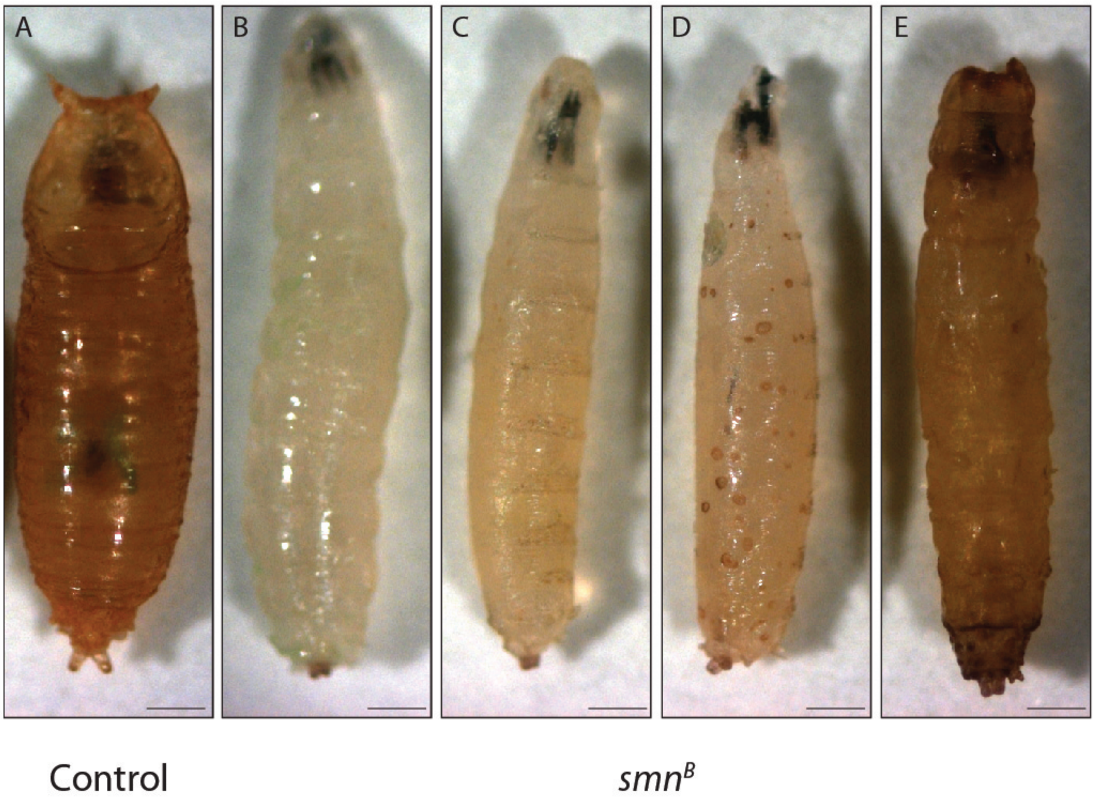


Figure 3.9: Defects in pupation in smn^B mutants - A. At 8 days AED, control *y,w* larvae have pupated. B. smn^B mutants remain in the wandering larval stage at 8 days AED, die or form a kind of aberrant 'pseudo' pupae (C-E) and are unable to proceed through to pupation. The varying stages observed at this time point is likely to reflect variations in SMN levels in individual larvae. All scale bars: 0.25mm

3.2.2 SMN is enriched in stem cells

Considering the defects observed in smn^B mutant larvae, it appears that SMN may function in growth control in *Drosophila*. It has been previously shown that SMN is highly expressed in stem cell populations in *Drosophila* (48). I was able to consistently repeat this finding in larval postembryonic neuroblasts as well as the stem cells of the adult and larval testis (Figure 3.10). In the larval CNS, SMN staining is highest in the postembryonic neuroblasts with lower levels observed in the daughter cells (Figure 3.10 A-D). SMN enriched punctate cytoplasmic structures are observed in the pNBs, likely corresponding to U bodies; proposed sites of snRNP assembly and storage (Figure 3.10 C, inset).

Similarly, in the larval (Figure 3.10 I-L) and adult (Figure 3.10 E-H) testis, SMN is highly expressed at the stem cell containing-tip of the testis and forms a gradient of expression, becoming reduced in levels as cells become more differentiated. A high density of U bodies is observed in the least differentiated cells types but these structures are not evident in differentiated cells containing low basal levels of SMN (Figure 3.10 F and J).

The enriched expression of SMN in stem cells suggests that stem cells have a greater requirement for SMN than differentiated cell types.

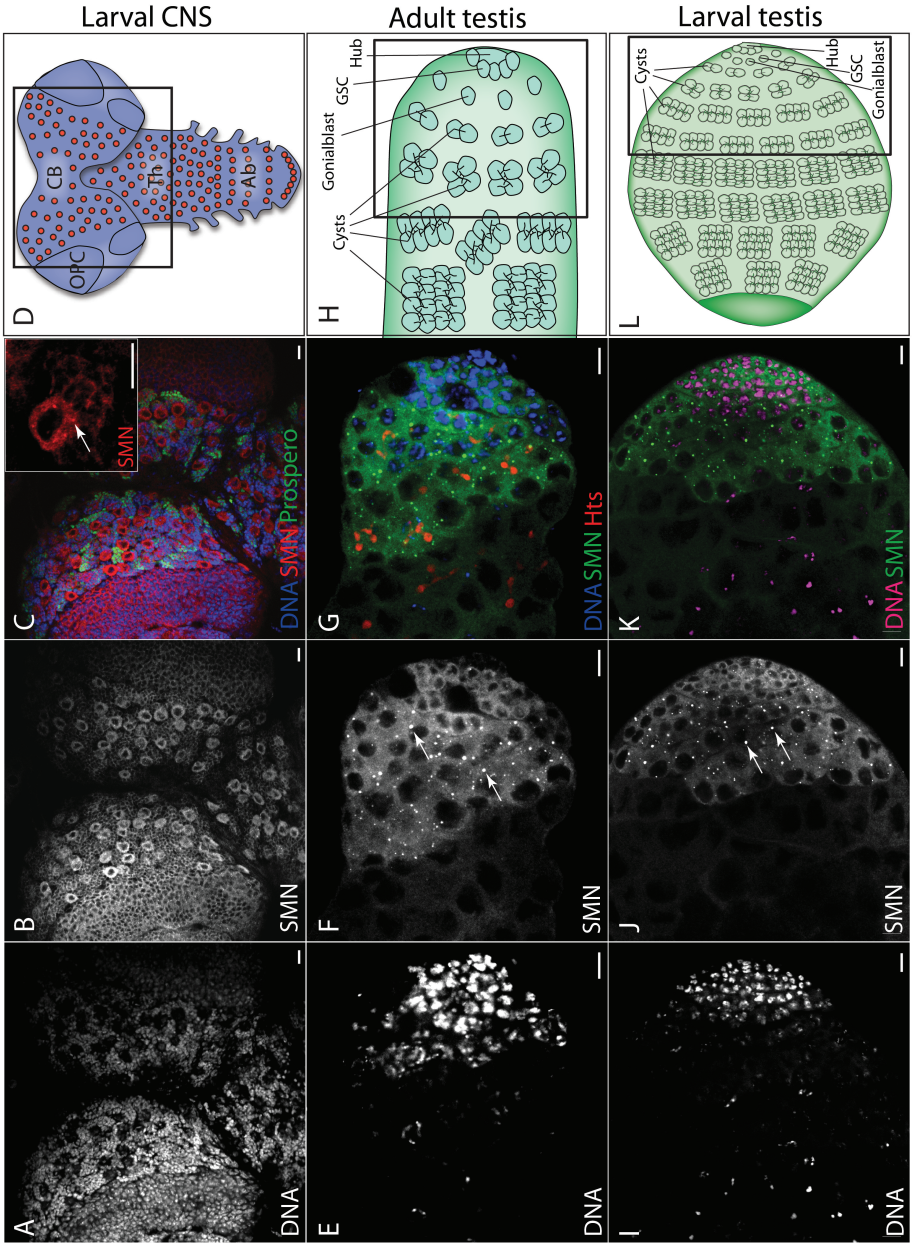


Figure 3.10: SMN is highly expressed in stem cells - A-D: SMN is highly expressed in the pNBs of the larval CNS. Immature differentiated daughter cells labelled with Prospero (green) have a lower basal level of SMN (red) than pNBs (C, inset). SMN can be also be observed in punctate cytoplasmic structures termed U bodies in the pNBs (C, arrow inset). D. The boxed region shows the area of the CNS imaged with red circles representing pNBs. E-H: SMN forms a gradient of expression in the adult testis. SMN levels are highest at the tip of the testis and become increasingly reduced as the cells become more differentiated (F, G). Schematic shows the tip of the testis with the boxed region denoting area of testis imaged (H). I-L: SMN also forms a gradient of expression in the 3^{rd} instar larval testis. SMN staining is highest at the tip of the testis (J, K). In the highly stained 'bands' of SMN expression, a high density of U bodies are observed in both adult (F arrows) and larval (J arrows) testis. Boxed region of schematic shows area of larval testis imaged (L). OPC: outer proliferation centre, CB: central brain, Th: thoracic ganglion, Ab: abdominal ganglion, GSC: germline stem cell. All scale bars: 10μm.

SMN functions in snRNP formation in complex with the Gemin proteins. Gemin3 and Gemin5 have *Drosophila* orthologues and have been shown to directly or indirectly interact with SMN (34). Using antibodies against Gemin3 and Gemin5 I analysed the expression levels of these SMN complex members in the adult testis.

Gemin5 was found to be expressed in a gradient equivalent to that observed for SMN (Figure 3.11 A-E). Gemin5 staining was higher near tip of the testis and lower in the more differentiated cell types. SMN and Gemin5 colocalise in U bodies in regions of high cytoplasmic Gemin5 and SMN staining (Figure 3.11 D). Gemin3 cytoplasmic staining was not obvious however U bodies could be detected as bright cytoplasmic foci (Figure 3.11 G and M). U bodies were observed in greater numbers in the region of the testis containing the dividing cysts, colocalising with SMN punctate staining (Figure 3.11 N). Given the similarities in expression patterns of SMN, Gemin5 and Gemin3, should the SMN expression gradient be functionally relevant, it is plausible that SMN is acting as part of the SMN complex rather than in an independent function.

3.2.3 SMN is highly expressed in mitotically dividing larval tissues

SMN was found to be highly expressed in the mitotically dividing larval tissues; the imaginal discs and the CNS (Figure 3.12). SMN is highly expressed in the neural stem cells of the CNS and is ubiquitously highly expressed in the eye disc and the wing disc.

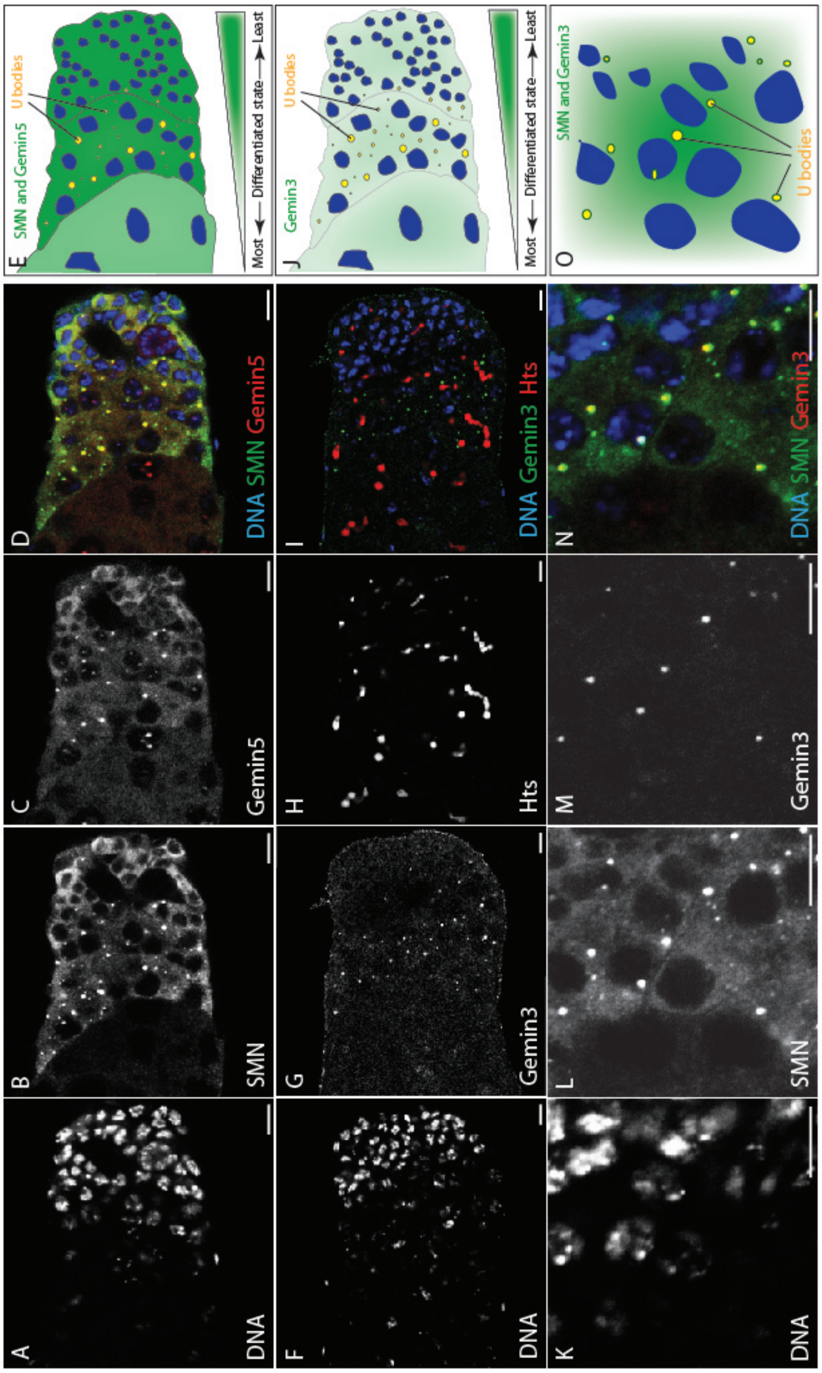


Figure 3.11: Gemin3 and Gemin5 are expressed in a gradient similar to that of SMN in adult testis - A-E: SMN expression (B) in the adult testis forms a gradient, having the highest expression in the stem cells and decreased expression in more differentiated cell types. Gemin5 is also more highly expressed in the stem cells with lower levels in the more differentiated cell types (C). The schematic (E) shows SMN and Gemin5 expression in green and U bodies in yellow. Nuclei are shown in blue. F-J: Gemin3 can be observed in punctate U-bodies that colocalise with SMN. K-O: Zoom of SMN (L) and Gemin3 (M) colocalising in U bodies (N). The schematics in J and O show Gemin3 expression in green and U bodies in yellow. Nuclei are shown in blue. All scale bars: 10μm.

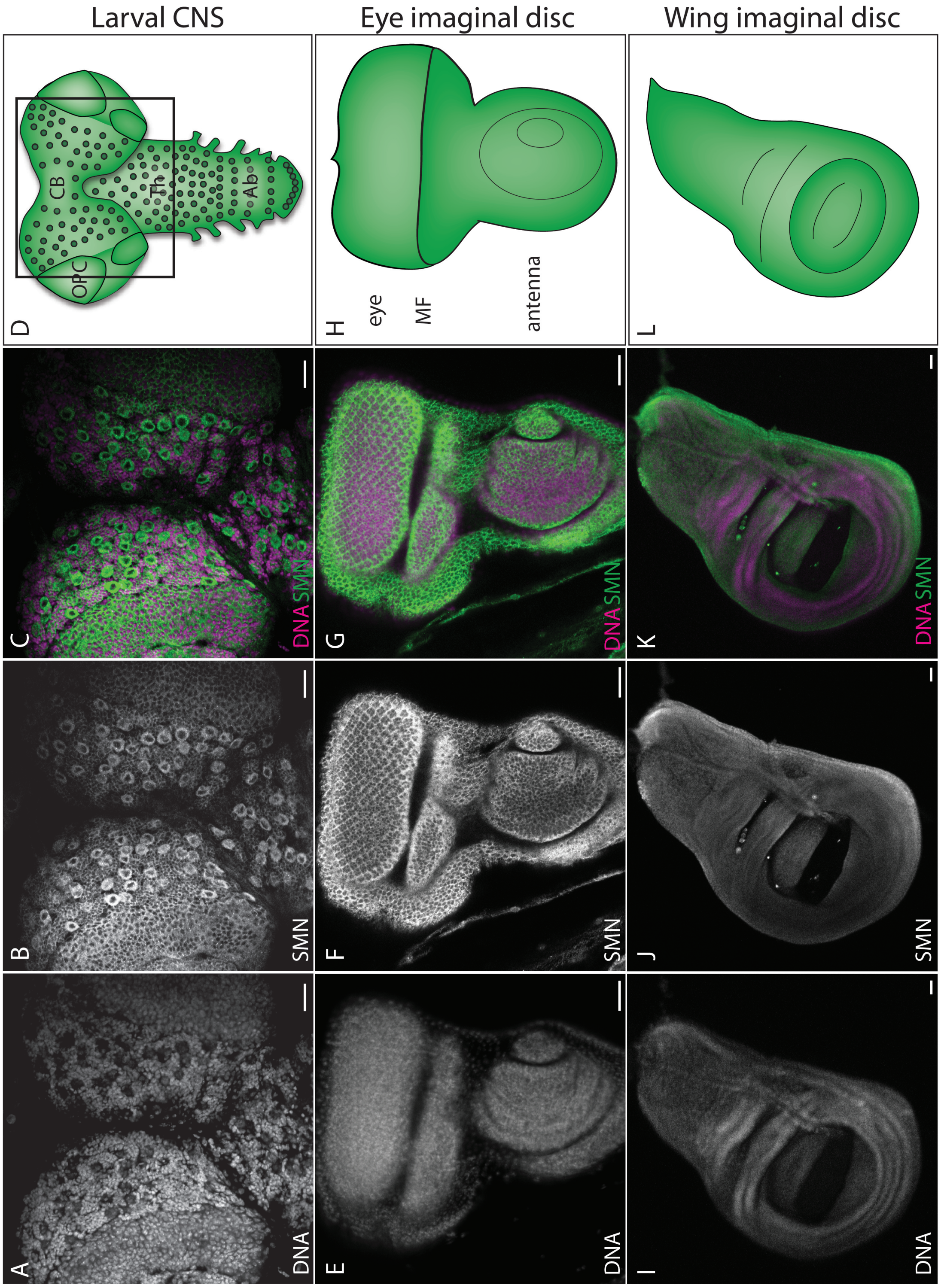


Figure 3.12: SMN is highly expressed in mitotically dividing tissues - A-D. SMN is highly expressed in the pNBs of the larval CNS. Boxed area in D denotes region of CNS shown. E-H. High SMN expression is observed in the eye imaginal disc. I-L. SMN expression in the wing disc. OPC: outer proliferation centre, CB: central brain, Th: thoracic ganglion, Ab: abdominal ganglion, MF: morphogenetic furrow. All scale bars: 20μm.

To ensure SMN staining is specific, SMN RNAi ‘flip-out’ clones were generated in the CNS and eye disc. GFP marked positive clones were found to have reduced levels of SMN (Figure 3.13).

3.2.4 SMN controls growth in *Drosophila*

3.2.4.1 *smn*^{B} mutant larvae have growth defects in their central nervous systems

SMN is highly expressed in the CNS. Therefore to analyse the effect of reduced levels of SMN on development I first studied the effect of SMN mutations on the growth of the CNS. I measured the diameter of the brain lobes of *smn*^{B} mutant larvae at two different time points (Figure 3.14). At 5 days after egg deposition (AED), corresponding to the wandering 3rd instar larval stage of wild type animals, the size of the *smn*^{B} mutant CNS is significantly reduced. At 8 days AED, unlike their control counterparts, *smn*^{B} larvae have not pupated and the CNS is increased in size being significantly larger than *smn*^{B} larvae at 5 days AED.

Interestingly, the OPC of the optic lobes that contains the neuroblasts of the optic lobes, is significantly reduced or absent in *smn*^{B} mutants (Figure 3.15).

3.2.4.2 Neural cells have aberrant sizes in *smn*^{B} mutants

The reduction in CNS size may be due to reduced cell proliferation or reduced cell size. To investigate the contribution of cell size to the growth defect in the CNS, I measured

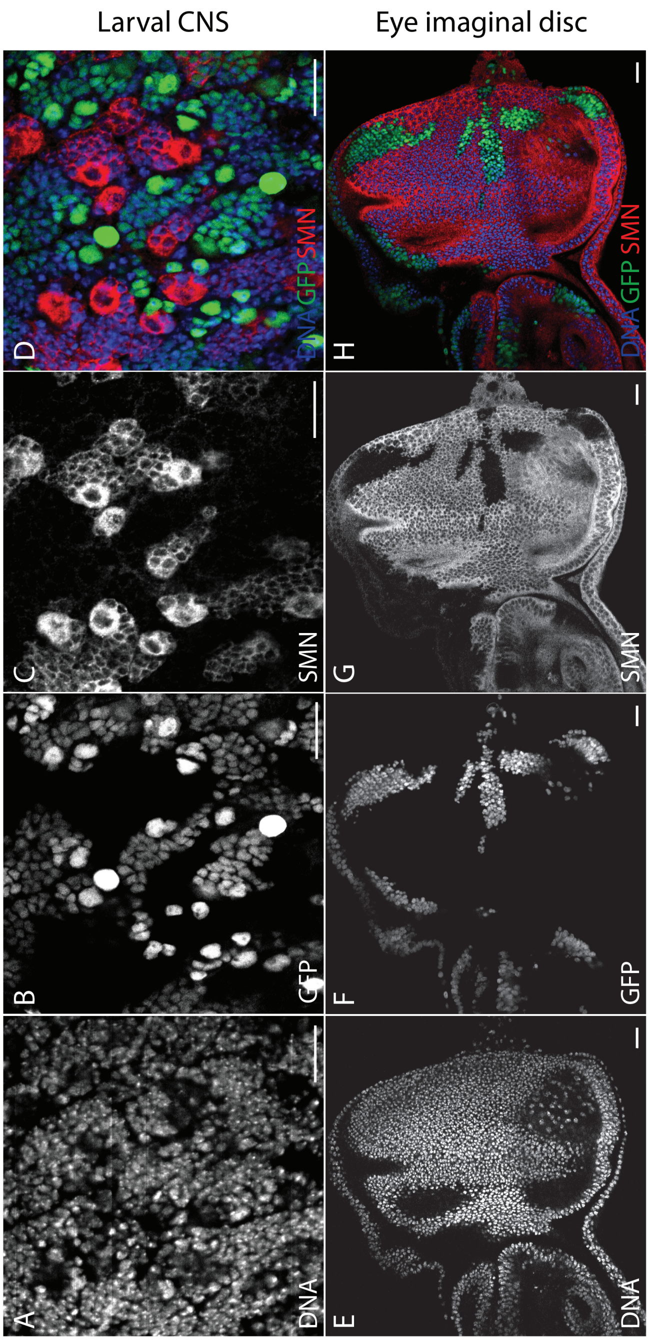


Figure 3.13: SMN expression in mitotic tissues - *UAS-Smn-RNAi*^{C24} 'flip-out' clones in the CNS (A-D) and the eye disc (E-H). 'Flip out' clones marked with GFP had reduced levels of SMN in comparison to neighbouring cells. All scale bars: 20μm.

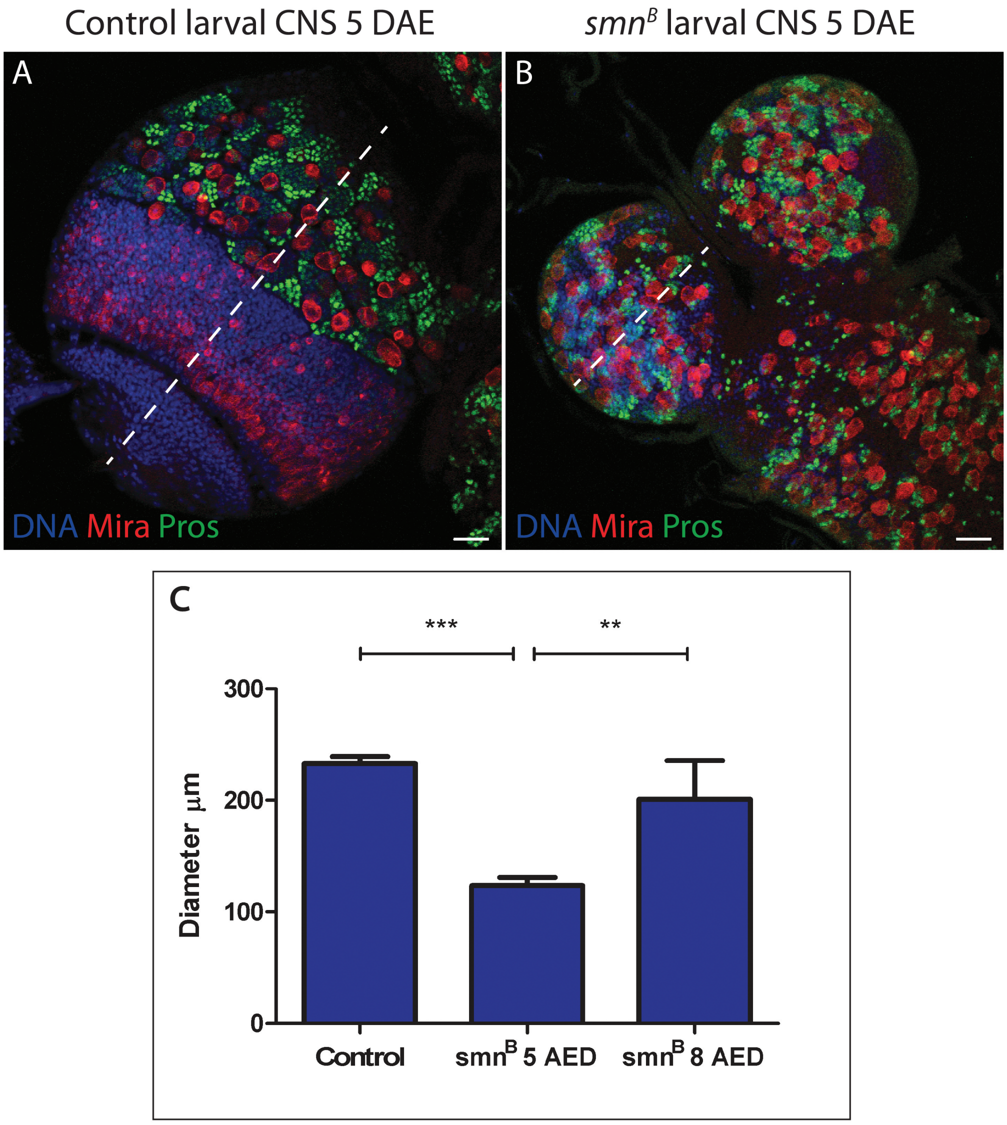


Figure 3.14: *smn*^{B} mutation causes growth defects in the *Drosophila* central nervous system - A-B. Images of the same magnification of control *y,w* (A) and *smn*^{B} mutant (B) larval CNS. Brain lobes were measured as shown by the dashed lines in A and B. C. Brain lobe measurements show *smn*^{B} CNS to be significantly smaller than control *y,w* CNS at 5 days AED. At 8 days AED, *smn*^{B} mutants are significantly larger than *smn*^{B} at 5 DAE. *y,w* control n=13, *smn*^{B} 5 days AED n=14, *smn*^{B} 8 days AED n=6. ANOVA multiple analysis, **P≤0.01, ***P≤0.001. Error bars show SEM. All scale bars: 20μm.

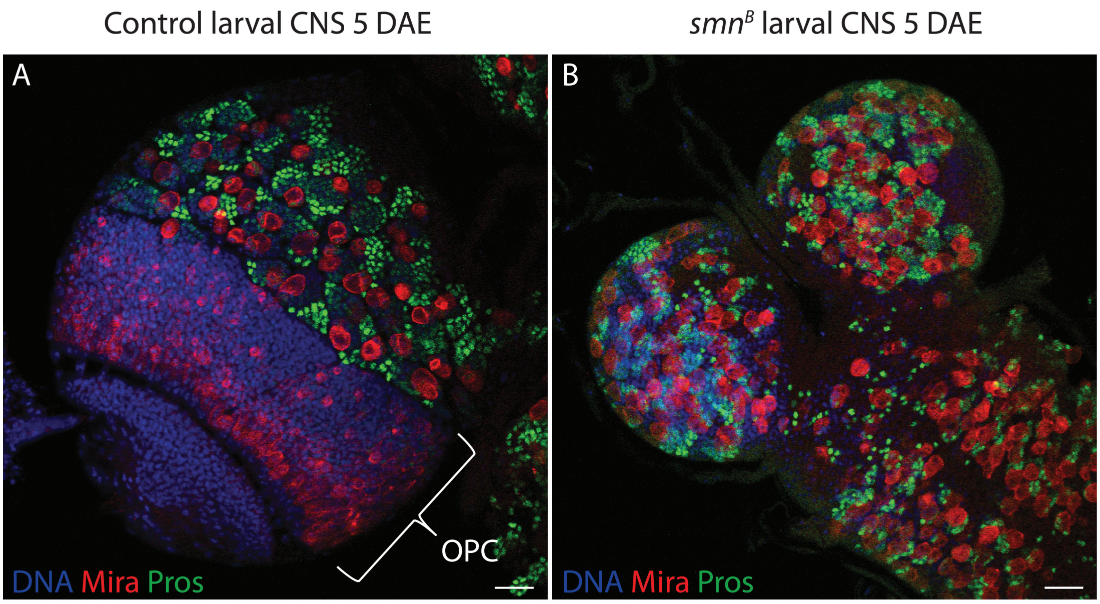


Figure 3.15: Loss of OPC in *smn*^{B} mutants - A. Control *y,w* wandering 3^{rd} instar larvae have a normally developed OPC, labelled. B. *smn*^{B} mutant larvae do not have a fully developed OPC therefore the neuroblasts of the OPC are absent. Scale bars: 20μm.

the sizes of post-embryonic neuroblasts (pNBs), the neural stem cells, in *smn*^{B} mutant larvae. pNBs are large cells that are easily identified by their location within the brain and by stem cell markers (66). pNBs in *smn*^{B} mutants dissected at 5 days AED were found to be significantly smaller than *y,s* control pNBs at 5 days AED (Figure 3.16).

pNBs generate cellular diversity and expansion in the CNS by asymmetric division, generating one large stem cell daughter and one smaller daughter, the ganglion mother cell (GMC), that undergoes terminal differentiation producing two neurons or glia. A reduced cell size can be the result of divisions becoming more symmetric in nature leading to a smaller pNB and a larger GMC. To test whether this reduction in size may be due to aberrant asymmetric division rather than deregulated growth control, I measured the relative sizes of dividing pNBs and their daughter GMCs. Similarly to pNBs, GMCs were significantly smaller in *smn*^{B} mutants at 5 days AED. The ratio of the sizes of the pNB and its GMC daughter is not significantly different in *smn*^{B} mutants compared with controls. The partitioning of the dividing pNB into stem cell and GMC daughters is therefore unaffected in *smn*^{B} mutants and the cell size defect is not due to asymmetric division defects or specific to pNBs (Figure 3.16).

At 8 days AED, the average size of pNBs and GMCs in *smn*^{B} mutants is normal although they are more variable at this time point (Figure 3.16). From these results it can be concluded that a decrease in cell size contributes to the reduced growth

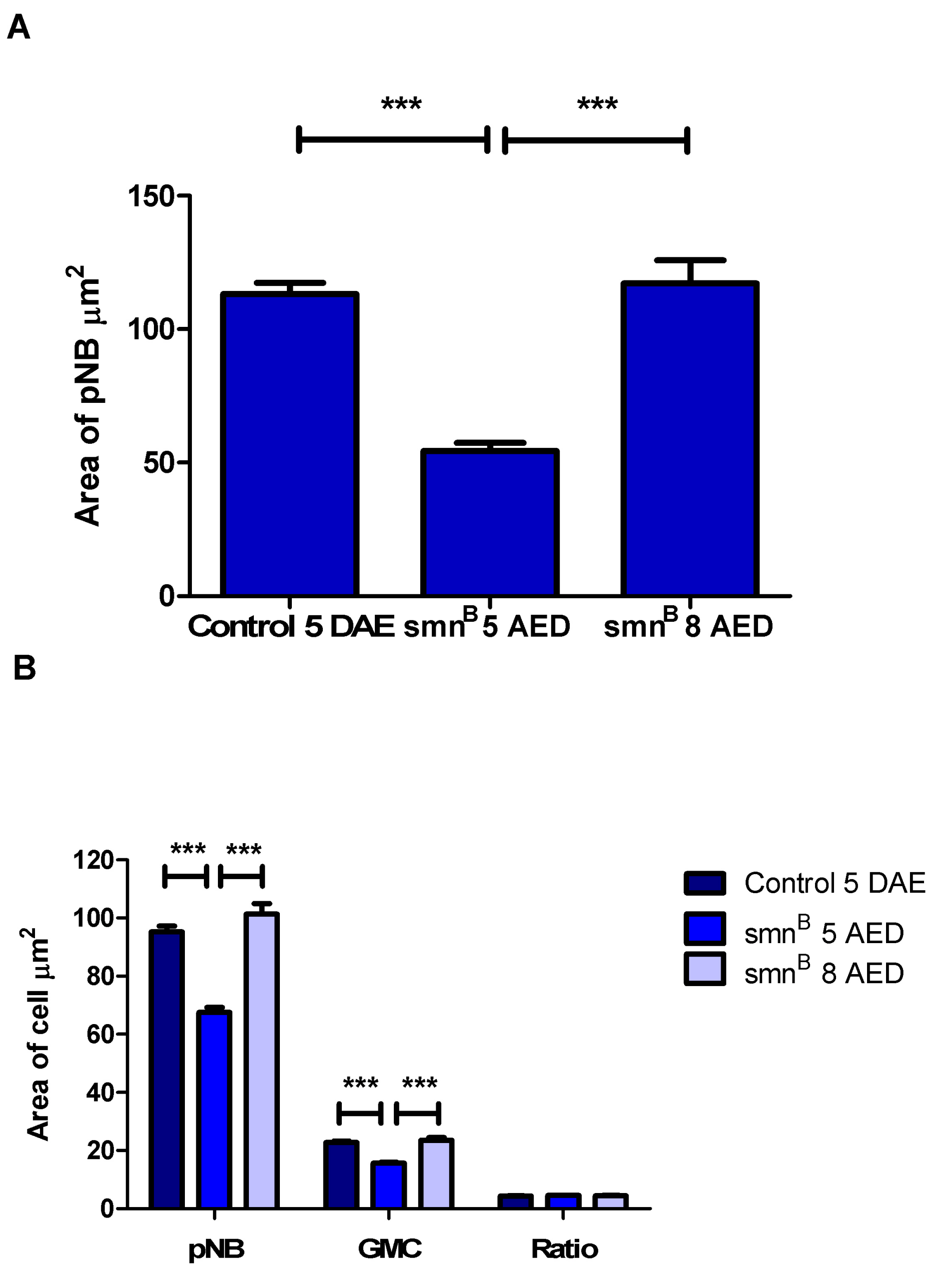


Figure 3.16: Growth defects are due, in part, to decreased cell size - *smn* mutation results in a decrease in neural cell size. A. pNBs are smaller in smn^B mutants at 5 days AED than in control *y,w* at 5 days AED. At 8 days AED, the neuroblasts have increased in size in smn^B mutants in comparison to *y,w* pNBs at 5 days AED. Number of neuroblasts:*y,w* control 5 days AED n=35, smn^B 5 days AED n=44, smn^B 8 days AED n=15. B. The GMC produced by pNB asymmetric division is also decreased in size in smn^B mutants at 5 days AED in comparison to *y,w* at 5 days AED, therefore the decrease is not specific to stem cells. The size of the GMC also increases at 8 days AED. The pNB to GMC cell size ratio is unaltered in smn^B mutants. Number of neuroblasts: *y,w* control n=244, smn^B 5 days AED n=122, smn^B 8 days AED n=44. ANOVA multiple analysis, ***P$\leq$0.001. Error bars show SEM.

phenotype of the CNS in smn^B mutants.

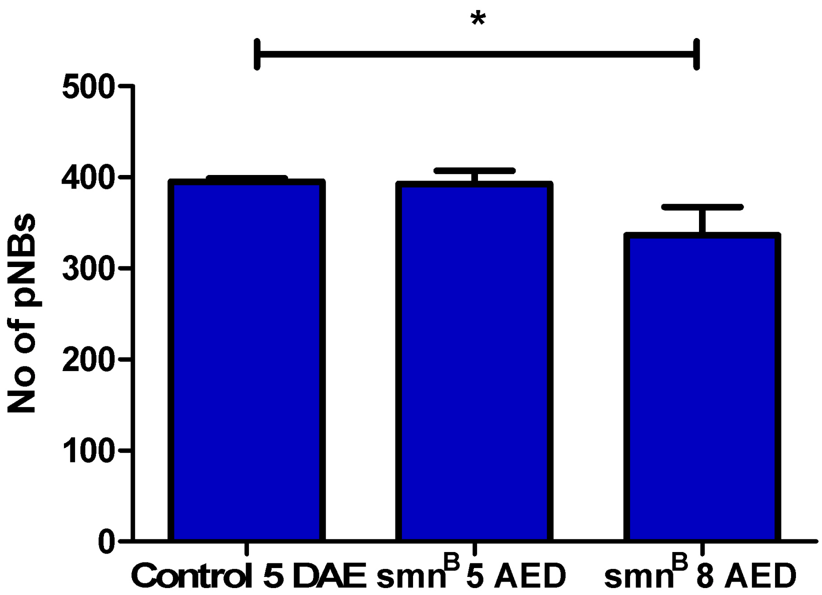
3.2.4.3 Increased variation in stem cell number and stem cell loss in smn^B mutants

Previous work has shown that germline stem cells are lost at a faster rate in smn^{73A0} mitotic clones than in wild type clones, implicating SMN in a role for stem cell survival (48). I have investigated neural stem cell loss in smn^B mutants in which maternally contributed SMN provides a low basal level of SMN protein.

At 5 days AED there is no stem cell loss in the CNS of smn^B mutants however at 8 days AED there is a significant loss of pNBs compared to control larvae at 5 days AED (Figure 3.17). The numbers of pNBs counted in control larvae were extremely consistent across all the animals tested, however the number of pNBs in smn^B mutants of both ages tested was more variable, particularly in the thoracic ganglion. It is possible that SMN has a role in neural stem cell maintenance and survival, however given that loss occurs only in later surviving larvae, further work must be carried out to determine this.

3.2.4.4 *smn* mutant cells have a reduced proliferative capacity in the eye disc

Reduced growth may be the result of reduced cell proliferation and/or reduced cell size. We have already observed that decreased levels of SMN result in reduced neural cell



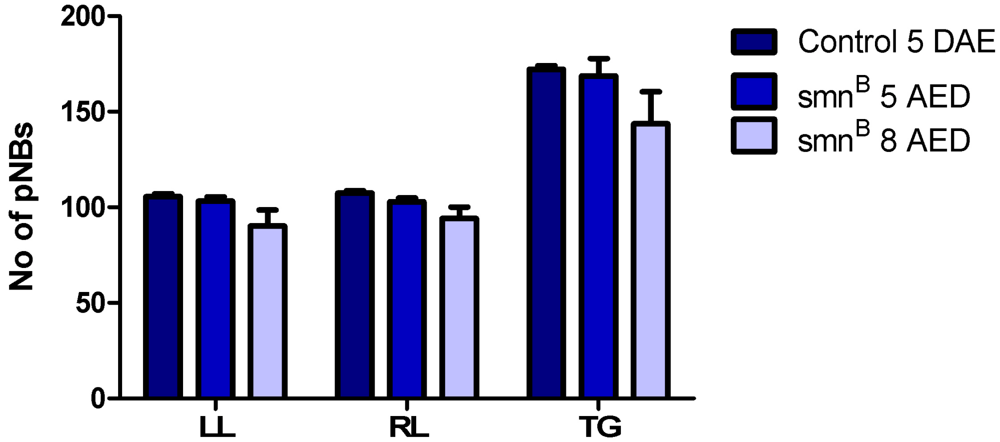


Figure 3.17: Decreased levels of SMN cause neural stem cell loss in late stages - A. Total numbers of pNBs in the CNS. pNBs were identified by their size, position in the brain and the presence of Miranda staining (66) (Figure 3.6). There is no loss of pNBs at 5 days AED in comparison to *y,w* controls at 5 days AED, however there is a significant loss of pNBs at 8 days AED in smn^B mutants when compared to *y,w* at 5 days AED. *y,w* control n=13, smn^B 5 days AED n=14, smn^B 8 days AED n=6. ANOVA multiple analysis, *P<0.05. B. Number of pNBs in each CNS compartment (LL, left lobe; RL, right lobe; TG, thoracic ganglion). Error bars show SEM.

sizes. To investigate the rate of cell proliferation in SMN mutants I have used mitotic clonal analysis in the eye imaginal disc.

Mitotic clonal analysis has the advantage of allowing GFP-negative mutant clones to be compared to their wild-type neighbouring GFP-positive twin-spot. This provides an elegant method of analysing growth competition between mutant and wild-type cells since the relative sizes of the clones produced from a single progenitor can be used as a measure of growth advantage or disadvantage.

SMN mutant clones in the eye imaginal discs were found to have a severe growth disadvantage, being out-competed by their wild-type twins (Figure 3.18 E-H). SMN mutant clones comprising a few cells are occasionally observed however the majority of clones fail to proliferate. In control mitotic clones, in which both twin-spots are wild-type, there is no growth advantage of GFP-positive clones over their GFP-negative twins (Figure 3.18 A-D).

The reduced proliferation of *smn*^{73A0} mutant cells can be rescued by generating clones in an SMN overexpression background; this was achieved using a *tub-smn* construct in which SMN expression is under the control of the tubulin promoter. Therefore we can confidently attribute the reduced growth of *smn* mutant clones to SMN reduction (Figure 3.18 I-L).

Given the striking growth disadvantage of *smn*^{73A0} clones in the eye disc, the abil-

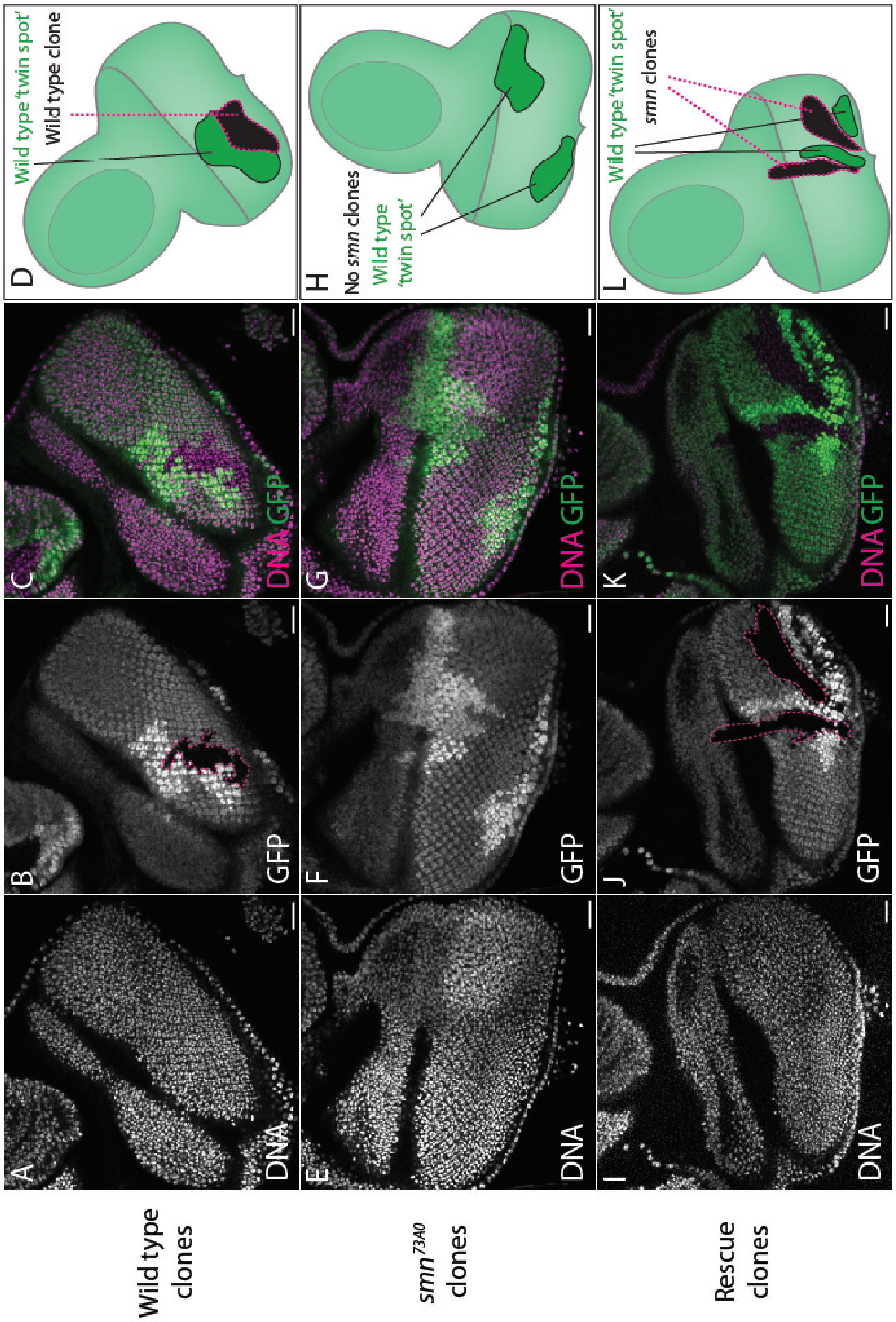


Figure 3.18: *smn*^{73A0} mutant cells have a reduced proliferative capacity in the eye disc - A-D: Control mitotic clones, in which both GFP-negative and -positive clones are wild type. GFP-negative clones are marked with a pink dashed line in (B). E-H: *smn*^{73A0} mitotic clones. GFP-negative, *smn*^{73A0} mutant clones have a severe growth disadvantage compared to their GFP-positive, wild-type twin spots, therefore no GFP-negative clone is observed. I-L: *smn*^{73A0} mutant clones are able to proliferate in an SMN overexpression background, marked with a pink dashed line in (J). All scale bars: 20μm.

ity of *smn*^{73A0} clones in the CNS to proliferate was investigated. Interestingly, unlike mutants clones in the eye disc, *smn*^{73A0} mutant clones in the CNS were able to proliferate (Figure 3.19). Eye discs and CNS from the same animals showed an absence of GFP-negative clones associated with twin-spots in the eye disc but proliferation of *smn*^{73A0} mutant clones in the CNS. Patches of GFP-negative *smn*^{73A0} clones were observed throughout the CNS suggesting that loss of SMN does not result in the same growth disadvantage in the CNS that occurs in the eye disc.

3.2.5 Cellular differentiation is influenced by SMN in specific tissues

In *Drosophila*, SMN is expressed in a gradient in the larval and adult testis, being most highly expressed in the stem cells and decreasing in expression as cells become more differentiated. This led to the hypothesis that SMN may be involved in stem cell division and differentiation. It has been previously observed that in testis, using coilin as a marker for spermatocytes, modifying the position of the gradient resulted in a shift in the coilin-positive spermatocyte band (48). Although a very interesting observation, of particular interest with regards to SMA pathology is whether SMN regulates cell division and differentiation in neural tissues.

SMN has been found to be involved in growth regulation in mitotic tissues, as discussed above. I have used the eye imaginal disc as a model to study the effect of

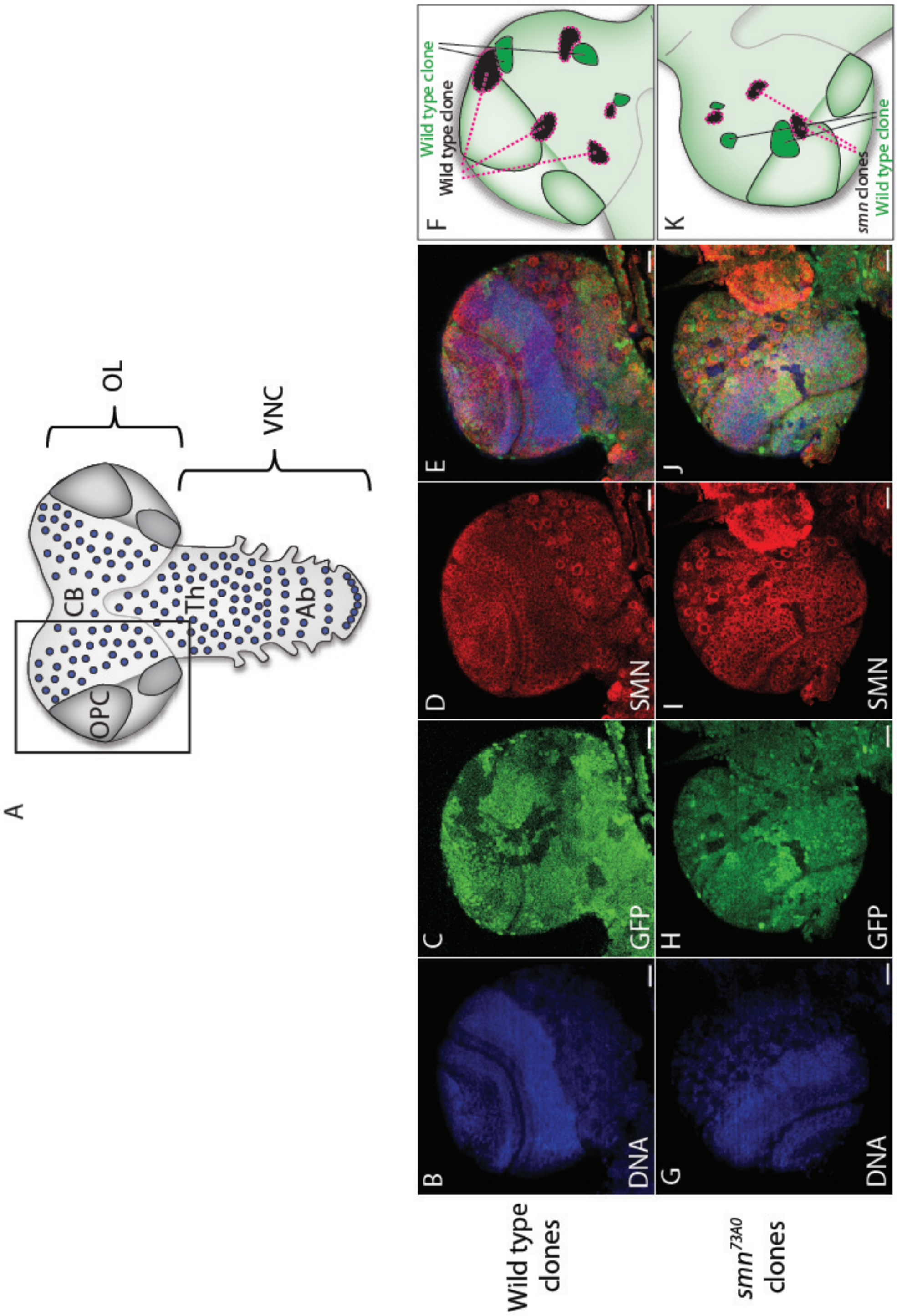


Figure 3.19: *smn*^{73A0} clones are able to proliferate in the CNS - A: Schematic of the 3rd instar larval CNS with boxed region denoting region of CNS imaged. B-F: Wild-type mitotic clones in the CNS have both GFP-positive and GFP-negative clones. SMN is ubiquitously expressed throughout the CNS (D). G-K: *smn*^{73A0} mitotic clones contain GFP-negative clones that show reduced levels of SMN (I). Therefore, unlike *smn* mutant clones in the eye disc, mutant clones are able to proliferate. OPC: outer proliferation centre, CB: central brain, Th: thoracic ganglion, Ab: abdominal ganglion, OL: optic lobes, VNC: ventral nerve cord. All scale bars: 20μm.

SMN reduction on the differentiation of neural cell types.

3.2.5.1 Differentiation defects are not observed in SMN deficient cells of the eye disc

smn^{73A0} mitotic clones are unable to proliferate in the eye disc, therefore to analyse the effect of SMN reduction on eye disc development I used an SMN specific RNAi line, *UAS-Smn-RNAi*^{C24}, previously used by Chang et al. (18) to show a reduction in bouton numbers in the NMJ when expressed using *act*-GAL4, *elav*-GAL4 and *how24B*-GAL4 . In order to generate RNAi clones within a wild-type background, allowing comparison of wild-type and RNAi cells in the same animal, I used a flip-out cassette generating GFP-positive flip-out clones with reduced levels of SMN.

SMN reduction in flip-out clones was tested using an antibody against SMN. Decreased levels of SMN can be clearly observed in the clones (Figure 3.20).

Eye imaginal discs, like the central nervous system, contain neuronal and glial cell types. Using antibodies against the glial marker, Repo, the neuronal marker Elav, and the cell-fate determinant Prospero, cellular differentiation was analysed in the eye discs. Elav and Repo expression was unaltered in SMN RNAi clones with no obvious defects in cell patterning in either neurons or glia. Similarly, Prospero expression was normal in flip-out clones (Figure 3.20).

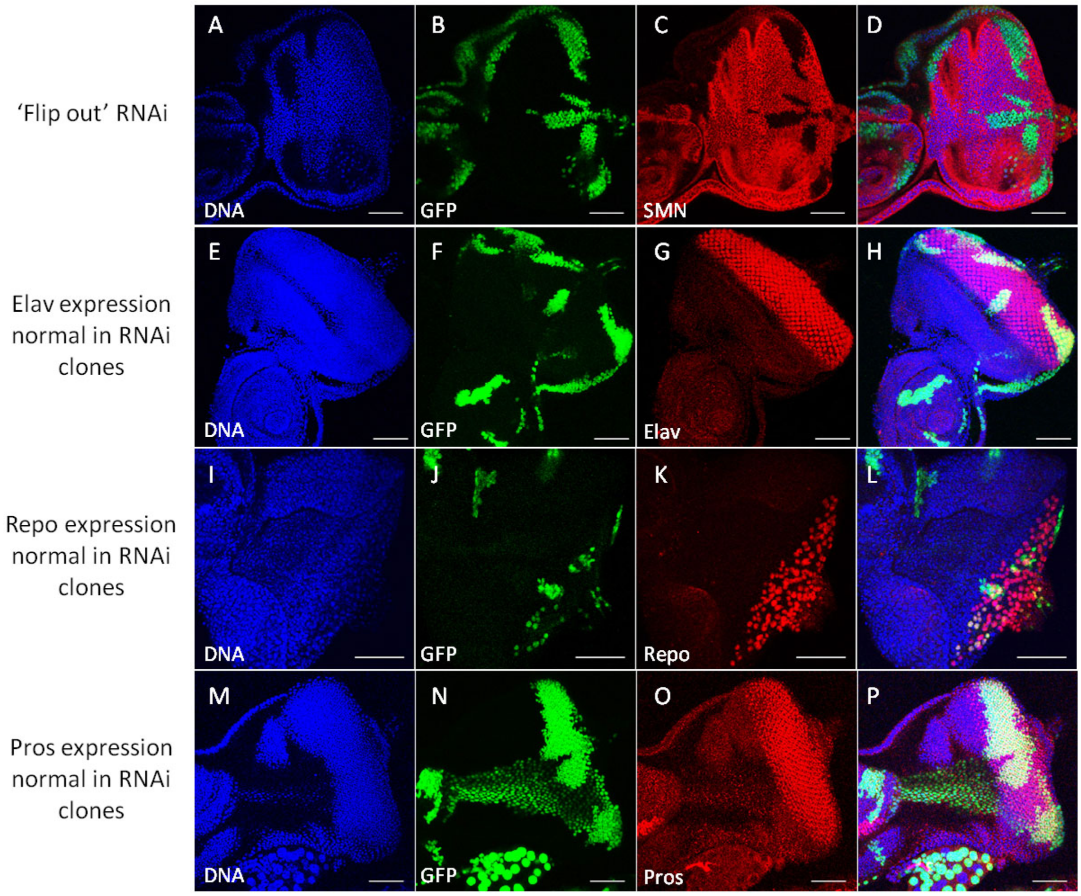


Figure 3.20: SMN RNAi clones do not display defects in differentiation in the developing eye - A-D: *UAS-Smn-RNAi*^{C24} clones marked with GFP have reduced levels of SMN. E-F: *UAS-Smn-RNAi*^{C24} clones have normal Elav localization indicating that the neurons of the eye disc differentiate normally both spatially and temporally. I-L: Repo staining is also normal in these clones. M-P: Prospero localization, which is disrupted in *smn*^{B} mutant neuroblasts, is unaffected in *UAS-Smn-RNAi*^{C24} clones. All scale bars: 40μm. Projected images are shown.

3.2.5.2 Differentiation defects are not observed in cells overexpressing SMN in the developing eye

SMN overexpression in the adult testis resulted in striking defects in cellular differentiation and growth (48). To analyse whether this phenomenon is unique to the testis I generated flip-out SMN overexpression clones in the eye imaginal disc.

SMN levels in the clones were observed using an antibody against SMN and shown to be increased in the GFP-positive flip-out clones. Using Elav, Repo and Prospero, I investigated the effect of SMN overexpression on the differentiation of eye disc progenitor cells into neurons and glia given that SMN overexpression in the adult testis results in inappropriate cellular differentiaion; overexpression in the adult testis appears to suppress cyst cell differentiation (48). SMN overexpression in eye disc clones however did not result in defects in cellular differentiation or growth, unlike the situation in the adult testis (Figure 3.21).

3.2.5.3 Modified SMN expression does not alter *Drosophila* adult eye development

SMN RNAi and overexpression flip-out clones displayed normal expression of differentiated cell markers in clones to the posterior and anterior of the morphogenetic furrow. Furthermore, no defects in cell growth were clear. To investigate the effect of prolonged modification of SMN levels and to remove potential trophic influences of neighbouring

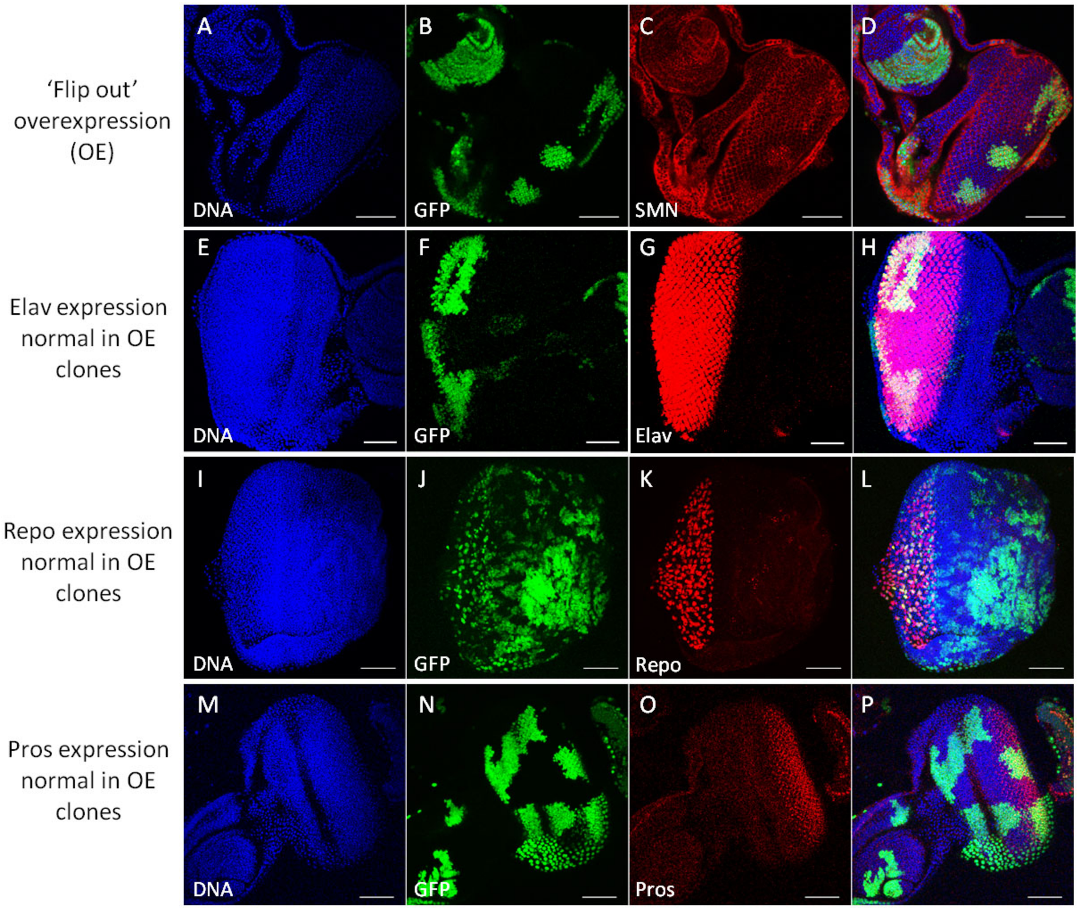


Figure 3.21: SMN overexpression clones do not display differentiation defects in the eye disc - A-D: *UAS-FLAG-Smn* clones marked with GFP have increased levels of SMN. E-F: *UAS-FLAG-Smn* clones have normal Elav localization. I-L: Differentiation into glia appears normal as shown by Repo staining. M-P: Prospero localization is also unaffected in *UAS-FLAG-Smn* clones. All scale bars: 40μm. Projected images are shown.

wild-type cells, defects in eye-specific SMN RNAi and overexpression in the adult were analysed.

Eye-specific expression was achieved using the eye-specific drivers, *eyeless*-GAL4 and *GMR*-GAL4. *eyeless*-GAL4 is a pre-mitotic driver, therefore using this driver, SMN levels are altered early in eye disc development. Temperature influences GAL4 expression levels with higher temperatures resulting in increased expression, therefore crosses were kept at 25 or 29°C. *eyeless*-GAL4 expression was found to cause severe developmental defects even at room temperature, therefore analysis of eye development using this driver was somewhat precluded, however no significant modification of the phenotype could be observed upon altering SMN levels (Figure 3.22).

GMR-GAL4 is a postmitotic driver, therefore SMN levels are modified following cell division. Eye-specific SMN RNAi and overexpression did not result in defects in growth, patterning or differentiation using a *GMR*-GAL4 driver (Figure 3.23).

3.2.5.4 The cell fate determinant Prospero is mislocalised in smn^B mutant CNS

Prospero is a transcription factor that plays a crucial role in the decision between self-renewal and differentiation in stem cells. *prospero* mRNA is localised to the basal side of dividing pNBs by its adaptor protein Miranda. Upon segregation into the GMC Miranda is degraded and Prospero translocates to the nucleus where it acts

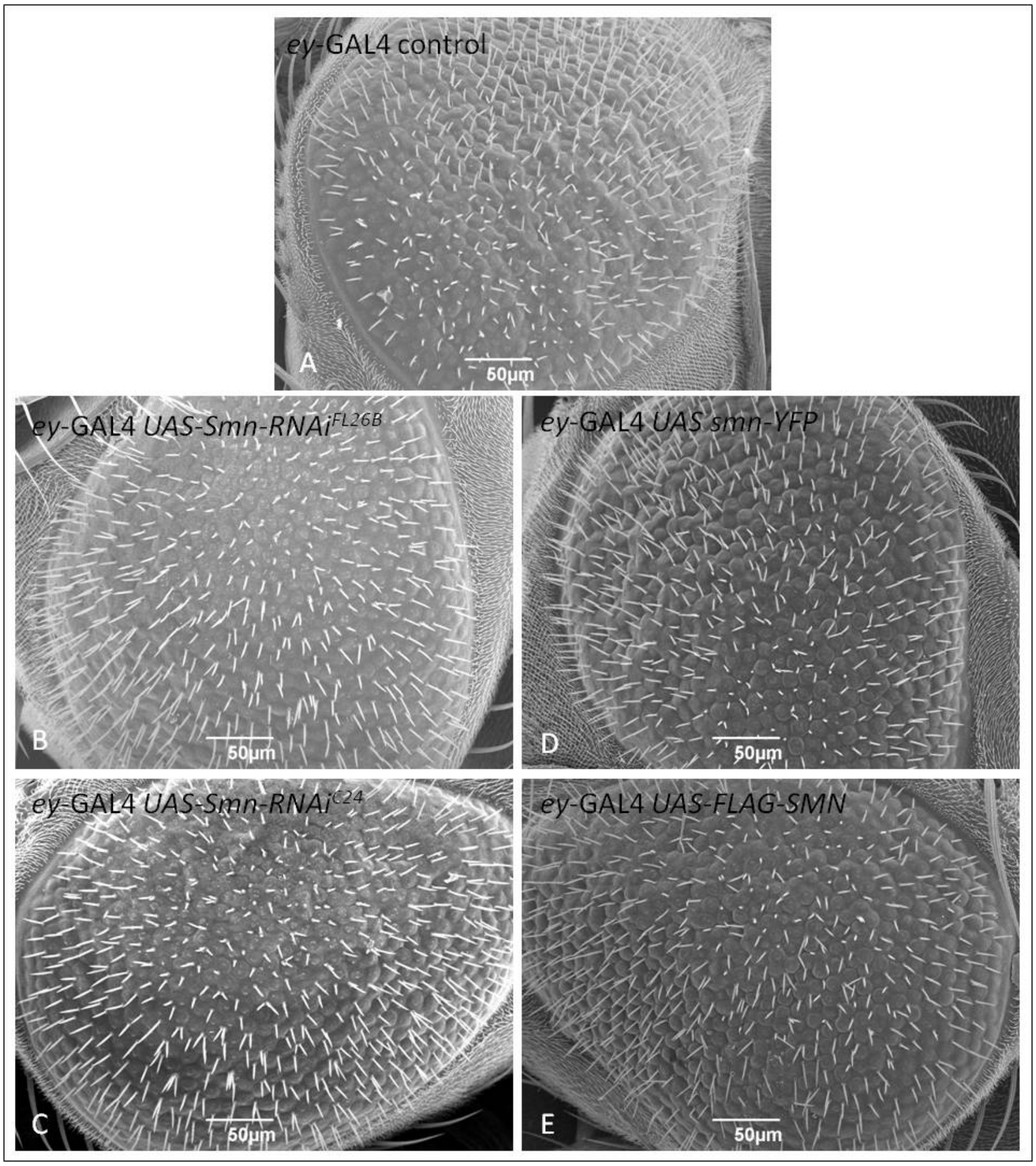


Figure 3.22: SMN RNAi or overexpression using a pre-mitotic driver does not result in aberrant growth or differentiation in the adult eye - A: ey-GAL4 control flies have severe adult eye defects. B, C: ey-GAL4 driven eye-specific SMN RNAi. D, E: SMN overexpression driven by ey-GAL4.

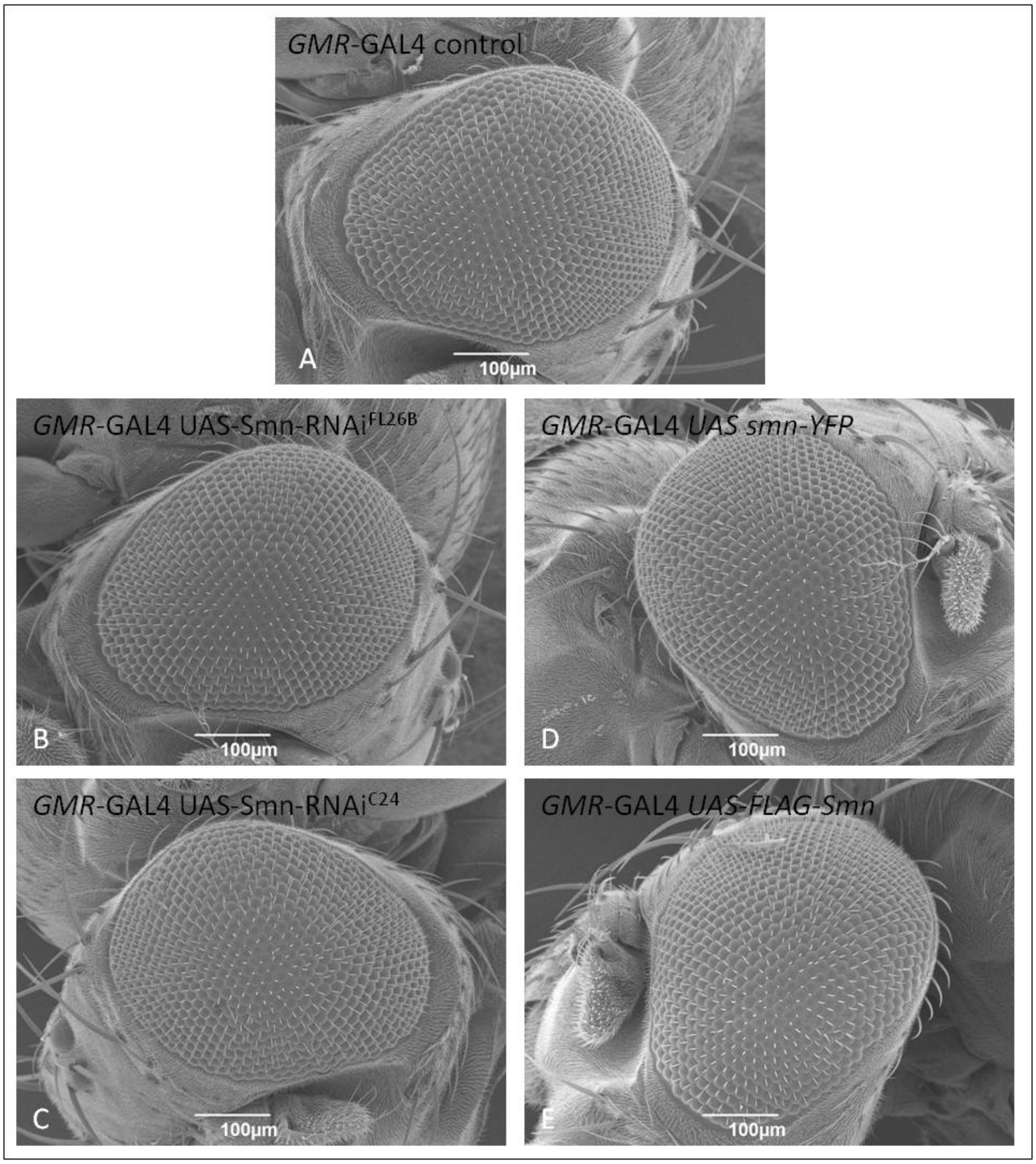


Figure 3.23: SMN RNAi or overexpression using a post-mitotic driver does not effect adult eye morphology - A: GMR-GAL4 control flies grown at 25°C have normal ommaditia. B, C: Similarly, eye-specific SMN RNAi does not affect eye development. D, E: SMN overexpression also does not cause abnormal eye development.

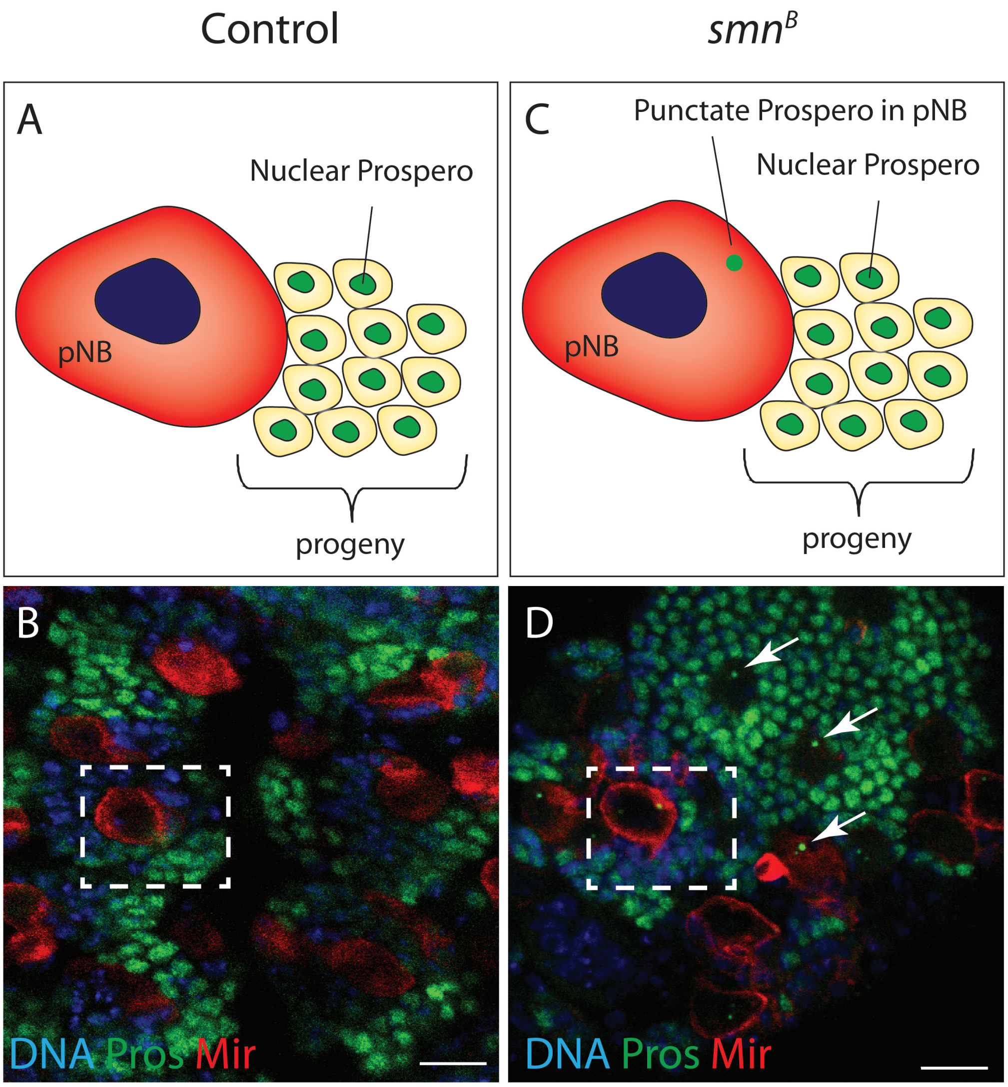


Figure 3.24: The cell fate determinant Prospero is mislocalised in late stage *smn*^{B} mutant pNBs - During asymmetric division, *prospero* is localised to the basal side of the dividing neuroblast by its adaptor protein, Miranda (red), to coordinate the complete segregation of Prospero (green) into the GMC. A. In control *y,w* larval CNS, Prospero protein (green) is not observed in the neuroblast but is expressed in the nuclei of GMCs and their progeny. B. Dashed box denotes the region illustrated by the schematic in (A). In *y,w* control neuroblasts Prospero protein expression (green) is not observed in the neuroblasts (red) but is present in progeny. C. In *smn*^{B} mutant neuroblasts, punctate Prospero staining is observed in addition to normal Prospero expression in pNB progeny. D. Dashed box denotes the region illustrated by the schematic in (C). Prospero is aberrantly localised to punctate structures (white arrows) in *smn*^{B} neuroblasts. Scale bars: 10μm.

to suppress stem cell identity genes and promote the expression of differentiated cell genes. Prospero protein is clearly observed in the nuclei of immature differentiated daughter cells and may also be visible at the basal side of pNBs in a cortical crescent at metaphase. *smn*^{B} mutants that remain in the larval stage rather than pupating have mislocalised Prospero protein expression. Prospero is found to be expressed in punctate inclusions in the pNBs as well as in daughter cells (Figure 3.24).

Interestingly, preliminary data suggests that the mutant pNB Prospero inclusions colocalise with punctate expression of the SMN complex members, Gemin5 and Gemin3 (Figure 3.25). Prospero was not found to colocalise with the U body marker Lsm11, the P body marker Me31B or the Cajal body marker, coilin (data not shown).

3.2.6 SMN function in the female germline in *Drosophila*

Drosophila oogenesis has been extensively studied and characterised allowing the female germline and the process of oogenesis to become an important model for investigating development. Previous work by our group has implicated SMN in the regulation of nurse cell chromosome morphology (33). The following experiments further this study of the role of SMN in the polyploid nurse cells of the female germline.

Drosophila females each contain two ovaries for egg production. Each ovary consists of structures termed ovarioles which are essentially egg production lines in which stem

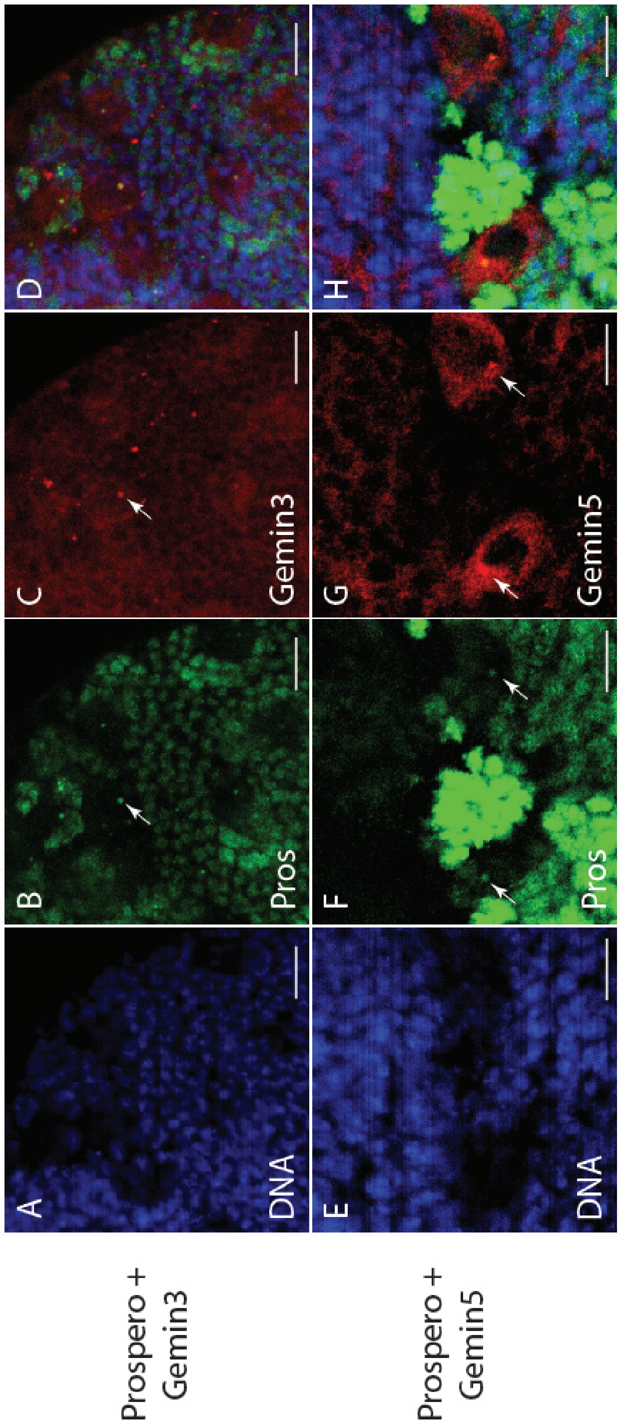


Figure 3.25: Mislocalised Prospero colocalises with the SMN complex members Gemin3 and Gemin5 - A-D: Prospero (green) positive punctate structures colocalise with Gemin3 (red) punctate expression (arrows). E-H: Aberrant Prospero (green) expression also colocalises with Gemin5 (red) punctate structures (arrows). All scale bars: 10μm.

cells divide in the germarium at the anterior end of the ovariole to produce a daughter cell, fated to become the mature egg following development and maturation along the ovariole (67). Throughout this development, nurse cells provide protein and RNA to the developing oocyte (67) (Figure 3.26). Endoreplication is adopted by the nurse cells in order to achieve polyploidy, thereby increasing DNA content to support the accumulation of protein and RNA required for the developing oocyte (68).

During oogenesis, the nurse cells go through 10-12 endocycles. These endocycles however, are not all identical with changes in endocycles regulating nurse cell chromosome morphology (69). During the first 4 endocycles, DNA replication is complete with the polytene euchromatin condensing during the late S-phases of each cycle to produce polytene chromosomes that have a 'blob-like' appearance. Endocycle 5 results in a dramatic change in chromosome structure. During this cycle, late S-phase is incomplete and a state resembling M-phase is achieved in that the 64 chromosomes in each nurse cell nucleus dissociate into 32 sister chromatid pairs held together by unreplicated regions (69). Following chromosome dissociation the nurse cell nuclei have a dispersed appearance. In summary, the nurse cell nuclei begin as condensed polytene nuclei but from stage 6 of egg chamber development become dispersed 32-ploid polytene nuclei (Figure 3.26).

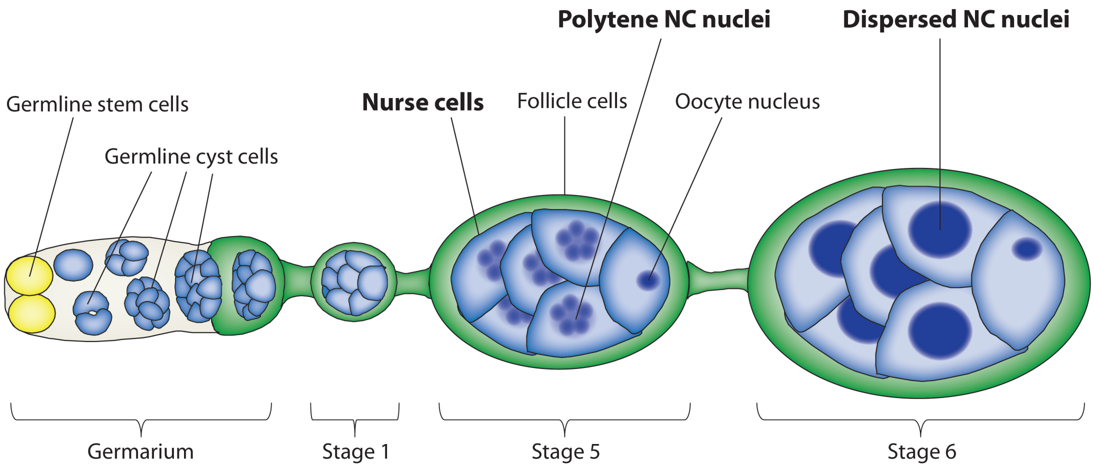


Figure 3.26: Oogenesis and nurse cell nuclear morphology - Germline stem cells (yellow) divide in the germarium to produce a daughter cell (blue) that undergoes 4 sequential mitotic divisions to produce a 2-, 4-, 8- and finally a 16-cell cyst (67). One of the 16 cells in the cyst becomes the oocyte whilst the remaining 15 become polyploid nurse cells (67). The cyst buds off from the germarium forming an egg chamber which develops through 14 stages, with stage 14 being the mature egg; stages 1, 5 and 6 only are depicted here (67). Nurse cell chromosome morphology is dependent on the stage of egg chamber development; prior to, and including stage 5, the nurse cell nuclei have a condensed 'blob-like' organisation, whereas from stage 6 onwards, the nuclei have a dispersed appearance (69). Figure adapted from (67).

3.2.6.1 Nurse cell chromosome morphology is disrupted in *smn* mutant egg chambers

SMN is highly expressed in the female germline. Antibodies against SMN show levels to be particularly high in the germarium in the cytoplasm of germline stem cells. This is consistent with the observation that SMN is expressed in a gradient in developing tissues, with the highest levels in stem cells and lowest in differentiated cell types (48). SMN remains highly expressed in the cytoplasm of wild type egg chambers, observed as both diffuse throughout the cytoplasm and enriched in U-bodies (Figure 3.27)

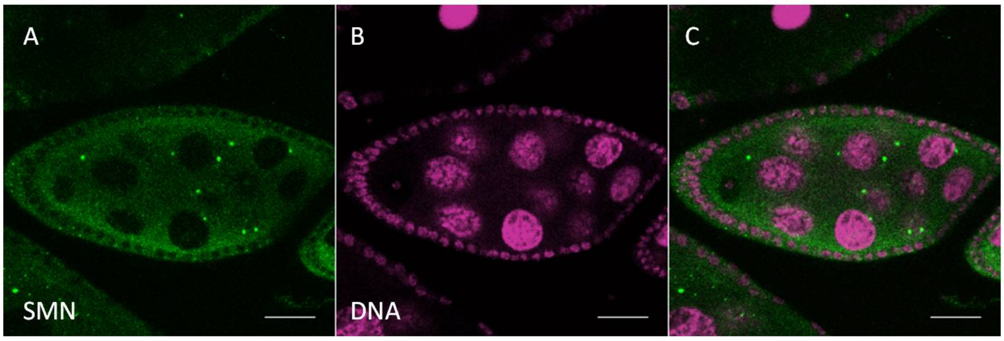


Figure 3.27: SMN is highly expressed in egg chambers - Egg chambers from y,w ovaries. SMN is expressed in egg chambers with enrichment in punctate cytoplasmic U bodies. SMN, green; DNA, magenta. All scale bars: 20μm.

Homozygous smn^{73A0} and smn^{B} mutants are unable to survive past the larval stages due to the exhaustion of maternally contributed SMN. Therefore SMN function in oogenesis cannot be studied in adults without the employment of genetic tools.

Germline clones using the FRT-*ovoD1* system allow the study of homozgous mutant cells in a heterozygous background. The *ovoD1* mutation is dominant negative, resulting in the degeneration of egg chambers that inherit this mutation. Clones are produced by the induction of the yeast FLP-recombinase which is under the control of the hsp20 promoter. The recombination targets (FRT) of this enzyme allow site-specific recombination. Cell that lack *ovoD1* and are homozygous for smn^{73A0} survive and effects of SMN loss can be analysed.

In smn^{73A0} germline clones, the nurse cell nuclei have an aberrant morphology. Unlike wild type nurse cell nuclei which have a dispersed organisation from stage 6 onwards, the nurse cell chromosomes of smn^{73A0} mutant egg chambers remain in a condensed state having a 'blob-like' appearance (Figure 3.28). The nuclei can appear to be doughnut-shaped, with the chromosomes being organised around the periphery of the nucleus. This observation has been previously published by our group using *y,w* as the control strain (33), however, due to concerns regarding the potential involvement of the genetic technique in chromosome morphology aberrations, I have repeated this finding using a germline clone control.

The nurse cell chromosome morphology defect described above was also observed in mitotic clones. This technique resembles the FRT-*ovoD1* system in that it relies on

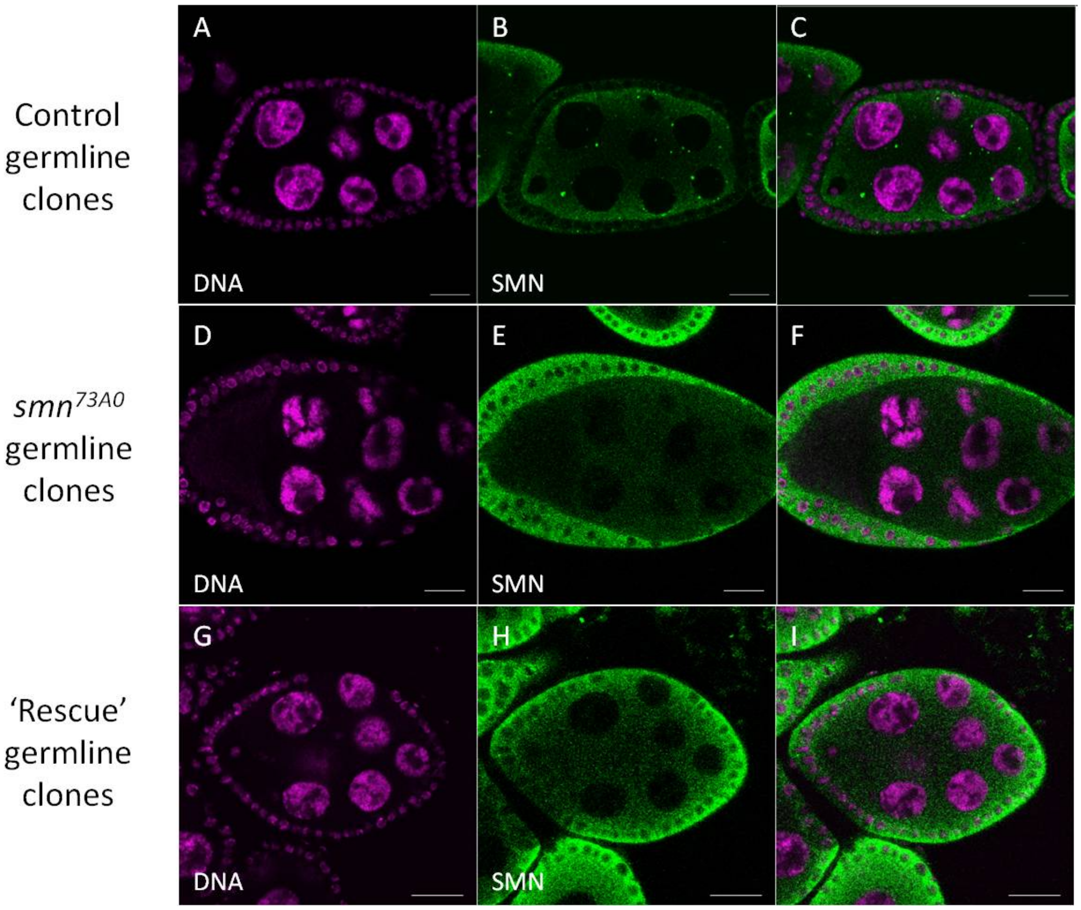


Figure 3.28: *smn* germline clones have aberrant nurse cell chromosome morphology - A-C: Egg chambers from control females show normal chromosome morphology (A) and SMN expression (B). D-F: *smn* germline clones display an aberrant condensed chromosome morphology (D) and decreased levels of SMN (E). G-I: Rescued germline clones regain correct nuclear architecture (G) and SMN levels (H). All scale bars: 20μm.

site-specific recombination controlled by the yeast FLP-recombinase, however, rather than using the degeneration of *ovoD1* cells to distinguish between *smn* homozgous cells and heterozygous cells, GFP is used. Homozygous *smn* egg chambers lack GFP, therefore an advantage of using mitotic clones as opposed to germline clones is that the use of GFP to distinguish between heterozygous and homozgous *smn* mutant egg chambers allows the comparison of mutant and wild type egg chambers in the same animal. Wild-type egg chambers consistently dispersed at stage 6 whereas *smn* egg chambers were unable to correctly perform chromosome dispersal at this stage (Figure 3.29).

The chromosome morphology defects observed in the nurse cells of *smn* germline and mitotic clones indicate that SMN is critical for nurse cell chromosome dispersal.

3.2.6.2 Aberrant nurse cell chromosome organisation can be rescued by exogenous SMN

smn^{73A0} germline clone nurse cell chromosome morphology defects were successfully rescued by the overexpression of a wild-type SMN construct under the control of a tubulin promoter, *tub-smn*. Rescued *smn* germline clones were no longer observed as condensed and 'blob-like' past stage 5 having regained a dispersed nuclear architecture from stage 6 onwards. Also, SMN levels are increased in the 'rescued' egg chambers in comparison to homozygous mutant egg chambers (Figure 3.28). These findings further

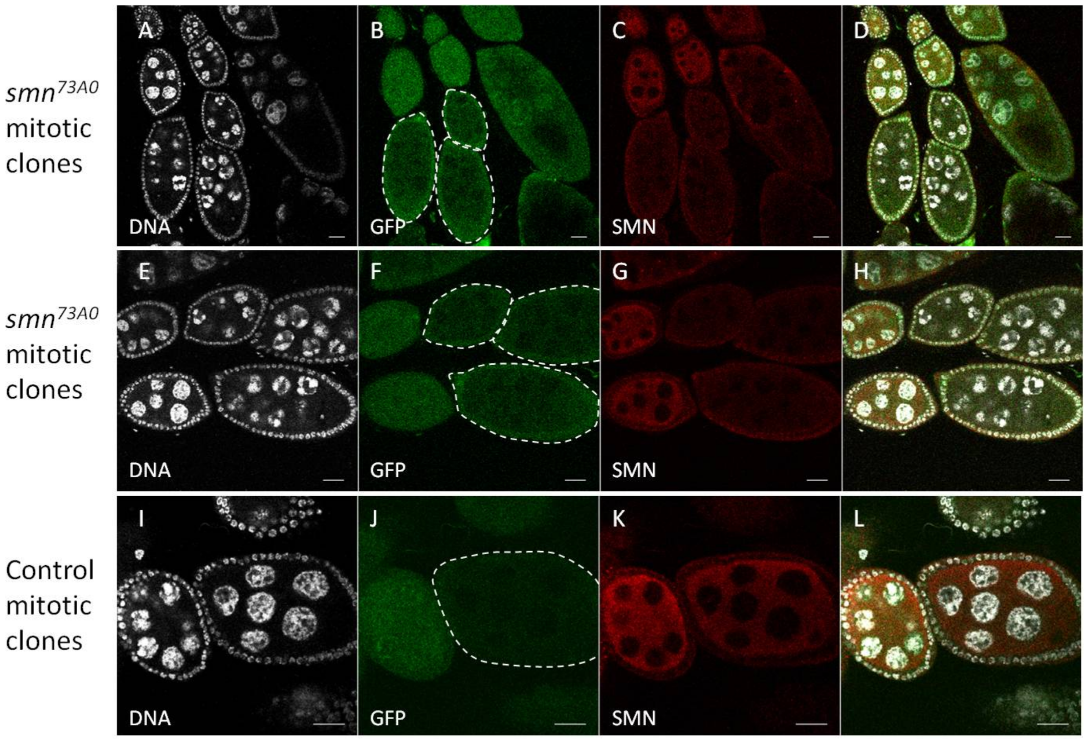


Figure 3.29: *smn*^{73A0} mitotic clones have disrupted nurse cell chromosome organisation - A-D: *smn* mitotic clones. GFP-positive, wild-type egg chambers have normal dispersed nuclear architecture following stage 5 whereas GFP-negative, smn mutant egg chambers retain their condensed appearance. SMN levels in GFP-negative egg chambers, shown by dashed lines, are reduced (C, G). E-H: Zoomed in image of A-D. I-L: Nuclear morphology is normal in both GFP-positive and negative (shown by dashed lines) egg chambers of control clones. SMN levels are also unchanged (K). All scale bars: 20μm.

implicate SMN as a regulator of nurse cell chromosome morphology during oogenesis.

3.2.6.3 Chromatin modification markers in *smn* mutant egg chambers

Chromatin modifications have an impact on chromosome morphology during cell cycle progression. In particular methylation and acetylation of certain histone proteins result in chromosomes having a more condensed or relaxed appearance. I studied the levels of chromatin modification markers in the nurse cell nuclei of *smn* mitotic clones compared to that in wild-type nurse cell nuclei to investigate whether chromatin modification could be influencing the chromosome morphology defect observed.

Histone 3 trimethylation at Lysine 4 and dimethylation at Lysine 9 are indicative of active and inactive chromatin states, respectively. Preliminary results show that in *smn* mitotic clones, H3K4me3 and H3K9me2 markers observed by immunofluorescence do not appear to be significantly changed in their expression. Their localisation is perturbed, with the markers being restricted to the 'blob-like' chromosomes therefore being more prevalent at the periphery of the nuclei in mutant egg chambers (Figure 3.30). This however is likely a consequence of aberrant chromosome morphology rather than an cause.

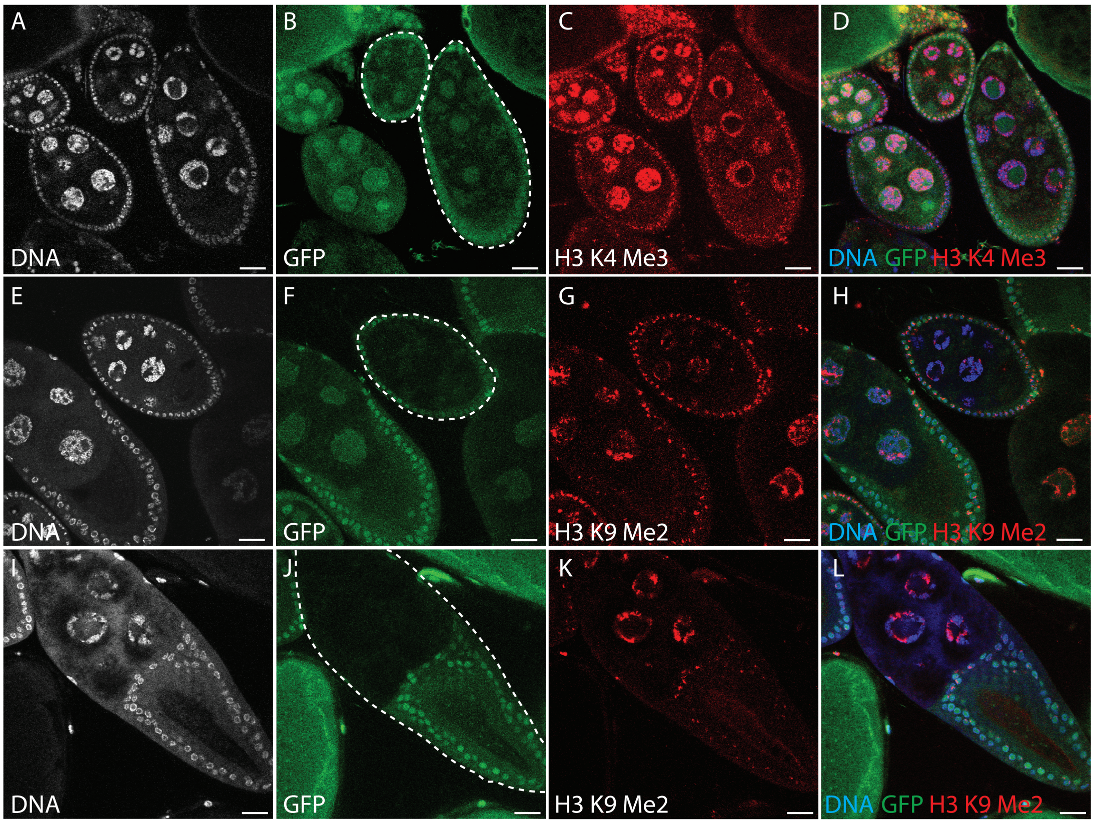


Figure 3.30: H3K4 trimethylation and H3K9 dimethylation in *smn*^{73A0} mitotic clones - A-D:H3K4 trimethylation. H3K4 trimethylation is representative of an active chromatin state. H3K4 Me3 expression is present and is altered in that is remains associated with the blob-like chromosome structures rather than having a dispersed appearance. E-L: H3K9 dimethylation. H3K9 Me2 is predominantly localised to euchromatic regions in *smn* mutant clones. Dashed lines surround GFP-negative, mutant egg chambers. All scale bars: 20μm.

3.3 Discussion

3.3.1 Does SMN function in the growth regulation?

A number of studies have drawn attention to the contribution of disrupted neuromuscular development to SMA pathogenesis. Morphological abnormalities in the neuromuscular system during development as well as altered gene profiles affecting neurodevelopmental pathways in animal models of the disease, preceding symptom onset, implicate imprecise establishment of the neuromuscular system in its eventual breakdown. Recent findings that SMN activity and expression is highest in early development and is downregulated in differentiated cell types similarly suggest a role for SMN in development and interestingly, a particular requirement for high SMN activity in stem cells. It has been my aim to elucidate whether SMN has a role in growth and differentiation in the *Drosophila*. The study of very early developmental processes including the regulation of growth and lineage and cell fate decisions in *Drosophila* may shed some light on how the earliest decisions in development can affect downstream pathways.

3.3.1.1 SMN is enriched in stem cells and mitotically dividing tissues

SMN is enriched in the stem cells of the developing 3^{rd} instar larval CNS, the larval testis and the adult testis. In these systems SMN forms an expression gradient, being more highly expressed in the stem cells and dividing daughters and decreasing in expression as cells become more differentiated. In the testis in particular, a higher density of U bodies is observed in the least differentiated cell types.

The SMN complex member Gemin5 similarly forms an expression gradient that correlates with that of SMN in the cytoplasm and in U bodies. Gemin3 cytoplasmic staining was not evident in the adult testis, however the highly enriched U bodies showed Gemin3 staining that colocalised with SMN. Therefore, in the testis, which presents a range of developmental stages at a particular time, SMN and the SMN complex members Gemin3 and Gemin5, correlate with the developmental gradient, having higher expression levels in the stem cells and dividing cysts and lower expression in more differentiated cell types as determined by immunohistochemistry.

In the larval stage of development, SMN was found to be highly expressed in the mitotically dividing tissues of the larvae; the CNS, wing discs and eye discs. Therefore, it is possible that the diploid tissues of the larvae have a greater requirement for SMN, represented by their higher levels.

3.3.1.2 SMN reduction results in growth and stem cell defects in the CNS

The larval stage of development is a period of rapid growth in which tissues grow by endoreplication becoming polyploid, with the exception of the mitotically dividing imaginal tissues and CNS. *smn* mutants survive until the late larval stages on maternally supplied SMN but do not progress through pupation at the appropriate time, remaining in the larval stage or forming 'pseudo-pupae'. The low levels of SMN however do not support the full growth potential of the larvae and *smn* mutants are reduced in size in comparison to age-matched controls.

Similarly, the larval CNS of smn^B mutants is smaller than that of age-matched controls, therefore SMN loss effects the growth of mitotically dividing tissues as well as the larval body tissues. At a later time point, the CNS of smn^B mutants is equal in size to the CNS of control larvae. This may be due to developmental delay in the mutants or be a reflection of the level of the maternal contribution of SMN. Animals that have a greater maternal supply of SMN may be able to survive for longer, continuing to grow during their extended larval phase.

The growth defect observed in the CNS may be due to defects in cell number, cell size or both. Analysis of the the contribution of cell size showed that that neural cells are significantly smaller in smn^B mutants, therefore, a reduced cell size contributes in part, to the decreased growth.

A striking observation in smn^B mutant 3^{rd} instar larvae is the loss or significant reduction of the OPC of the optic lobes. The OPC contains neuroblasts that are generated during the larval stages of development, at which point maternal SMN is becoming exhausted, unlike the pNBs of the CB and VNC which are derived during the embryonic stage. CB and VNC pNBs are preserved in smn^B mutants at 5 days

AED however after prolonged SMN reduction, a decrease in number is observed. It is possible that threshold SMN levels are required for the generation of the OPC but that the low basal levels of SMN in the late larval stages are sufficient for stem cell survival in the CB and VNC, with only prolonged SMN reduction resulting in loss. The OPC is observed in smn^B mutants at 8 days AED, therefore OPC development may be delayed in *smn* mutants with the late stage selecting out animals having the highest maternal contribution of SMN, sufficient to support OPC development.

Taken together, it appears that SMN is required for stem cell derivation and surivival, however prolonged reduction of SMN is necessary for the loss of existing stem cells. It is necessary to test with an apoptotic marker, whether prolonged SMN reduction results in loss that is specific to the stem cells or whether widespread cell death occurs at this time point. This finding is consistent with SMN having a role in stem cell division and maintenance as shown by SMN being required for germline stem cell survival (48).

3.3.1.3 SMN is required for eye disc proliferation

SMN reduction results in a decrease in cell proliferation, therefore cell number. smn^{73A0} mitotic clones in the eye imaginal disc are unable to proliferate or occasionally form very small clones in comparison to their wild-type twin-spots, therefore the growth defect caused by SMN loss is cell-autonomous in the eye disc. The generation of *smn* mitotic clones in an SMN overexpression background was able to restore clone growth.

In contrast, *smn*^{73A0} mutant clones in the CNS were able to proliferate. *smn*^{73A0} clones in the CNS were observed, whereas *smn*^{73A0} clones in the eye disc were unable to grow in tissue dissected from the same larvae. Given that in the instance of systemic loss in the eye disc and larval CNS, both tissues display reduced growth, although it is possible that SMN loss has a more active role in growth in the eye, I believe it is more likely that the discrepancies in clone growth between the two tissues reflects differing regulation of growth competition in these tissues. Growth competition refers to the active removal of slowly proliferating cells when positioned near neighbouring faster growing cells (70). Cell competition may be more acute in the eye imaginal discs, with slow growing *smn* clones eliminated more efficiently than in the CNS. Alternatively, trophic influences provided by wild-type tissue in the CNS may support the growth of mutant clones in this tissue.

3.3.2 SMN regulates cellular differentiation in specific tissues

Modification of SMN levels has been previously shown to have a dramatic effect on cell differentiation in the testis (48). I analysed whether the regulation of differentiation by SMN was specific to the testis or more widespread, regulating the differentiation of neural tissues. The production of all neural cell types and importantly the timing of their differentiation is crucial for the establishment and maintenance of the neuromuscular system. If the reduction of SMN results in defects in differentiation and cell fate decisions in the developing CNS, this may have major consequences for the establishment and maintenance of the neuromuscular system. To investigate neural cell differentiation, I used the eye imaginal disc as a model since it contains both neurons and glia and has an extremely well defined pattern of differentiation.

Eye differentiation and development was unaffected upon altering SMN levels by RNAi or overexpression. It is possible that high levels of SMN expression is important at particular time points of development and that moderate alterations in levels are well-tolerated outside of these stages. Flip out clones generated during the early larval stages may not reduce SMN to the required threshold levels, or at the correct time, in order to produce differentiation defects. Similarly, moderate modification of SMN levels in post-mitotic cells may not result in defects, in contrast to alterations in pre-mitotic cells. SMN overexpression and RNAi using an eye-specific pre-mitotic driver however did not result in observable defects.

When considering loss of SMN function in contributing to SMA pathogenesis, the extent of SMN reduction is an important factor. In humans, low levels of functional SMN protein is produced by the SMN2 gene, therefore in individuals with SMA low basal levels of SMN remain. However, SMN levels must be depleted by approximately

80 percent before manifestation of the disease. It is possible that the levels reached by RNAi do not meet these threshold targets. It was surprising that SMN RNAi clones were able to proliferate in contrast to mutant clones, potentially due to an insufficient knockdown of SMN.

However, it must also be considered that SMN does not function as a master regulator of differentiation, rather its role is tissue specific. Although the mechanisms of differentiation of tissues in *Drosophila* contain universal signalling molecules and rely on many common regulators, the intricacies of differentiation in different tissues involve distinct regulatory pathways. SMN may regulate a testis specific mechanism of differentiation and is not required for the control of eye disc differentiation.

3.3.2.1 Neural stem cell defects are observed in *smn* mutants

Sustained SMN loss resulted in mislocalised Prospero protein expression, being expressed in punctate inclusions in the pNBs. Interestingly, the mislocalised Prospero was found to colocalise with the SMN complex members, Gemin5 and Gemin3 in punctate structures in the pNB. The identity of these punctate structures is unclear however preliminary data (not shown) shows that these structures do not colocalise with Coilin, Lsm11 or Me31B, therefore do not correspond to P bodies or the SMN containing Cajal bodies and U bodies.

The adaptor protein, Miranda, that tethers *prospero* mRNA to the basal side of the pNB during asymmetric division, is frequently mislocalised in *smn*^{73A0} and *smn*^{B} mutant pNBs (48). This may result in the mislocalisation of Prospero observed in *smn*^{B} neuroblasts. Given the function of Prospero in repressing the cell cycle and inducing differentiation, the aberrant localisation of Prospero may contribute to a reduced proliferative capacity of smn mutant neuroblasts.

In summary, SMN functions in growth regulation in *Drosophila* larvae as displayed by the larval growth and developmental defects discussed in this study. SMN has previously been shown to be involved in cell differentiation in the testis however differentiation defects were not observed in the eye in this study, suggesting that its role in differentiation is tissue specfic.

3.3.3 Does SMN function in endocycle regulation in the *Drosophila* female germline?

The major players in endocycle regulation display nurse cell chromosome morphology defects upon mutation. The nurse cell nuclei phenotypes observed in CycE, APC/C, DAP and E2F1 mutants have distinct differences between them, but are common in that they all result in a condensed chromosome morphology that is retained past stage 6 of oogenesis (71)(72)(73)(74). The mutations result in an inability to switch from a condensed to dispersed nuclear morphology reflecting aberrant transition from the polytene endoreplicating cycles during the first stages of oogenesis through the pseudomitotic cycle of endocycle 5 to polyploid polytene endocycles.

smn germline and mitotic clones also retain a condensed chromosome organisation in nurse cell nuclei following the polytene-polyploid transition at endocycle 5. This aberrant condensed chromsome morphology may similarly be the result of an inability of SMN deprived nurse cells to enter or complete the pseudomitosis that occurs at this stage of oogenesis.

Given the similarities in the nurse cell nuclear morphology in *smn* mutants and mutations in endocycle regulators, it is possible that SMN plays a role in endocycle regulation, either directly or indirectly, at the polytene-polyploid switch. Cyclin E and Dacapo levels were investigated in this study however the findings were inconclusive and must be investigated further. It would be interesting to investigate defects in endocycle regulators in *smn* germline and mitotic clones.

3.3.3.1 SMN regulates nurse cell chromosome organisation through the U body-P body pathway?

SMN is essential for small nuclear ribonucleoprotein, snRNP, assembly, functioning in assembling small nuclear RNAs, snRNAs, onto their associated Sm proteins. snRNPs are the constituents of the both the major and minor spliceosome and therefore in turn, SMN is required for pre-mRNA splicing. Interestingly, other RNA regulatory proteins have been shown to regulate nurse cell chromosome morphology, including *fs(2)B*, *ovarian tumour (otu)*, *squid*, *cup*, *half pint* and *hrb27C* (75) (76) (77). Mutations in these genes result in the common phenotype of nurse cell nuclei that are unable to switch from condensed, polytene structures to dispersed, polytene-polyploid chromosomes, instead retaining a condensed polytene morphology. The chromosome morphology defects observed in *smn* germline and mitotic clones phenocopies that of these RNA regulatory proteins.

ovarian tumour (otu), *squid*, *cup*, *half pint* and *hrb27C* have been implicated in a common pathway in *Drosophila* oogenesis in *gurken* RNA localisation and nurse cell chromosome dispersal (75). This pathway presents another potential method through which SMN could regulate chromsome morphology during oogenesis.

In support of this hypothesis is the interaction between processing bodies (P bodies) and U bodies in the egg chamber (33). P bodies are punctate subcellular structures that function in mRNA decay, surveillnace, translational repression and RNA silencing (33) (78) (79). U bodies similarly are punctate cytoplasmic structures and are sites of SMN enrichment. The function of U bodies in the cell is unclear however it has been suggested that they act as sites of snRNP assembly or storage. It has been observed that P bodies often physically associate with U bodies whereas U bodies invariably interact with P bodies (33) (80).

Cup and Otu both localise to P bodies in egg chambers. Upon SMN mutation however, P body distribution in egg chambers is disrupted, visualised by Cup and Otu as well as other P body components. Conversely, U bodies are disrupted in *cup* and *otu* mutant egg chambers (33). Given the relationship between U bodies and P bodies, the disruption of their patterns in U body and P body mutants, taken together with the nurse cell chromosome morphology defect shared between mutants of particular U body and P body components, it is feasible that these subcellular bodies act in a common pathway to regulate chromosome morphology.

A model for the action of Squid, Otu and Hrb27C in the control of chromosome dispersal has been put forth in which they form an RNP complex that functions in the processing, localisation and translational regulation of an unknown RNA target (75). This RNP complex therefore regulates the unknown RNA target both spatially and temporally to ensure correct chromosome dispersal during oogenesis.

SMN functions in snRNP assembly but has been implicated in the organisation of other RNP complexes. A potential role for SMN in this pathway could be in the assembly of the Squid, Otu, Hrb27C, unknown RNA RNP complex.

3.3.3.2 Function of chromatin modifications in chromosome morphology changes

Chromatin modifications control chromosome morphology by acting as biding sites for proteins that regulate chromatin structure (81) (82). Chromatin compaction is an important factor in gene regulation as it determines the accessibility of the DNA to the transcriptional machinery. Therefore more compact chromatin, heterochromatin, results in a transcriptionally 'silent' state whereas less compacted chromatin, euchromatin, is transcriptionally 'active'. Histone modifications, particularly lysine methylation and acetylation, are an important mechanism of regulating chromatin organisation.

In *Drosophila*, heterochromatin silencing is dependent on small RNA regulation (83). Mutations in *piwi* and *aubergine*, the germline-specific RNAi machinery results in a partial loss of H3K9 methylation, a marker of a transcriptionally 'inactive' state. Piwi and Aubergine expression levels are reduced in *smn* germline and mitotic clones and piRNA regulated transposon silencing is disrupted (Jieni Cao, personal communication). Therefore decreased levels of SMN may indirectly control chromatin organisation via methylation regulation by the piRNA proteins Piwi and Aubergine.

H3K9 methylation in nurse cell nuclei, however, was not obviously disrupted in *smn* mitotic clones, as was the case for H3K4 methylation. Further work regarding the role of SMN in chromatin modification must be carried out to fully investigate this prospect.

3.3.4 Summary

In humans, mutations or deletions in the housekeeping protein SMN results in the motor neuron-specific disease SMA, that causes muscle atrophy (23). Whether this disease is due to neurodegeneration of aberrant development is a point of contention in the field, and the observation of extra-neuromuscular abnormalities in SMA patients including congenital heart (50) and bone (51) defects has raised the question of whether reductions in SMN levels may result in more widespread developmental defects.

The effect of SMN depletion on neuromuscular development in *Drosophila* has been investigated, with a reduction in SMN most prominently resulting in aberrant NMJ structure and function (17) (40) (18). The role of SMN in development however, with the exception of muscle, has not been extensively studied in *Drosophila*. More recently SMN has been implicated in growth and differentiation in *Drosophila* (48) and mouse (49) models, and growth pathways have been found to modify SMN-dependent phenotypes in *Drosophila* (18)(54) (58). Therefore, in this chapter I aimed to extend our understanding of role of SMN in growth and differentiation in developing *Drosophila* tissues.

SMN was found to be highly expressed in dividing tissues and SMN mutation resulted in growth defects including a reduction in cell size and proliferation, neural stem cell abnormalities and larval developmental delay. Modification of SMN levels has been previously shown to result in aberrant differentiation in the testis in *Drosophila* (48), however SMN depletion did not cause defects in differentiation in the developing eye, therefore the role of SMN in differentiation appears to be tissue specific. Finally, SMN was shown to be highly expressed in the developing egg chambers of the female germline and the results described in this chapter implicate SMN in the regulation of the chromosome morphology of the endocycling nurse cells in the ovary.

In addition to my work on the function of SMN in *Drosophila*, I have used the *Drosophila* model organism and the genetic techniques described in this thesis to investigate a putative modifier of a *Drosophila* model of Parkinson's disease that will be discussed in Chapter 4.

Chapter 4

Nedd4, a putative modifier of a *Drosophila* model of Parkinson's disease

In this chapter I will discuss my work on Parkinson's disease that formed part of a collaborative project with George Tofaris. Previous work has implicated the aggregation of α-synuclein protein as an important mechanism in Parkinson's disease pathogenesis and in turn α-synuclein degradation pathways as potential avenues for therapy. More recently, the E3 ubiquitin ligase, Nedd4, has been shown to affect α-synuclein clearance in yeast and human cell lines (84). In this study I have used a previously characterised *Drosophila* model of Parkinson's disease to investigate whether Nedd4 is able to affect α-synuclein-dependent phenotypes in an animal model.

4.1 Introduction

4.1.1 Clinical features and etiology of Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder. In contrast to SMA, which generally manifests earlier in life, PD is a disease of later life affecting 1% of the population over the age of 65 (85). The clinical features of the disease are the loss of dopaminergic neurons from the substantia nigra (SN) pars compacta and the presence of intraneuronal cytoplasmic inclusions called Lewy bodies (85). The striatal dopamine deficiency that results from the loss of dopaminergic neurons causes the motor function defects typical of PD, including tremor at rest, rigidity and bradykinesia (86) (85). In addition to the movement disorder, patients also experience non-motor symptoms such as depression, sleep disturbance and cognitive decline (86).

The events characterising the pathogenesis of PD are not fully understood. Unlike SMA which is a monogenic disease, the etiology of PD is multifactorial and a number of genes have been implicated in its pathogenesis. Importantly, neuropathological and genome-wide association studies have implicated some of these genes in sporadic PD, which accounts for the majority of cases (85) (87). Therefore the study of known mutations in familial cases may shed light on the mechanisms of pathogenesis in sporadic forms of the disease. There is a body of evidence implicating the role of protein misfolding and aggregation as an important factor in PD pathogenesis.

4.1.2 Protein misfolding and aggregation in PD pathogenesis

Lewy bodies are eosinophilic cytoplasmic structures that are defining neuropathological features of PD (88). The primary constituent of Lewy bodies is aggregated α-synuclein protein (88) (89).

α-synuclein was the first PD gene to be discovered, with missense mutations and gene triplication resulting in autosomal dominant familial PD (86). α-synuclein is expressed throughout the nervous system and is thought to function in synaptic vesicle function (90). Both wild-type and mutant forms of α-synuclein have a propensity to aggregate, due to a central hydrophobic region (85), however it has been proposed that mutated forms may do so more readily through increased misfolding (89).

Parkinson's disease is not unique in featuring protein aggregates in its pathology; a number of neurodegenerative diseases are proposed to result from aggregate formation including Alzheimer's disease, Huntington's disease and ALS. These observations implicate proteotoxic stress in disease progression. The formation of proteinaceous inclusions may cause neurotoxicity by physically deforming the cell or via the disruption of normal cell processes such as intracellular trafficking (89).

The primary cause of protein accumulation in the case of familial forms of PD in which α-synuclein is mutated may result from mutated forms of the protein having a greater propensity for aggregation than the wild type protein; mutant α-synuclein has been shown to result in increased toxicity in *Drosophila* (20) and mutations found in familial PD cause enhanced aggregation *in vitro* (91).

In the case of sporadic PD the cause of proteotoxic insult is unclear. Protein modifications resulting in increased misfolding may be caused by oxidative stress. Lewy bodies contain oxidatively modified α-synuclein that aggregates more readily *in vitro* (92). Aberrant oxidised protein content increases with age as does protein clearance dysfunction (89). Therefore increased protein misfolding in combination with impaired protein degradation may result in the accumulation of toxic protein species with increasing age.

Although α-synuclein-containing Lewy bodies are characteristic neuropathological features of PD, the role of Lewy body formation in disease progression is contentious. Much speculation surrounds the identification of the precise nature of the toxic α-synuclein species; whether Lewy body formation is pathogenic, an epiphenomenon or even protective, remains unclear (Figure 4.1).

An animal model of PD has been developed in which rats were injected with a recombinant adenoassociated viral vector containing human wild-type or mutant α-synuclein directly into the substantia nigra pars compacta (93). This resulted in the progressive appearance of α-synuclein-containing cytoplasmic inclusions, the larger of

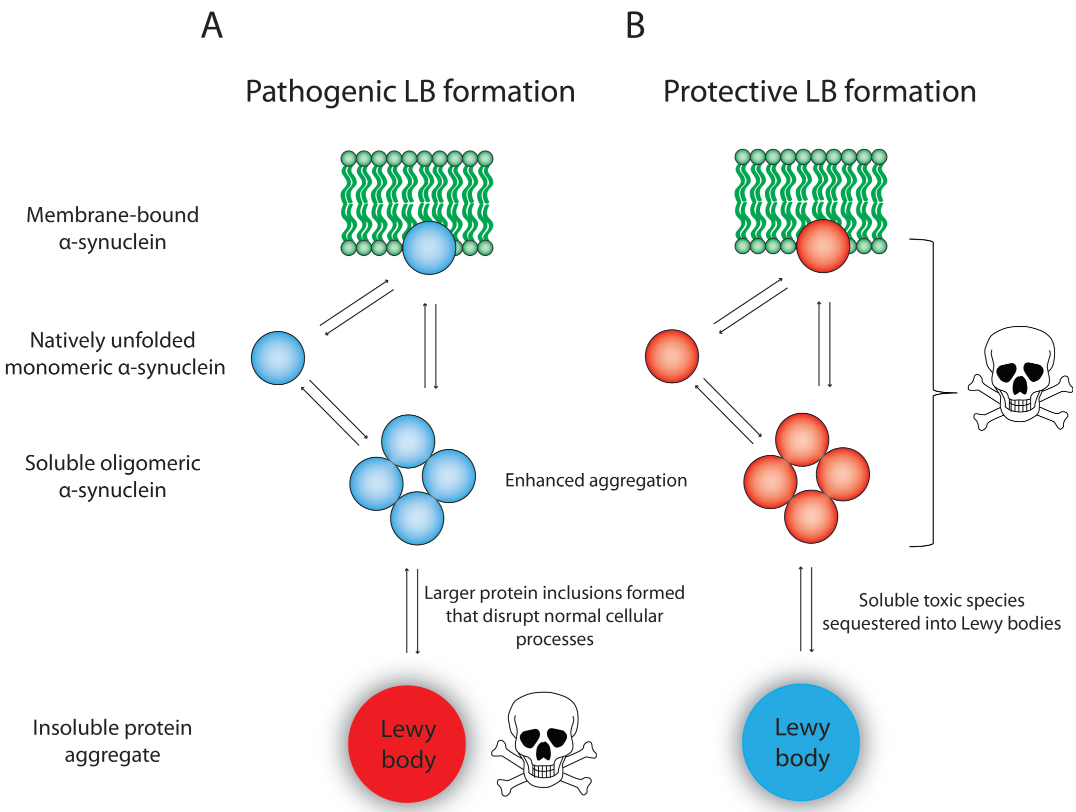


Figure 4.1: Lewy body formation: pathogenic or protective? - The presence of α-synuclein-containing Lewy bodies (LBs) is a defining feature of PD, however whether Lewy bodies are pathogenic is unclear. A. LBs as toxic species: α-synuclein may be membrane-bound, in a soluble unfolded monomeric state or soluble oligomeric states (shown in blue) due to the propensity of α-synuclein to aggregate. In the diseased state, enhanced aggregation leads to the formation of large protein inclusions i.e. Lewy bodies (red), that are pathogenic and disrupt normal cellular processes. B. LB formation as defensive: toxic soluble species (red) are actively sequestered into Lewy bodies (blue) thereby protecting cells from toxic α-synuclein species.

which resemble Lewy bodies (93). This aggregation correlated with the selective loss of dopaminergic neurons from the substantia nigra strongly implicates α-synuclein aggregation in neurotoxicity (93).

The human α-synuclein protein contains a central hydrophobic region called the non-β-amyloid component (NAC) that has been shown both *in vitro* (94) and *in vivo* in *Drosophila* (95) to be required for α-synuclein aggregation. Conversely, the acidic C-terminus of the protein appears to prevent aggregation since C-terminally truncated forms of the protein aggregate more readily *in vivo* (95). Using a *Drosophila* PD model (20), the relative effect on toxicity of individual protein domains has been investigated (95). *Drosophila* pan-neuronally overexpressing α-synuclein with a deletion in the NAC region, α-synuclein$^{\Delta 71-82}$ and a C-terminally truncated form, α-synclein$^{1-120}$ were analysed for loss of the dorsomedial dopaminergic neurons (20).

In aged flies, animals overexpressing the wild-type form of α-synuclein displayed DA neuron loss. This reduction was not observed in flies overexpressing α-synuclein with a deletion in the NAC region suggesting that this region is important for neurotoxicity (95). Interestingly, inclusions were absent upon disruption of the NAC domain and high molecular weight aggregates could not be observed by Western blotting (95). Therefore the loss of extensive α-synuclein aggregation was found to correlate with abrogation of dopaminergic neuron loss thereby implicating inclusion formation in neurotoxicity.

Conversely, C-terminal truncation of α-synuclein resulted in a modest increase in dopaminergic neuron toxicity that was accompanied by a significant increase in inclusion formation and the production of high molecular weight α-synuclein species (95). Taken together, the results of this study strongly implicate protein aggregation and inclusion formation in neurodegeneration.

In contrast, phosphorylation at Ser129 has been shown to increase α-synuclein oligomer formation and enhance dopaminergic neuron loss in *Drosophila*, whilst Tyr125 phosphorylation reduced the levels of soluble oligomeric α-synuclein (96). The substitution of Ser129 to an alanine residue completely abrogated dopaminergic neuron loss whilst the blocking of Tyr125 phosphorylation enhanced both dopaminergic neuron loss and locomotor dysfunction (96).

Although phosphorylation at Ser129 results in an increase in soluble oligomeric species of α-synuclein, blocking of this phosphorylation, as well as protecting against neuron loss, significantly increases aggregation and inclusion formation (97). This result may provide an explanation for the inconsistencies in the findings regarding protein aggregation and toxicity in *Drosophila* PD models. α-synuclein aggregation has been shown to both rescue and enhance neuronal degeneration in *Drosophila* models. It is possible that whilst the production of large aggregates and inclusions are protective, smaller aggregated α-synuclein species are toxic and result in neurodegeneration (95)

(Figure 4.1 B).

In support of this, *Drosophila* models overexpressing the chaperone protein, Hsp70, abrogated dopaminergic neuron loss in the absence of a decrease in inclusion formation thereby implicating soluble misfolded forms of α-synuclein in toxicity rather than insoluble aggregated forms (98).

However, regardless of the specific identity of the toxic α-synuclein species, the presence of Lewy bodies in sporadic PD, the correlation of α-synuclein levels with disease severity in cases with α-synuclein gene multiplications, and the fact that overexpression of α-synuclein has been shown to be sufficient for neurodegeneration in animal models (85) suggests that promoting α-synuclein clearance is likely to be an important process in disease prevention, and identification of factors involved in aggregate degradation essential for the development of putative therapeutic targets.

4.1.3 Methods of α-synuclein clearance

There are 2 protein degradation systems in cells; proteasomes and lysosomes. Inappropriately folded proteins are targeted for destruction by the proteasome via ubiquitination. Ubiquitination involves the coordination of 3 classes of enzyme; E1, ubiquitin-activating enzymes, E2, conjugation enzymes and E3, ubiquitin ligases described in Figure 4.2. E3 ligases fall into two categories, RING E3 ligases and HECT E3 ligases. Members of the HECT family of E3 ubiquitin ligases are able to directly catalyse transfer of ubiquitin onto the substrate (99). The attachment of at least four ubiquitin monomers to the substrate is required for targeting for degradation therefore additional ubiquitin molecules are attached to Lys residues on the ubiquitin monomers to form polyubiquitin chains (100). The ubiquitinated substrate is then transported to the 26S proteasome for degradation.

Inactivation of the 26S proteasome in the substantia nigra has been shown to cause the accumulation of α-synuclein-positive aggregates (102). The PSMC1 subunit of the 26S proteasome is essential for function (102) and knock-out of this gene has been shown to result in 26S proteasome depletion in mice (102). Proteasomal inactivation of *Psmc1* specifically in the substantia nigra using the Cre-Lox system in mice results in neurodegeneration that is accompanied by the formation of Lewy body-like inclusions that are positive for both ubiquitin and α-synuclein, implicating ubiquitination and targeting to the 26S proteasome in α-synuclein clearance (102). Consistent with 26S proteasome function in α-synuclein clearance, α-synuclein has been shown to interact with the proteasome and mutation or overexpression to inhibit proteasomal function (89) (103) (104). Proteasomal inhibitors, including environmental toxins have been shown to impair UPS function (105) (106) and cause parkinsonian phenotypes, including neurotoxicity (100).

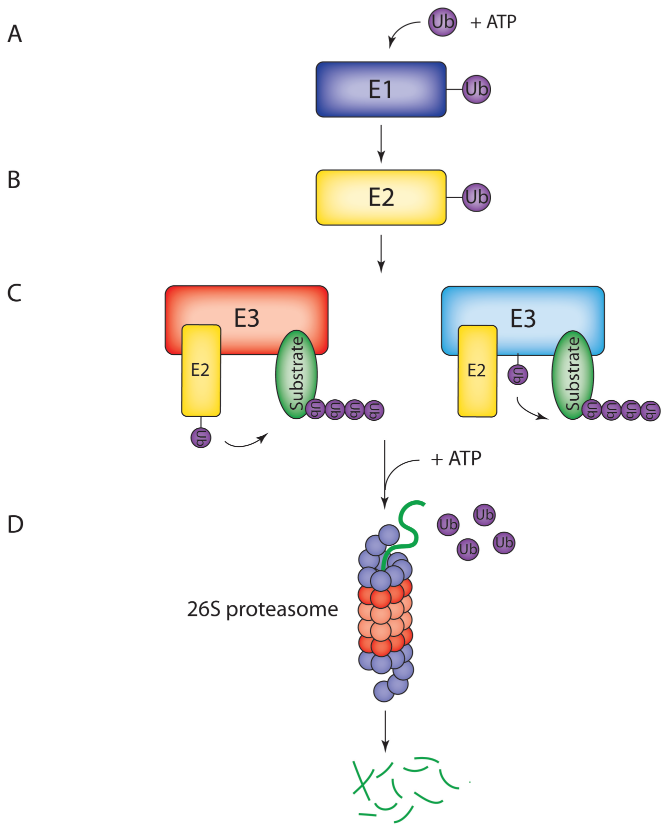


Figure 4.2: Ubiquitin proteasome system - A. Ubiquitin is attached via its C-terminal glycine residue to a cysteine residue in an E1 ubiquitin-activating enzyme. B. The ubiquitin moiety is then transferred to the E2 ubiquitin-conjugating enzyme. C. E3 ubiquitin-ligases confer substrate specificity. The E3 binds to the protein substrate destined for ubiquitination and ubiquitin is transferred from E2 to an internal lysine residue in the substrate. The formation of this isopeptide bond is catalysed by E2 in conjunction with an E3 ligase or directly by E3 (99). D. The ubiquitinated substrate is then transported to the 26S proteasome for degradation. Figure adapted from (101)

Given the effect of impaired proteasomal function on α-synuclein levels and neurotoxicity, it appears that α-synuclein is degraded, at least in part, by ubiquitination and its consequent targeting to the 26S proteasome.

There is evidence that ubiquitin-*independent* proteasomal degradation contributes to α-synuclein clearance. Proteasome inhibition in a dopaminergic neuroblastoma cell line resulted in the accumulation of non-ubiquitinated α-synuclein and increased observation of ubiquitin-negative α-synuclein-containing inclusions (107). α-synuclein was found to be efficiently degraded by the proteolytic 20S particle in the absence of ubiquitination (107).

It has been proposed that the ubiquitination of α-synuclein present in Lewy bodies is a disease-specific event (108). In diseased tissue, a modified 22-24kDa α-synuclein species was found to undergo ubiquitination whereas the monomeric form was not, despite normal proteasomal function. A mechanism was suggested in which the accumulation of the modified species in the diseased state overpowers ubiquitin-independent proteasomal degradation resulting in α-synuclein ubiquitination and inclusion formation (108) (Figure 4.3).

There is much evidence implicating the proteasome as a component of α-synuclein degradation however the mechanism of degradation in normal and diseased states remains contentious.

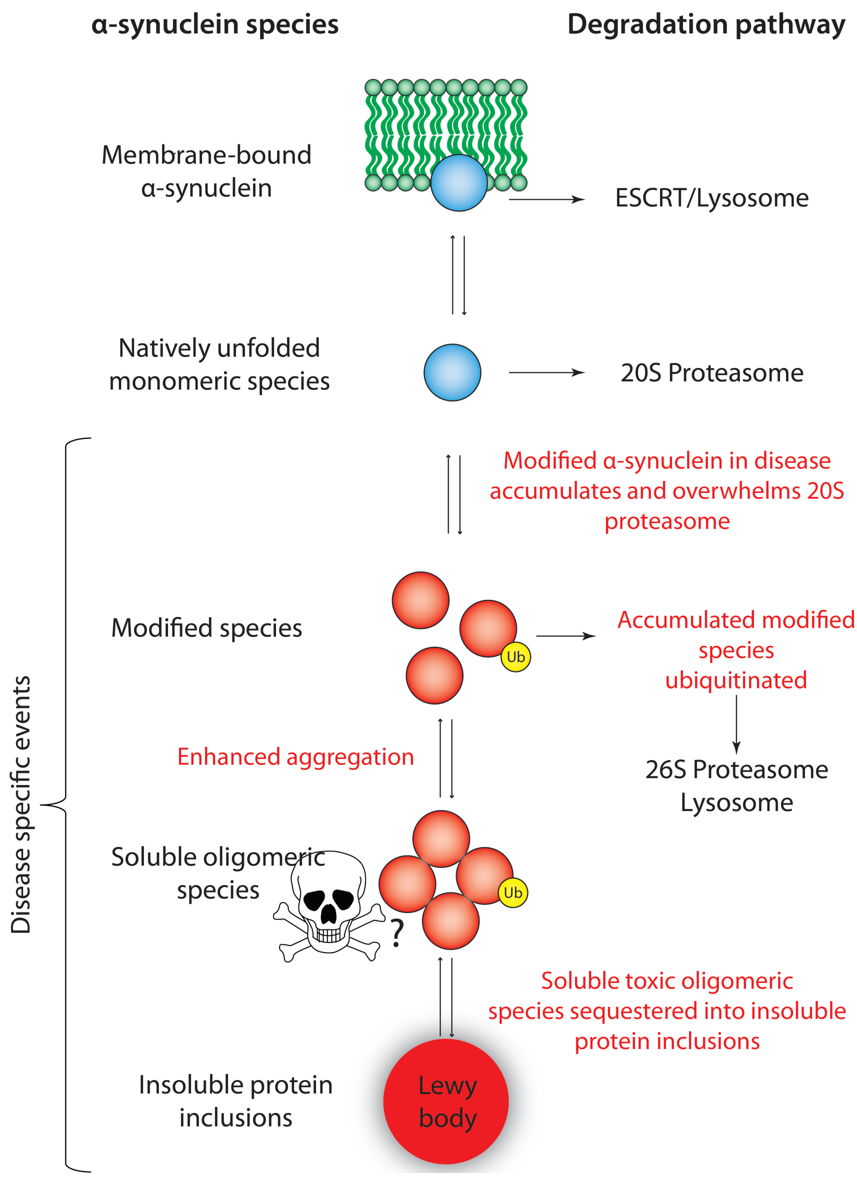


Figure 4.3: Proposed roles of degradation pathways in α-synuclein clearance - Membrane bound α-synuclein is targeted to the lysosome for degradation by ubiquitination and the ESCRT pathway. Under normal conditions, unfolded monomeric α-synuclein is efficiently degraded by the proteolytic 20S particle in the absence of ubiquitination. However in diseased tissue, a modified α-synuclein species is unable to be efficiently cleared by the 20S proteasome and is subsequently ubiquitinated targeting for degradation by the 26S proteasome or lysosome. Enhanced aggregation results in the formation of toxic oligomeric species and the sequestration of toxic species into Lewy bodies.

A second proteolytic system employed in protein clearance is the lysosome. Proteins may be delivered to lysosomes via macroautophagy, microautophagy, chaperone-mediated autophagy (103) (Figure 4.4) and the endosomal pathway. Microautophagy involves the bulk capture of cytosolic regions by the lysosomal membrane (103). Similarly, macroautophagy results in sequestration of bulk regions of the cytosol, however, in this case via the formation of a *de novo* vesicle called an autophagosome (103). CMA is a selective form of autophagy in which substrates are recognised by molecular chaperones and targeted to the lysosome for degradation.

The endosomal sorting complex required for transport (ESCRT) functions in the endocytic internalisation of ubiquitinated membrane proteins and their subsequent endosomal sorting (109). Substrate ubiquitination in the targeting of proteins for proteasomal destruction is well characterised. However, ubiquitination by specific E3 ubiquitin ligases also targets specific substrates to the lysosome for degradation. Substrates can be monoubiquitinated, multi-monoubiquitinated or polyubiquitinated. Ubiquitin contains 7 lysine residues, each of which can form isopeptide bonds which other ubiquitin moieties to form polyubiquitin chains. The type and extent of substrate ubiquitination determines its fate; polyubiquitin chains linked through lysine residue 48 signals for proteasomal degradation whereas muliti-monoubiquitination and lysine-63 linked polyubiquination signals membrane-associated proteins for endosomal sorting

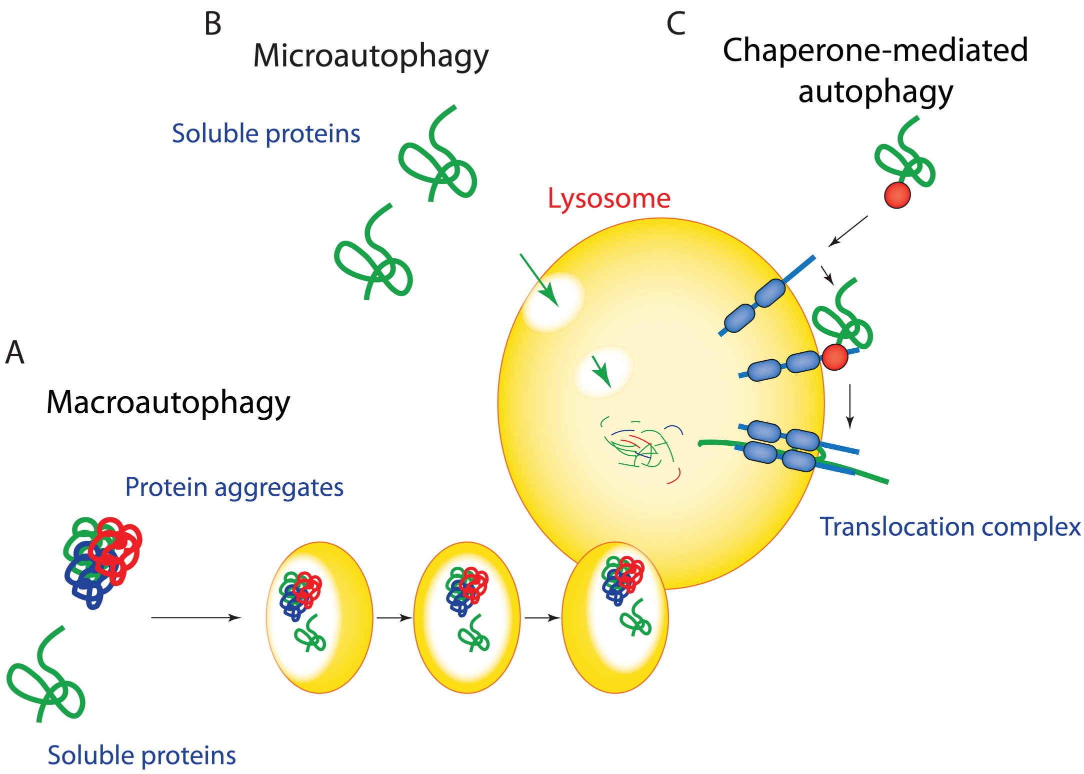


Figure 4.4: Autophagic pathway - The autophagic pathway targets substrates to the lysosome for degradation to di- and tri-peptides and free amino acids. Substrates are delivered to the lysosome by the following routes: A. Macroautophagy results in sequestration of bulk regions of the cytosol via the formation of a *de novo* vesicle called an autophagosome. The autophagosome fuses with the lysosome exposing its contents to the proteolytic machinery. B. Microautophagy involves the bulk capture of cytosolic regions by the lysosomal membrane. C. Finally chaperone-mediated autophagy, CMA, is a selective form of autophagy in which substrates are recognised by molecular chaperones and targeted to the lysosome for degradation. Substrates are also targeted to the lysosome via the endosomal pathway, not shown in this diagram. Figure adapted from (101)

and transport to the lysosome (109).

In the endosomal pathway, specific E3 ubiquitin ligases ubiquitinate aberrantly folded proteins at the plasma membrane or membrane-associated receptors. This results in their internalisation by endocytosis and transportation to the lysosome inside multivesicular endosomes (MVEs) (109). In the endosomal membrane, the ESCRT machinery specifically targets ubiquitinated substrates and prevents their trafficking back to the plasma membrane. Following substrate capture, it is deubiquitinated and sequestered into invaginations in the endosomal membrane formed by ESCRT components. The continued invagination of the endosomal membrane forms an intraluminal vesicle (ILV) which breaks off containing the selected cargo. Upon MVE fusion with lysosomes, the ILV membrane is degraded exposing the sorted cargo to lysosomal proteases (109).

α-synuclein appears to be degraded by both the proteasome and lysosome. It is possible that these pathways may degrade different types of protein species and may be involved to differing extents in the diseased state (Figure 4.3). The clearance of the soluble pool of α-synuclein can occur via CMA-mediated transport to the lysosome and by proteosomal degradation. Aggregated forms of the protein however, that cannot be unfolded are cleared by macroautophagy (105).

A recent study has identified ESCRT targeting of ubquitinated α-synuclein for lysosomal degradation as another mechanism of α-synuclein clearance in addition to proteasomal degradation, macroautophagy and CMA (84). Nedd4 has been identified as the E3 ubiquitin ligase required for α-synuclein ubiquitination and consequent degradation via the endosomal-lysosomal pathway (84).

4.1.4 Nedd4 is a potential modifier of alpha-synuclein

Neuronal precursor cell-expressed developmentally downregulated gene 4, or Nedd4, is a member of the Nedd4 family of HECT E3 ubiquitin ligases. Phylogenetic analysis of Nedd4 family members within and across species show that they are closely related (110)(Figure 4.5). Members of this family are generally involved in the ubiquitination and endocytosis of membrane-associated proteins (99).

α-synuclein contains a C-terminal proline-rich motif, implicating it as a substrate for HECT E3 ligases such as Nedd4. This was tested by incubating recombinant human Nedd4 with recombinant α-synuclein in the presence of E1, the E2 UbcH5, ubiquitin and ATP (84). Nedd4 was found to ubiquitinate recombinant α-synuclein as shown by the identification of oligo-ubiquitinated and poly-ubiquitinated species using antibodies against α-synuclein (84). In contrast, the other Nedd4 family members, Nedd4-2, SMURF1 and SMURF2 were unable to ubiquitinate α-synuclein (84). The deletion of only the C-terminal α-synuclein region bearing the proline-rich motif resulted disrupted

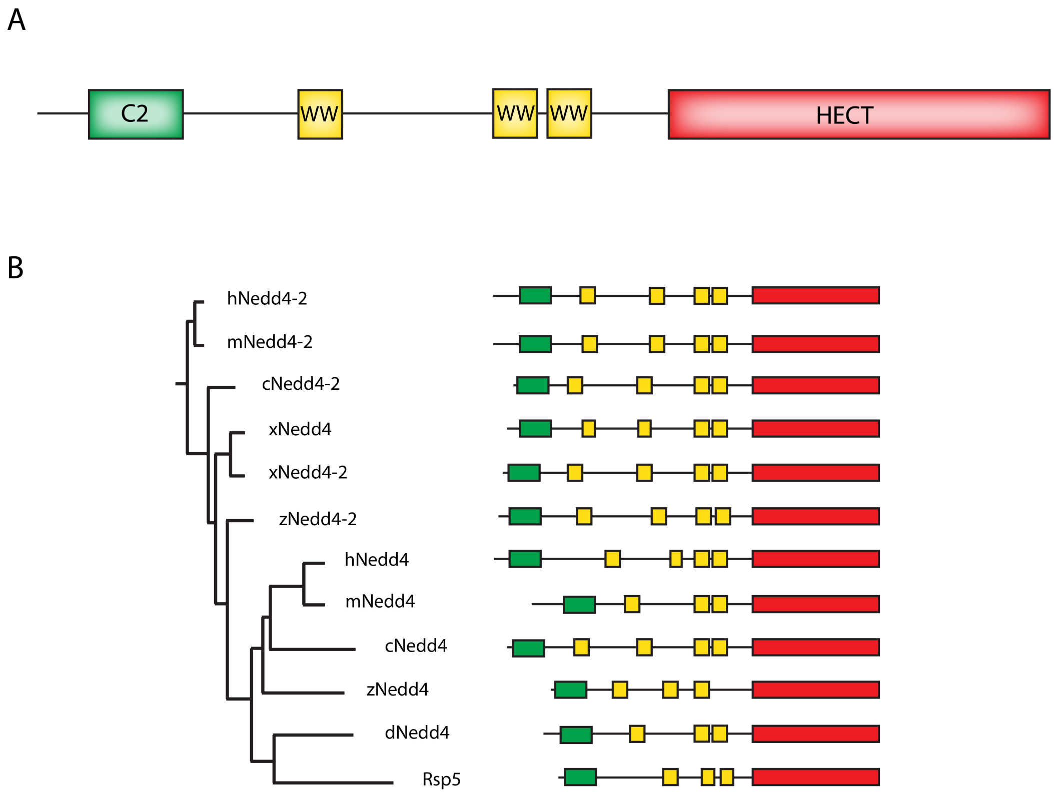


Figure 4.5: Nedd4 family domains and phylogenetic relationship between species - A. Nedd4 family functional domains. The N-terminal C2 domain is responsible for membrane binding (99) (111). Nedd4 family members contain between 2 and 4 WW domains that bind proline-rich sequences in the substrate. Finally, the C-terminal catalytic HECT domain catalyses the transfer of ubiquitin to the substrate. B. Comparison of protein sequence and domain structure between family members. The C2 domain is depicted in green, WW in yellow and the HECT domain in red. The phylogenetic relationship was taken from (110).

ubiquitination by Nedd4 (84).

Endogenous Nedd4 is also able to bind α-synuclein as is recombinant human Nedd4 and the yeast Nedd4 orthologue, Rsp5p. Therefore Nedd4 appears to be a *bona fide* binding partner of α-synuclein (84).

In human dopaminergic neuroblastoma cell lines, the overexpression or knockdown of Nedd4 levels reduced or increased endogenous α-synuclein levels, respectively (84). Given that Nedd4 is a ubiquitin ligase and the function of ubiquitination in protein clearance, it was proposed that Nedd4 ubiquitinates α-synuclein to signal its degradation.

Nedd4 preferentially catalyses the formation of lysine 63-linked polyubiquitin chains on α-synuclein, a ubiquitination signal predominantly used to target substrates to the lysosome for degradation. The inhibition of proteasomal function, lysosomal function and macroautophagy resulted in increased levels of α-synuclein in all cases in cell lines (84). This is consistent with previous studies implicating the proteasome (106) (105) (107) (108), CMA (112) (113) and macroautophagy (105) in α-synuclein clearance. However enhanced α-synuclein degradation by Nedd4 overexpression was only abrogated in the instance of lysosomal inhibition. The blocking of proteasomal function or macroautophagy did not effect accelerated α-synuclein clearance by Nedd4 (84); therefore Nedd4 does not target α-synuclein for degradation via the proteasomal pathway but to the lysosome, via a mechanism other than macroautophagy.

α-synuclein is predominantly membrane-associated, being localised to the synaptic terminals, therefore, given the function of the ESCRT pathway in targeting membrane-associated lysine-63 linked polyubiquitinated proteins to the lysosome, this pathway was investigated for a role in α-synuclein degradation. The downregulation of ESCRT components in cell lines was found to abrogate Nedd4 overexpression-induced acceleration of α-synuclein clearance thereby implicating the endosomal-lysosomal pathway in clearance of α-synuclein (84).

Finally, the *S.cerevisiae* Nedd4 orthologue, Rsp5p was able to promote human α-synuclein degradation and importantly, decrease α-synuclein-induced cytotoxicity. This protection was lost upon the mutation of the active cysteine residue in Rsp5p to an alanine resulting in an inability to catalyse ubiquitination. Conversely, a reduction in Rsp5p levels increased α-synuclein-containing inclusion formation and increased α-synuclein-induced toxicity (84).

This study (84) provides compelling evidence for a role of Nedd4 in the ubiquitination of α-synuclein thereby targeting it to the endosomal-lysosomal pathway for degradation, enhancing α-synuclein clearance.

The effect of Nedd4 on α-synuclein-dependent phenotypes such as dopaminergic neuron loss and locomotor dysfunction in an animal model is required to further our understanding of the relationship between Nedd4 and α-synuclein *in vivo* and the validity of Nedd4 as a potential therapeutic target.

4.1.5 *Drosophila* models of Parkinson's disease

In 2000, Feany and Bender characterised a *Drosophila* model of PD that effectively mimics the clinical features of PD; namely aggregation of α-synuclein and dopaminergic neuron loss leading to locomotor dysfunction (20). There is no *Drosophila* homologue of α-synuclein, therefore this model utilises ectopic expression of wild-type or mutant protein using the GAL4/UAS system. Overexpression of wild-type and mutant forms of the protein using the pan-neuronal driver *elav*-GAL4, results in a punctate expression pattern suggesting aggregate formation (20).

Overexpression of human α-synuclein in *Drosophila* results in normal brain development with no widespread neuronal loss coinciding with aging (20). Selective DA neuron loss, however, is observed (20). Dopaminergic neuron loss was investigated using an antibody specific to tyrosine hydroxylase. Analysis of the dorsomedial clusters of dopaminergic neurons, consisting of 4-5 TH-positive cells, revealed an age-dependent loss of neurons when wild-type or mutant α-synuclein is overexpressed pan-neuronally by *elav*-GAL4, or specifically to the dopaminergic neurons by *Dcd*-GAL4. Significant loss of DA neurons is evident between 30-60 DAE (20) and is generally restricted to the dorsomedial clusters.

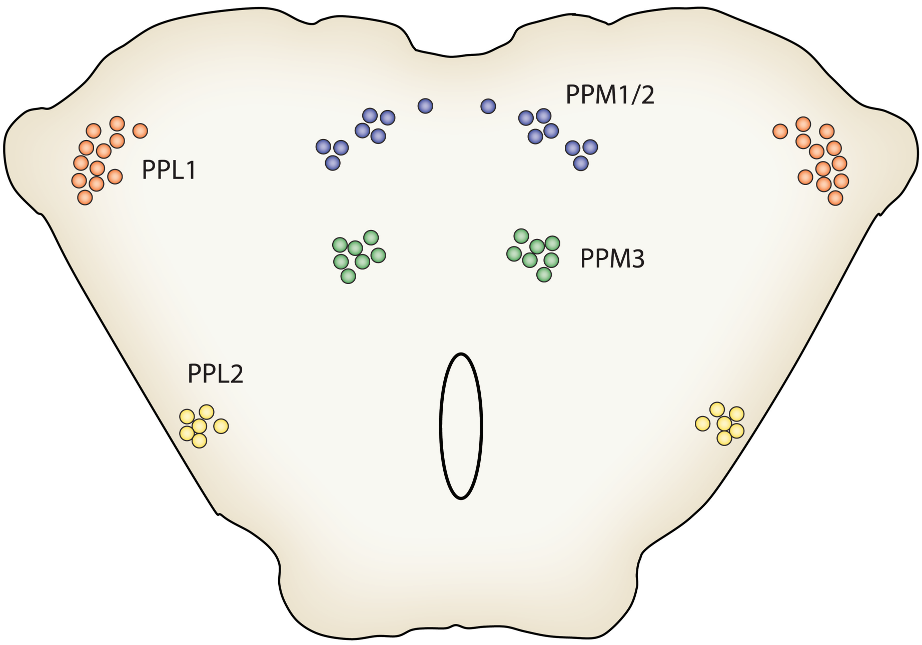


Figure 4.6: Dopaminergic neuron system in *Drosophila* - The posterior view of the dopaminergic neurons in the adult brain. The dorsomedial posterior protocerebral clusters, PPM1, PPM2 and PPM3, the dorsolateral posterior protocerebral cluster, PPL1 and the lateral posterior protocerebral cluster, PPL2. The anteriorly positioned dorsolateral anterior protocerebral, PAL cluster is not shown (22). The dorsomedial cluster affected in *Drosophila* models of PD is a subset of the PPM1/2 cluster.

A climbing assay revealed that the loss of DA neurons correlated with a increased rate of locomotor dysfunction when α-synuclein is overexpressed (20). This decreased climbing ability can be rescued by the adminstration of L-DOPA (114), widely used to treat parkinsonian symptoms in PD patients. Ectopic expression of mutant forms of the protein results in an enhanced locomotor dysfunction phenotype, however only a marginal difference in severity is observed (20).

Significantly, the impact of α-synuclein on neuronal degeneration has been shown to be not exclusive to DA neurons in *Drosophila.* It should be noted that mild retinal degeneration has also been detected with ectopic expression of α-synuclein by *GMR*-GAL4 (20). However, when expressed in the *Drosophila* brain, neurodegeneration was only observed within a subpopulation of the dopaminergic neurons; the dorsomedial cluster. Therefore α-synuclein induced neuronal degeneration is not specific to DA neurons however they are more susceptible to degeneration, given that other neurons examined in the brain are unaffected (20).

In summary, this *Drosophila* model (20) recapitulates the major hallmarks of the human disease, specifically the formation of α-synuclein containing inclusions, adult onset neurodegeneration that is largely restricted to dopaminergic neurons and age-related loss of motor function. This model is therefore ideally suited for the identification of modifiers of parkinsonian phenotypes including genetic interactors and drug screens.

Following the publication of the above model, *Drosophila* has been used in a number of studies to identify modifiers of α-synuclein-dependent phenotypes and to further investigate modes of PD pathogenesis, in particular that of aggregation formation and proteotoxic insult.

4.1.6 Aim of chapter

The enhancement of α-synuclein clearance is an important process to consider with regards to PD prevention, and the identification of factors involved in aggregate degradation is essential for the identification of putative therapeutic targets. Human Nedd4 and the yeast Nedd4 orthologue, Rsp5p, have been shown to robustly ubquitinate α-synuclein thereby targeting it for lysosomal degradation (84). Using a characterised *Drosophila* model of PD (20), in collaboration with George Tofaris, I have investigated the effect of modification of dNedd4 levels on α-synuclein-dependent phenotypes in the fly.

4.2 Results

4.2.1 Verification of transgene expression

Drosophila does not contain an α-synuclein orthologue, therefore to examine the relationship between dNedd4 and α-synuclein *in vivo* I used the bipartite GAL4/UAS system (9) to overexpress α-synuclein in a targeted fashion, using a *UAS-α-synuclein*^{WT} line (20). To modify dNedd4 levels we obtained dNedd4 lines in which the transgenes are under the control of a UAS promoter; *UAS-dNedd4* (21) and *UAS-dNedd4-RNAi*.

Firstly, 'flip-out' clones were generated in the larval 3^{rd} instar eye disc from these lines to confirm the ability of the transgenes to modify α-synuclein and dNedd4 levels.

'Flip-out' clones are positively marked by the expression of GFP. The appropriate up-regulation of wild-type α-synuclein was observed in *UAS-α-synclein*^{WT} 'flip-out' clones and increased and decreased levels of dNedd4 were observed in *UAS-dNedd4* and *UAS-dNedd4-RNAi* clones respectively (Figure 4.7).

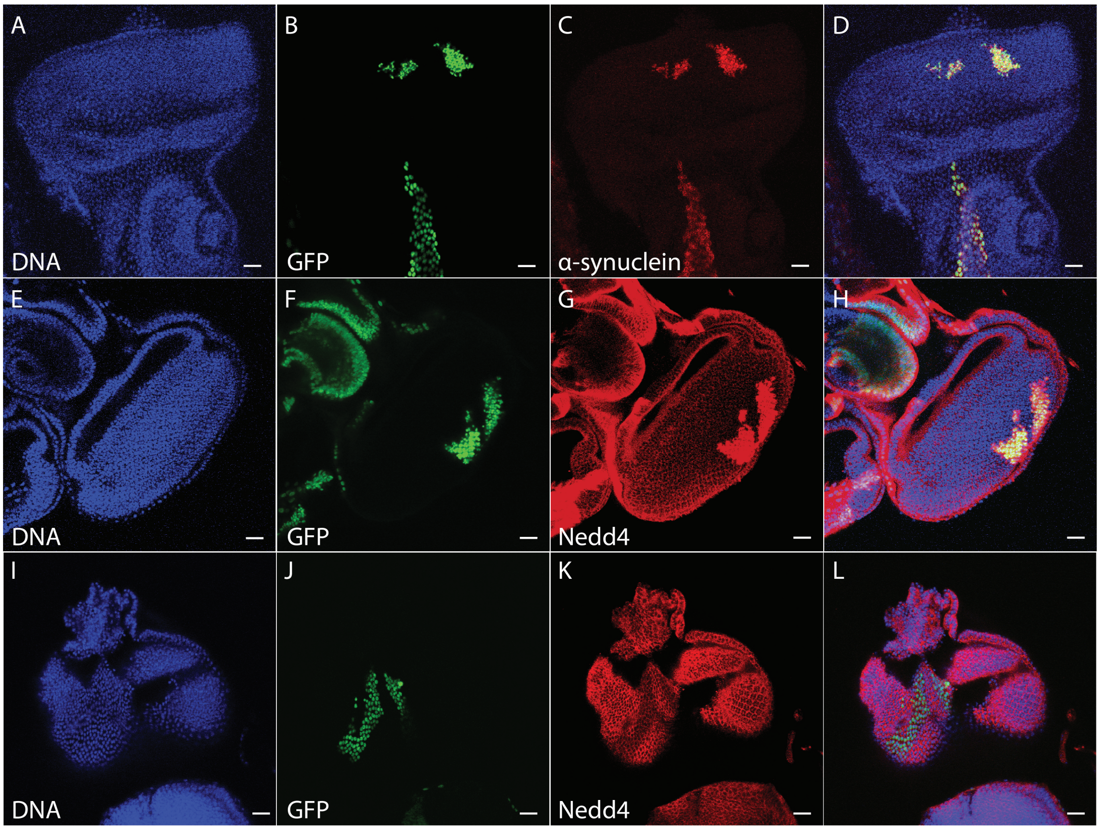


Figure 4.7: Clonal expression of α-synuclein and dNedd4 transgenes - Clones are marked with GFP (B, F, J). A-D: Clonal expression of *UAS-α-synclein*^{WT} shows upregulation of α-synuclein protein levels in positive clones (C). E-H: Overexpression of dNedd4 is observed in *UAS-dNedd4* 'flip-out' clones (G). I-L: Downregulation of Nedd4 by RNAi in *UAS-dNedd4-RNAi* clones (K). All scale bars: 20μm

4.2.2 Alteration of dNedd4 levels does not cause toxicity in the adult eye

The *Drosophila* adult eye is not required for survival, therefore is an appropriate system in which to manipulate gene expression, bypassing developmental aberrations that may occur if expressed pan-neuronally. In the cases of transgenes whose reduction or over-expression results in neuronal degeneration, a 'rough eye' phenotype may be observed. Suppression or enhancement of the rough eye phenotype can be used to readily identify interactors. Therefore, to investigate whether α-synuclein and dNedd4 genetically interact *in vivo*, the *GMR*-GAL4 driver was used to induce eye-specific expression of our transgenes.

Importantly, overexpression or knockdown of dNedd4 does not appear to be toxic in the adult eye. The overexpression of a catalytically inactive form of dNedd4 in which the active Cys residue is replaced with Ala, *UAS-dNedd4*$^{C-A}$ (21), results in a severe rough eye phenotype, suggesting the catalytically inactive form acts in dominant negative fashion (Figure 4.8). A dominant negative action for this allele is supported by the fact that the overexpression of wild type Nedd4 rescues the rough eye phenotype (Figure 4.9).

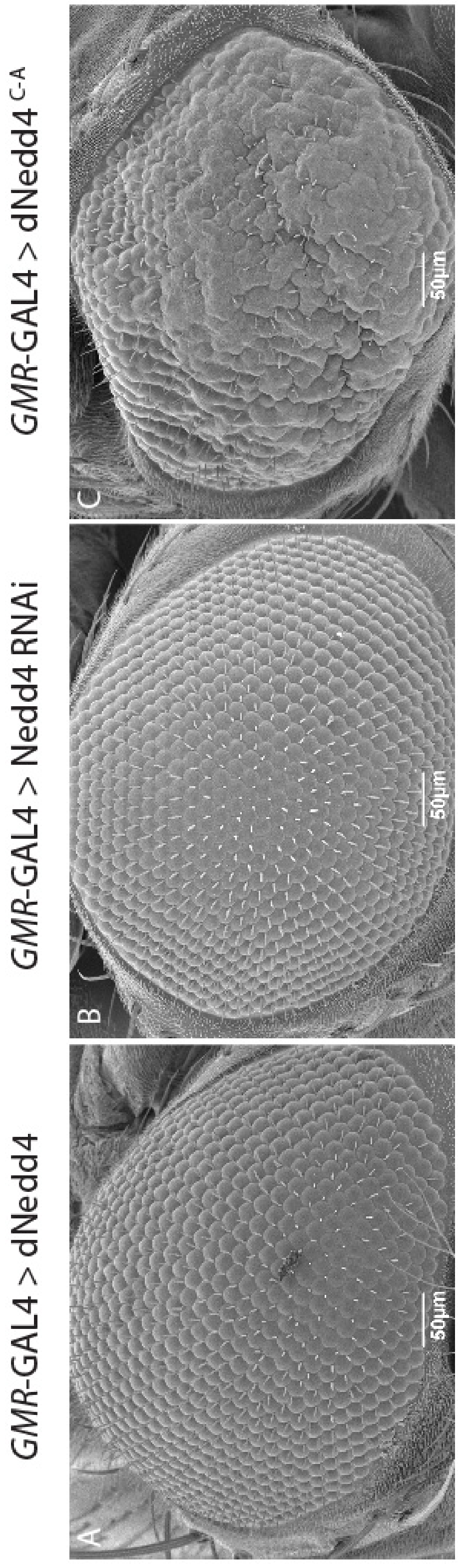


Figure 4.8: Alterations in Nedd4 levels do not cause toxicity in the adult eye - A-B: The overexpression of Nedd4 or downregulation of Nedd4 by RNAi, A and B respectively, results in normal adult eye development. C. The overexpression of a catalytically inactive form of Nedd4, *UAS-dNedd4*$^{C-A}$, by *GMR*-GAL4 however, results in a severe rough eye phenotype at 25°C.

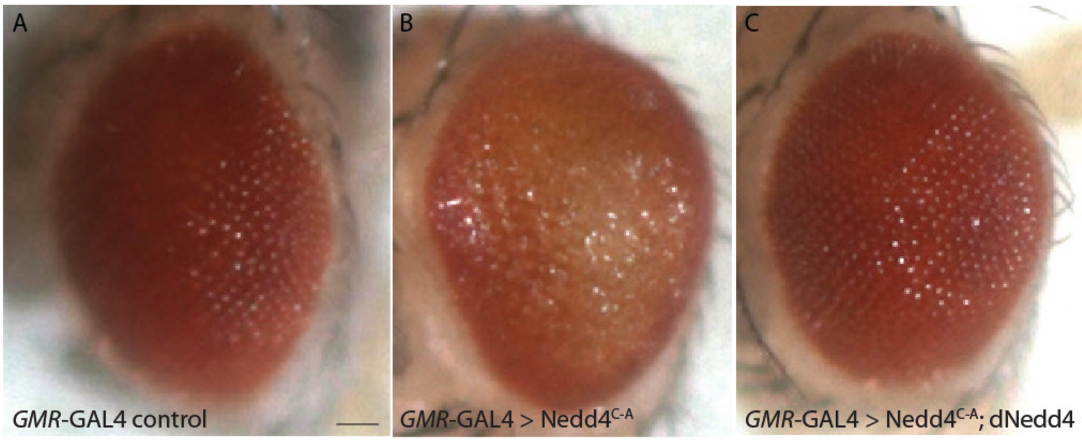


Figure 4.9: Overexpression of wild type Nedd4 rescues the dNedd4^{C-A} rough eye phenotype - A. *GMR*-GAL4 control. B. The overexpression of a catalytically inactive form of Nedd4, *UAS-dNedd4*$^{C-A}$, results in a severe rough eye phenotype and pigmentation loss. C. This defect is rescued by the overexpression of dNedd4. Scale bar: 50μm for all images.

4.2.3 α-synuclein causes an age-dependent rough eye phenotype in the adult eye

The overexpression of human α-synuclein using *GMR*-GAL4 resulted in a mild rough eye phenotype in aged flies crossed at 29°C. In newly eclosed flies, at 2 DAE, the flies had a normal eye phenotype, implying that lens development is unaffected. At 20 DAE, areas showing aberrant patterning of the ommitidia were observed (Figure 4.10). It should be noted that at 29°C, a mild rough eye phenotype can be observed in *GMR*-GAL4 heterozygote controls, however, the rough eye of α-synuclein expressing flies shows a marked enhancement of this rough eye in aged flies. Therefore the rough eye phenotype observed upon eye-specific α-synuclein overexpression appears to be age-related.

4.2.4 α-synuclein-induced rough eye phenotype is rescued by dNedd4 upregulation

In view of the toxicity conferred by α-synuclein overexpression in the adult eye, the ability of alterations in dNedd4 levels to modify the rough eye phenotype was investigated. dNedd4 was overexpressed or knocked down by RNAi in an α-synuclein overexpression background using *GMR*-GAL4. At 20 DAE the reduction of Nedd4 by RNAi did not result in any appreciable modification of the rough eye phenotype. However, a rescue of the rough eye phenotype was observed when dNedd4 is overexpressed (Figure

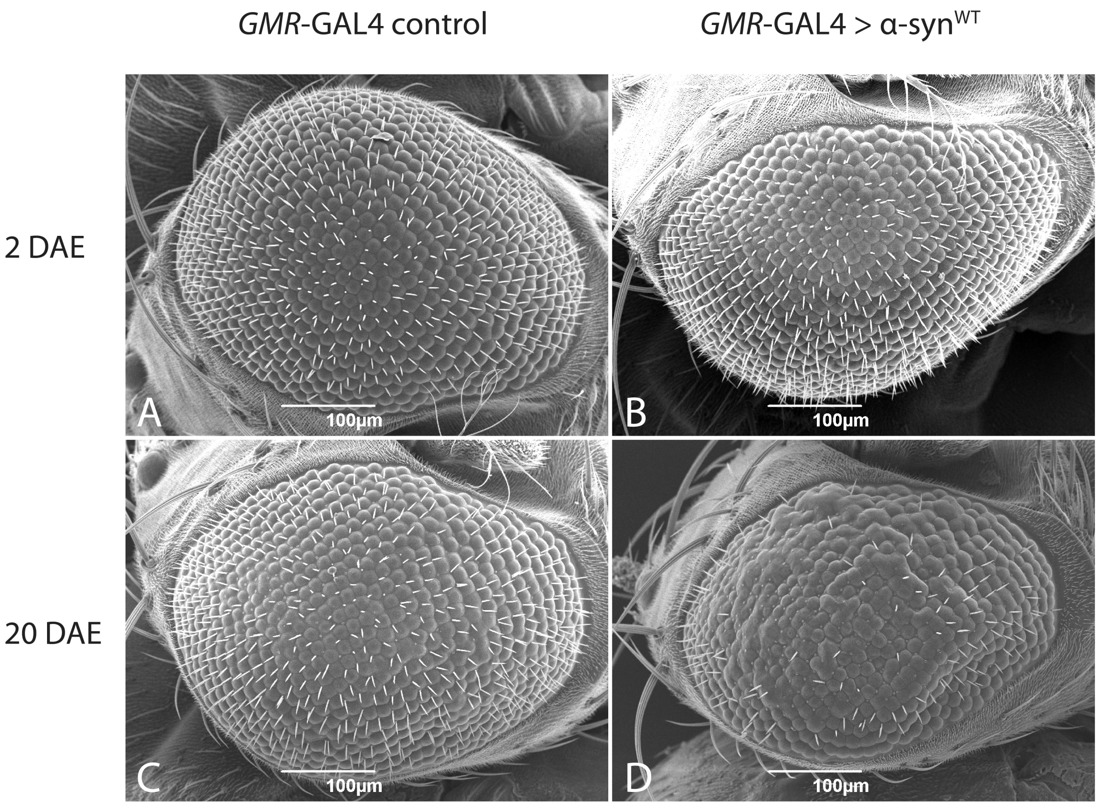


Figure 4.10: α-synuclein-induced toxicity in the adult eye is age-dependent - Control flies and those overexpressing α-synuclein in the eye do not have aberrant eye development at 2 DAE (A and B). α-synuclein overexpression results in a rough eye phenotype in aged flies (D) whereas control flies appear normal (C). Genotypes depicted are as follows: *GMR*-GAL4/+ (A and C), *UAS-α-synuclein*^{WT}/+; *GMR*-GAL4/+ (B and D). All crosses were raised and maintained at 29°C.

4.11). Preliminary data suggests this is also the case at 30 DAE however confirmation is required. The alteration of dNedd4 levels in the eye without α-synuclein did not result in any rough eye phenotype. This exciting finding suggests that dNedd4 and α-synuclein genetically interact and that dNedd4 upregulation can indeed modify α-synuclein toxicity *in vivo*.

Due to the modification of α-synuclein toxicity by dNedd4 overexpression in the *Drosophila* adult eye, we wished to know whether alterations in dNedd4 levels could modify the dopaminergic neuron loss and locomotor dysfunction phenotypes previously reported in *Drosophila* PD models (20).

4.2.5 Pan-neuronal overexpression of α-synuclein and dNedd4 transgenes does not affect lifespan

α-synuclein was pan-neuronally overexpressed using the *Elav*-GAL4 driver as performed by Feany and Bender (20). dNedd4 levels were modified by ectopically expressing *UAS-dNedd4* and *UAS-dNedd4-RNAi* using *Elav*-GAL4. Western blotting of adult head lysates showed overexpression of α-synuclein and the appropriate overexpression and downregulation of dNedd4 (Figure 4.12).

dNedd4 has a number of different splice isoforms, including two major isoforms, dNeddS and dNedd4Lo (115). Both isoforms contain the same functional Nedd4 family domains; a conserved C2 domain, 3 WW domains and the catalytically active HECT

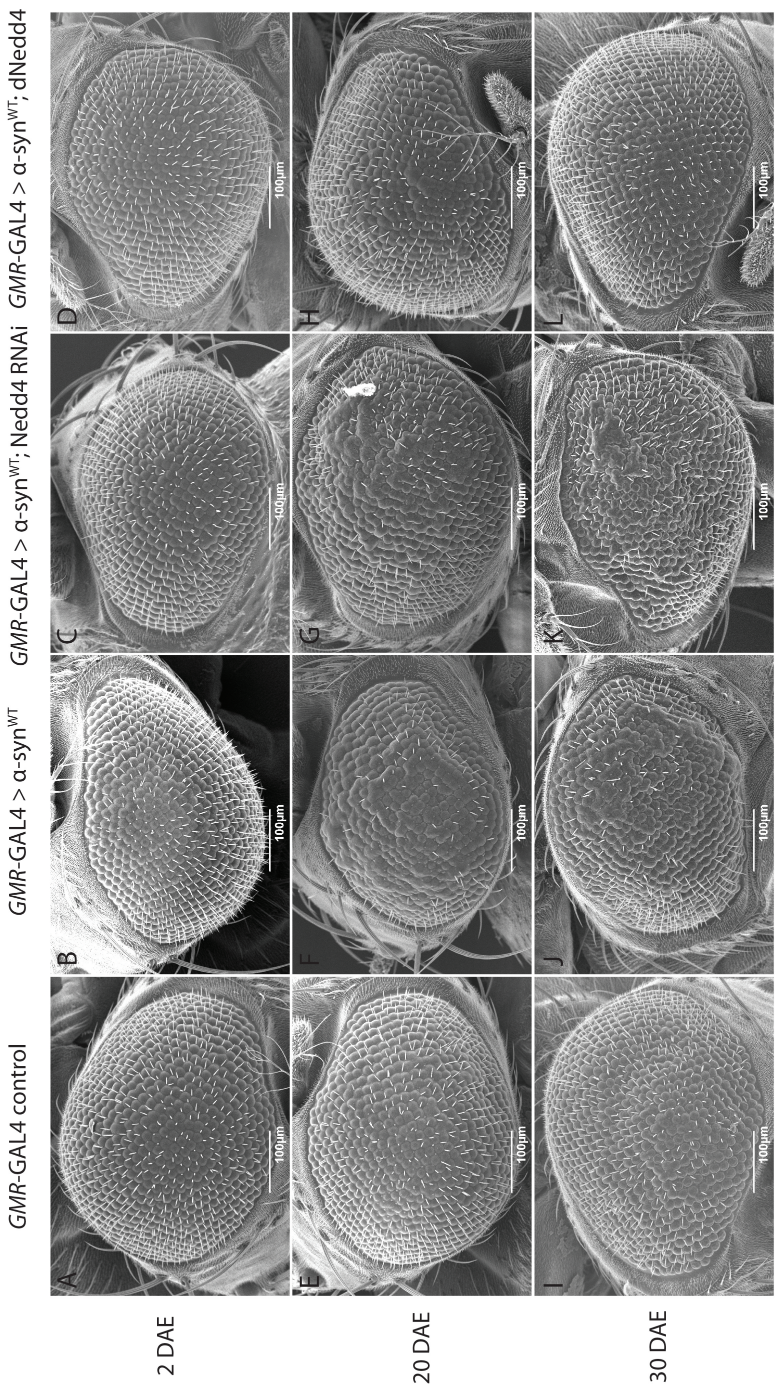


Figure 4.11: α-synuclein-induced rough eye phenotype is rescued by Nedd4 upregulation - Control flies, *GMR*-GAL4/+, do not display an appreciable rough eye phenotype at 2 days after eclosion (DAE)(A), however may develop a mild rough eye in aged flies (E, I). Overexpression of α-synuclein (B, F, J) results in an enhanced rough eye phenotype at later time points (F, J). This phenotype is not appreciably worsened by a reduction in dNedd4 levels (C, G, K) however, it is ameliorated by the overexpression of dNedd4 (H, L). Genotypes depicted are as follows: *GMR*-GAL4/+ (A, E, I), *UAS-α-synuclein^{WT}*/+; *GMR*-GAL4/+ (B, F, J), *UAS-α-synuclein^{WT}*/+; *GMR*-GAL4/*UAS-dNedd4-RNAi* (C, G, K) and *UAS-α-synuclein^{WT}*/+; *GMR*-GAL4/*UAS-dNedd4* (D, H, L). All crosses were raised and maintained at 29°C.

domain (115). However, the two isoforms have different roles; dNedd4S enhances neuromuscular synaptogenesis in *Drosophila* through the regulation of Comm ubquitination and endocytosis (116), whilst dNedd4Lo has a negative effect on neuromuscular synaptogenesis, with its overexpression resulting in synapse defects and larval locomotor dysfunction (115). Also, the overexpression of dNedd4S, but not dNedd4Lo, is able to partially rescue the embryonic lethality of dNedd4 mutants (115). The *UAS-dNedd4* line used in this study, generated by Myat et al. (21), upregulated the levels of dNedd4S but did not show increased levels of dNedd4Lo. The transgenic RNAi line used resulted in the downregulation of both dNedd4S and dNedd4Lo.

Interestingly, the overexpression of *UAS-dNedd4*$^{C-A}$ results in upregulation of a higher molecular weight dNedd4 species, possibly corresponding to dNedd4Lo. Due to the dominant negative phenotype observed upon overexpression of dNedd4^{C-A} in the adult eye and those previously described in the wing (117), in combination with the specific upregulation of a higher molecular weight dNedd4 isoform only, the use of dNedd4^{C-A} as a catalytically inactive dNedd4 control was abandoned. This line is inappropriate to use for the investigation of abrogation of the dNedd4 protective effect in the eye due to secondary phenotypes and due to the lack of upregulation of dNedd4S.

Importantly, quantification of Western blots from head lysates showed that at 20 DAE, the pan-neuronal overexpression of dNedd4 resulted in a decrease in levels of

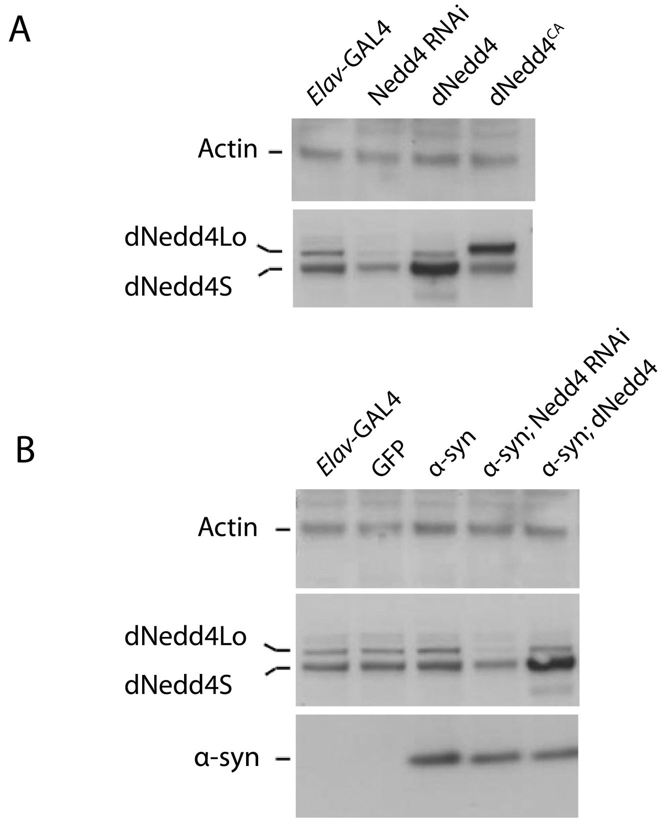


Figure 4.12: Western blots from adult head lysates showing pan-neuronal transgene expression - The *Elav*-GAL4 driver was crossed to each line to achieve pan-neuronal expression. A. Expression of dNedd4 transgenes. The expression of each of our Nedd4 lines by *Elav*-GAL4 resulted in appropriate upregulation or downregulation of protein levels. dNedd4 has two splice isoforms, dNedd4Lo and dNedd4S. B. *Elav*-GAL4 driven *UAS-α-synuclein^{WT}* expression results in upregulation of α-synuclein protein levels. Overexpression of α-synuclein is observed in the double transgenics, *Elav*-GAL4/+; *UAS-α-synuclein^{WT}/+*; *UAS-Nedd4-RNAi/+* and *Elav*-GAL4/+; *UAS-α-synuclein^{WT}/+*; *UAS-dNedd4/+*, along with downregulation and upregulation of dNedd4, respectively. Genotypes depicted are as follows: A, left to right, *Elav*-GAL4/+, *Elav*-GAL4/+; *UAS-Nedd4 RNAi/+*, *Elav*-GAL4/+; *UAS-dNedd4/+*, *Elav*-GAL4/+; *UAS-dNedd4^{C-A}/+*. B, left to right, *Elav*-GAL4/+, *Elav*-GAL4/+; *UAS-GFP/+*, *Elav*-GAL4/+; *UAS-α-synuclein^{WT}/+*, *Elav*-GAL4/+; *UAS-α-synuclein^{WT}/+*; *UAS-Nedd4-RNAi/+* and *Elav*-GAL4/+; *UAS-α-synuclein^{WT}/+*; *UAS-dNedd4/+*. The driver control was crossed to w^{1118}, the background strain of all the transgenes used, to produce the *Elav*-GAL4/+ heterozygote control. All crosses were raised and maintained at 25°C.

monomeric α-synuclein compared to α-synuclein only overexpressing flies. Nedd4 RNAi however, did not significantly alter α-synuclein protein levels (Figure 4.13). The mild knockdown of Nedd4 produced by Nedd4 RNAi is therefore insufficient to significantly alter α-synuclein levels.

Modulating dNedd4 levels did not result in reduced survival of adult flies in preliminary experiments. During early time points the overexpression of dNedd4 resulted in a slight reduction of survival however this did not continue to later time points. After 40 DAE the percentage of survivors was more distinct between the lines however altering dNedd4 levels did not cause reduced survival, each line having a greater percentage of surviving flies that the control line. It is possible that the knockdown of dNedd4 and the overexpression of dNedd4^{C-A} results in increased survival at very late time points, after 40 DAE, however further experiments would be required to perform the statistical analysis required to test the significance of this variation. However, from this preliminary experiment, it appears that alterations in dNedd4 levels does not effect adult survival (Figure 4.14).

Similarly, pan-neuronal overexpression of human α-synuclein did not result in increased mortality in adult flies. The downregulation of dNedd4 in an α-synuclein background also had normal survival rates however the overexpression of dNedd4 resulted in increased mortality at very late time points (between 50 and 60 DAE) in this

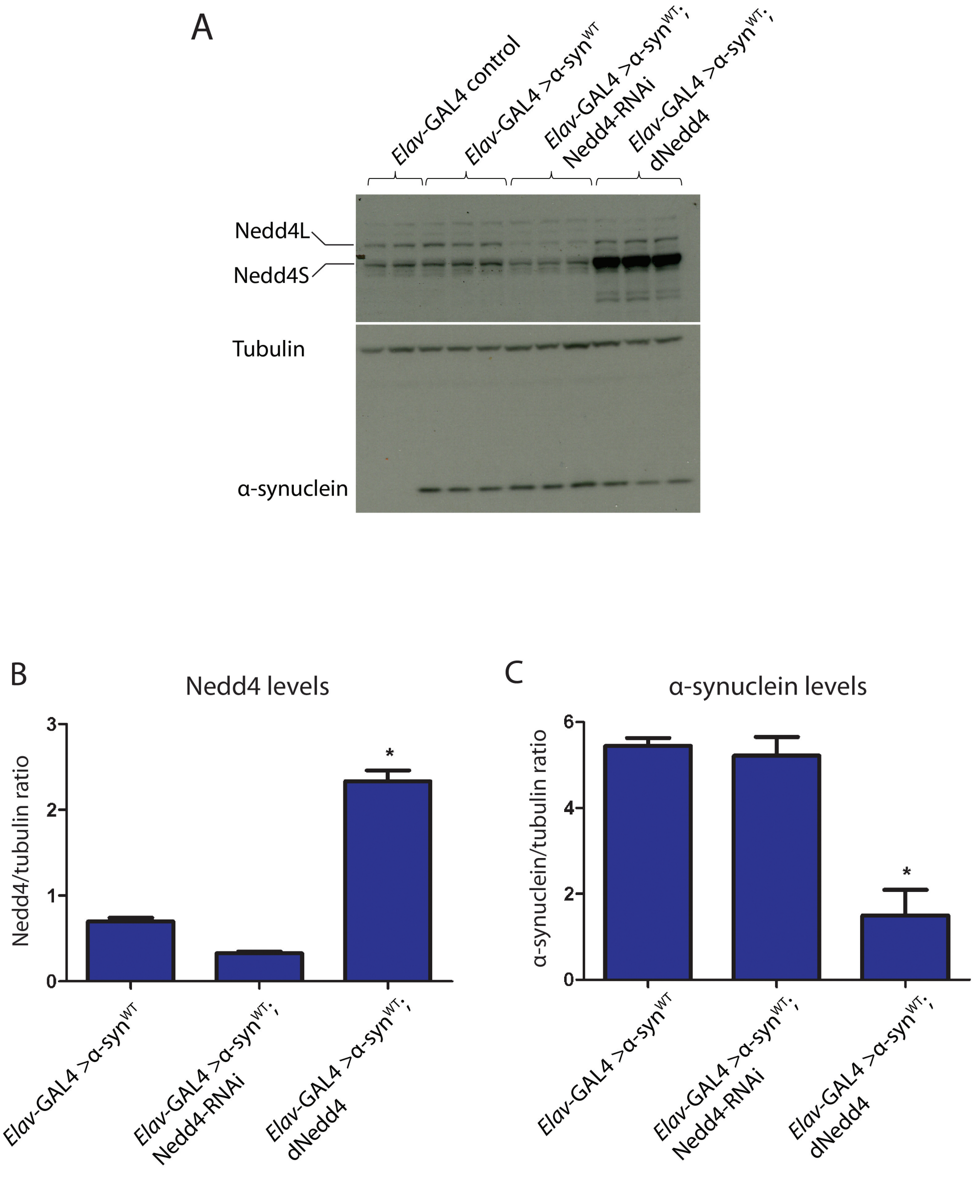


Figure 4.13: Quantification of Nedd4 and α-synuclein levels at 20 DAE - A. Western blot showing levels of Nedd4, α-synuclein and a tubulin control from adult head lysates. B. Nedd4 and α-synuclein levels were determined by quantifying band intensities from western blotting of adult head lysates and calculating the ratios compared to a tubulin control. The genotypes depicted are: α-synuclein; *Elav*-GAL4/+; *UAS-α-synuclein*^{WT}/+, RNAi DT; *Elav*-GAL4/+; *UAS-α-synuclein*^{WT}/+; *UAS-Nedd4-RNAi*/+ and OE DT; *Elav*-GAL4/+; *UAS-α-synuclein*^{WT}/+; *UAS-dNedd4*/+. n=3 for all samples. Western blotting was performed by the author and the quantification by George Tofaris. ANOVA multiple analysis, *P$\leq$0.05

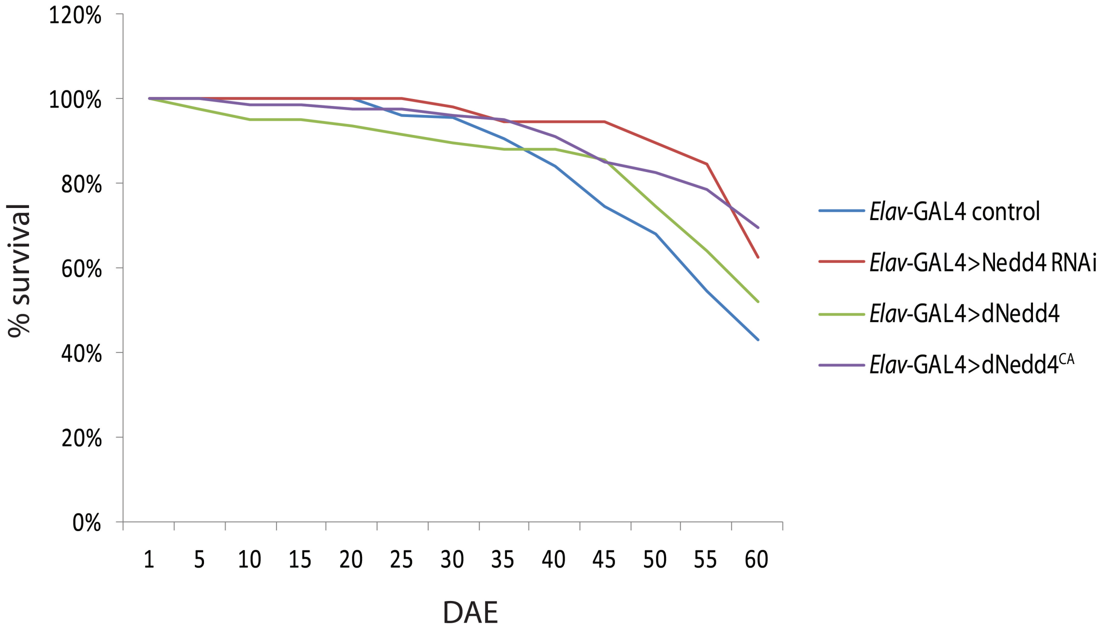


Figure 4.14: Alterations in dNedd4 levels does not reduce lifespan - Pan-neuronal overexpression of dNedd4, dNedd4^{C-A} and knockdown of dNedd4 by RNAi did not cause appreciable changes in survival. At very late time points, knockdown of dNedd4 and up-regulation of dNedd4^{C-A} may result in increased survival. The number of flies at the start of the experiment were as follows: *Elav*-GAL4 control, n=140; *Elav*-GAL4>Nedd4RNAi, n=49; *Elav*-GAL4>dNedd4, n=139; *Elav*-GAL4>dNedd4^{C-A}, n=130. All crosses were raised and maintained at 25°C.

preliminary test (Figure 4.15). Whether this result is of significance requires repeating of the survival assay to allow for robust statistical analysis.

However, these assays it appears that alterations in α-synuclein and dNedd4 levels does not effect the mortality rate of adult flies.

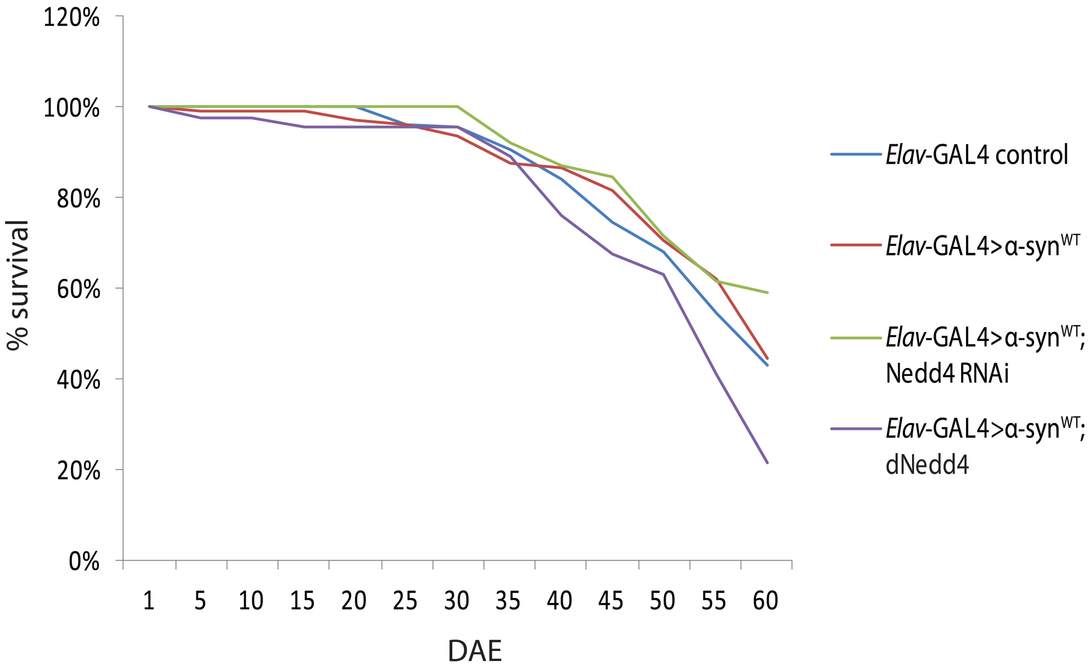


Figure 4.15: Overexpression of α-synuclein does not increase mortality - Pan-neuronal overexpression of α-synuclein does not result in decreased survival rate of adult flies. Knockdown of dNedd4 similarly does not increase mortality compared to control flies. The overexpression of dNedd4 results in decreased survival at very late time points in this preliminary analysis. The number of flies at the start of the experiment were as follows: *Elav*-GAL4 control, n=140; *Elav*-GAL4>α-synuclein^{WT}, n=100; *Elav*-GAL4>α-synuclein^{WT}, Nedd4RNAi, n=40; *Elav*-GAL4>α-synuclein^{WT}, dNedd4, n=48. All crosses were raised and maintained at 25°C.

4.2.6 Analysis and modification of locomotor dysfunction in a *Drosophila* PD model

Overexpression of α-synuclein in *Drosophila* was previously shown to cause enhanced age-related locomotor dysfunction (20). Preliminary analysis of climbing ability in α-synuclein transgenic flies showed a similar enhancement of age-dependent locomotor dysfunction in comparison to control flies (Figure 4.16) albeit less striking. Interestingly, this decreased climbing ability was abrogated by the overexpression of Nedd4. However, the knockdown of Nedd4 levels by RNAi similarly restored climbing ability, therefore this result should be treated with caution. The reduction in Nedd4 levels may not be sufficient to enhance the reduced climbing ability, especially given that Nedd4 RNAi did not result in an increase in α-synuclein protein levels. However, the suppression of the α-synuclein-dependent locomotor dysfunction observed is unexpected. It is necessary for this experiment to be repeated with the inclusion of another double transgenic control in order to fully understand this outcome.

The Nedd4 transgenics, apart from $UAS\text{-}dNedd4^{C-A}$, did not display reduction in climbing ability, however, the large variations in climbing ability observed warrants repetition of this experiment (Figure 4.16).

I reanalysed the climbing assay data measuring the distance travelled by individual flies in constrast to the previous analysis that quantified the percentage of flies able

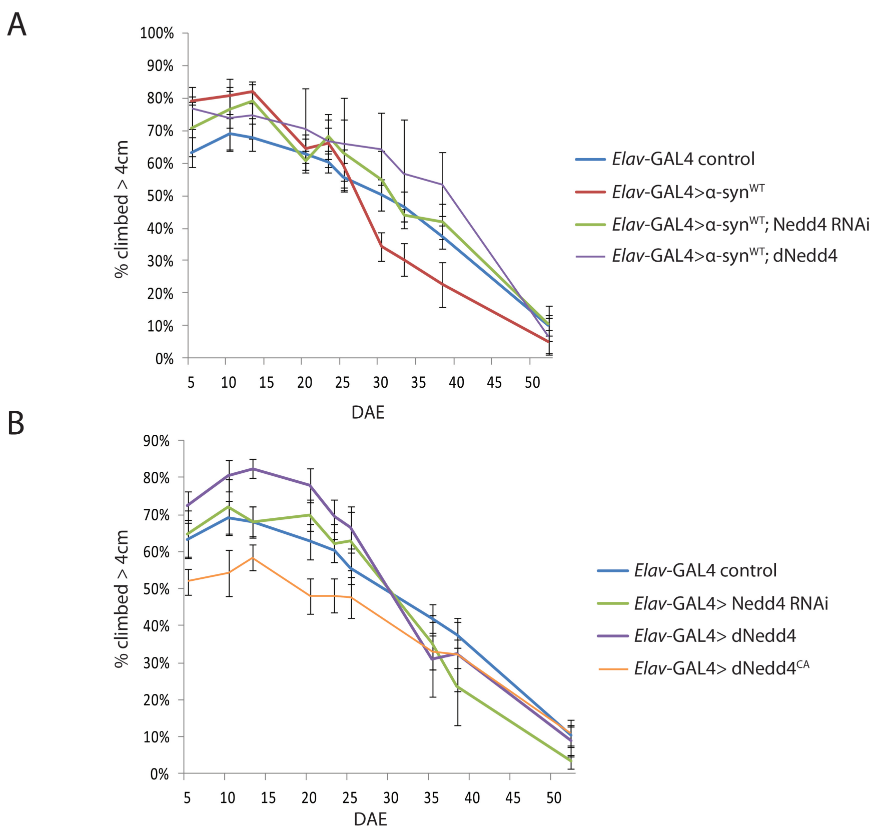


Figure 4.16: Enhanced decline in climbing ability in α-synuclein transgenic flies is modified by Nedd4 levels - A. α-synuclein transgenic flies show enhancement of age-related locomotor dysfunction. This phenotype was modified by the knockdown and overexpression of Nedd4. B. Nedd4 transgenic flies do not display an enhancement of age-dependent decline in climbing ability. The starting number of flies for this experiment were as follows: *Elav*-GAL4 control, n=100; *Elav*-GAL4>α-synuclein^{WT}, n=70; *Elav*-GAL4>α-synuclein^{WT}, Nedd4RNAi, n=30; *Elav*-GAL4>α-synuclein^{WT}, dNedd4, n=40; *Elav*-GAL4>Nedd4RNAi, n=30; *Elav*-GAL4>dNedd4, n=130; *Elav*-GAL4>dNedd4^{C-A}, n=100. No significance was observed from ANOVA multiple analysis.

to climb a particular distance. Interestingly, enhanced age-related decline in climbing ability was not observed when calculated in this manner (Figure 4.17). Therefore it is important to note for further analyses that the method of quantification of climbing ability may have an effect on the outcome.

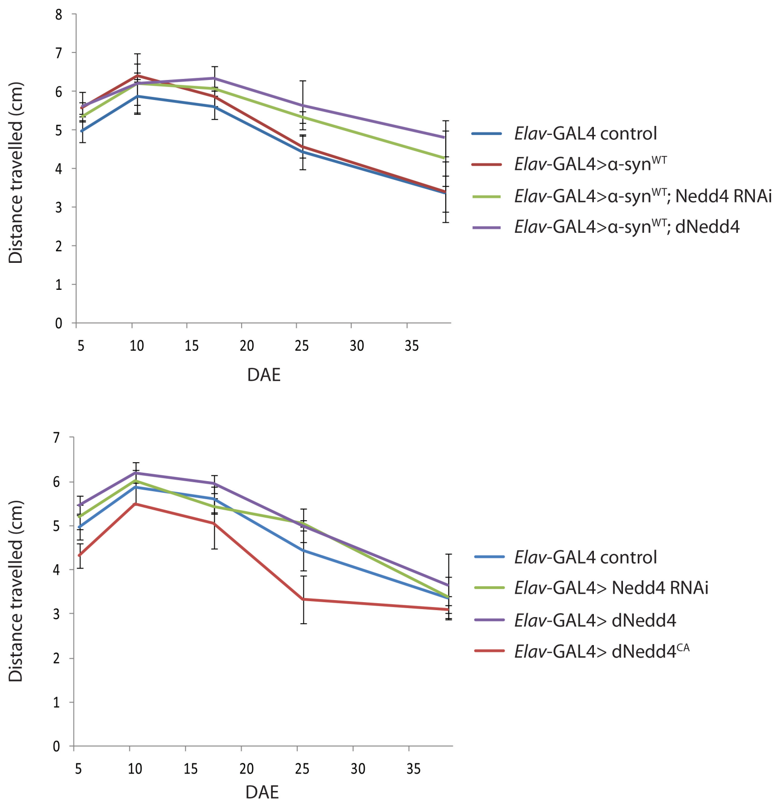


Figure 4.17: Climbing ability determined by distance travelled by transgenic flies - A-B. Reanalysis of the climbing assay measuring the distance travelled by individual flies as a measure of climbing ability did not result in any significant defects in climbing ability.

4.2.7 Analysis of dopaminergic neuron loss

The overexpression of α-synuclein has previously been shown to result in the age-dependent selective degeneration of the dorsomedial clusters of dopaminergic neurons in adult *Drosophila* (20) (95) (98) (97) (96) (118). If dNedd4 is protective against α-synuclein toxicity, it is possible that the overexpression of dNedd4 may abrogate α-synuclein-dependent dopaminergic neuron loss and conversely, a reduction in dNedd4 levels may enhance neurotoxicity.

The dopaminergic system in *Drosophila* is well defined and dopaminergic neurons can be identified by immunostaining with an antibody against tyrosine hydroxylase (TH), an enzyme that catalyses the conversion of tyrosine to the dopamine precursor, L-3,4-dihydroxyphenylalanine (L-DOPA). The dopaminergic neurons in the adult brain are predominantly localised to bilaterally symmetrical clusters; the posteriorly located dorsomedial posterior protocerebral clusters, PPM1, PPM2 and PPM3, the dorsolateral posterior protocerebral cluster, PPL1 and the lateral posterior protocerebral cluster, PPL2 and the anteriorly positioned dorsolateral anterior protocerebral, PAL cluster. Each cluster contains a stereotypical number of neurons and specifically projects to functionally distinct regions of the adult brain (22).

To investigate adult dopaminergic neuron loss, our α-synuclein and dNedd4 transgenes were overexpressed pan-neuronally using the *Elav*-GAL4 driver, and specifically to the dopaminergic system using *TH*-GAL4. Dopaminergic neurons were identified as being positive for an anti-TH antibody.

The overexpression of α-synuclein using *TH*-GAL4 did not result in significant dopaminergic neuron loss in any of the clusters in the adult brain at 40 DAE. Similarly, modifying dNedd4 levels did not significantly effect neurotoxicity (Figure 4.19).

Adult dopaminergic neurons were unexpectedly also largely resistant to pan-neuronal overexpression of α-synuclein at 60 DAE. No significant loss of dopaminergic neurons was observed in any of the α-synuclein transgenic lines. However *Elav*-GAL4>α-synuclein flies and α-synuclein overexpression in combination with dNedd4 knockdown resulted in large variations in neuron number. In the double transgenics, some brains retained the full complement of dopaminergic neurons in each cluster with others having very few, causing the large variation observed (Figure 4.19). A decrease in dopaminergic neuron number was also not observed for the single Nedd4 transgenics (data not shown).

Previous studies showed dopaminergic neuron loss in *Drosophila* to be specific to the dorsomedial clusters (20)(98), however, surprisingly the dorsomedial clusters did not show a marked loss of dopaminergic neurons, inconsistent with previously reported results (20)(98). Western blotting analysis of siblings confirmed appropriate overex-

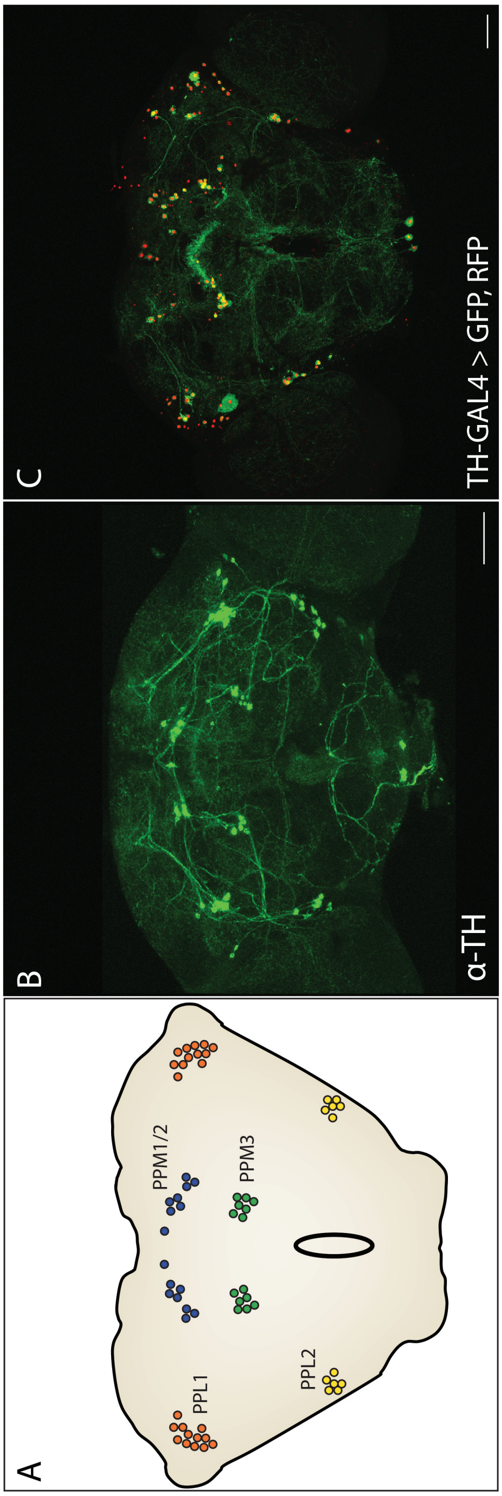
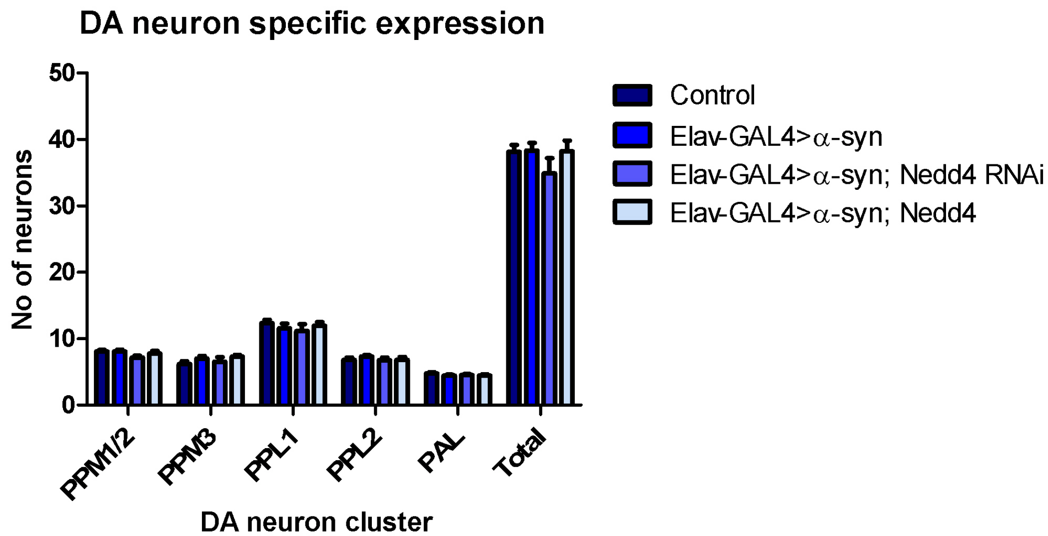


Figure 4.18: Dopaminergic neuron detection in the *Drosophila* adult brain - A. Schematic showing the locations of the major clusters of dopaminergic neurons in the posterior side of the adult brain. The anteriorly located PAL cluster has also been examined in this study, not present in diagram. B. Detection of dopaminergic neurons in the adult brain at 60 DAE with an antibody against TH. TH-positive cells are labelled in green. C. Expression pattern of *TH*-GAL4. Dopaminergic neurons are labelled with nuclear RFP and cytoplasmic GFP. Scale bars: 50μm.



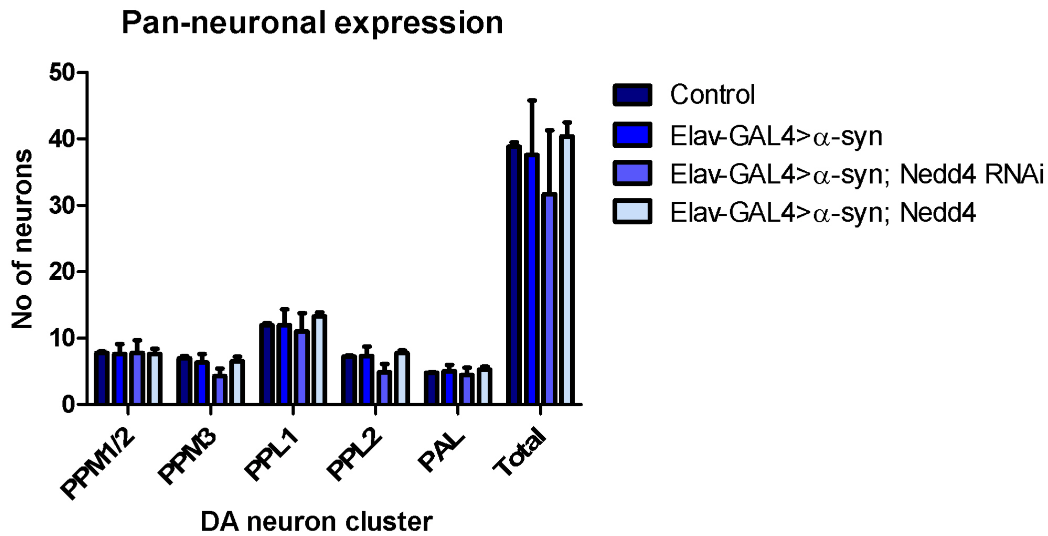


Figure 4.19: Dopaminergic neuron loss was not observed in α-synuclein transgenic flies - Dopaminergic neurons were analysed in transgenic flies using: A. *TH*-GAL4 specific expression at 40 DAE. Control *TH*-GAL4 heterozygotes n≐30 hemispheres, *TH*-GAL4>α-syn n≐22 hemispheres, *TH*-GAL4>α-syn; Nedd4RNAi n≐8 hemispheres, *TH*-GAL4>α-syn; Nedd4 n≐16 hemispheres and B. *Elav*-GAL4 expression at 60 DAE. No significant decrease in dopaminergic neuron number was observed. Control *Elav*-GAL4 heterozygotes n≐39-46 hemispheres (n values for different clusters vary), *Elav*-GAL4>α-syn n≐21-25 hemispheres, *Elav*-GAL4>α-syn; Nedd4RNAi n≐11-16 hemispheres, *Elav*-GAL4>α-syn; Nedd4 n≐8 hemispheres.

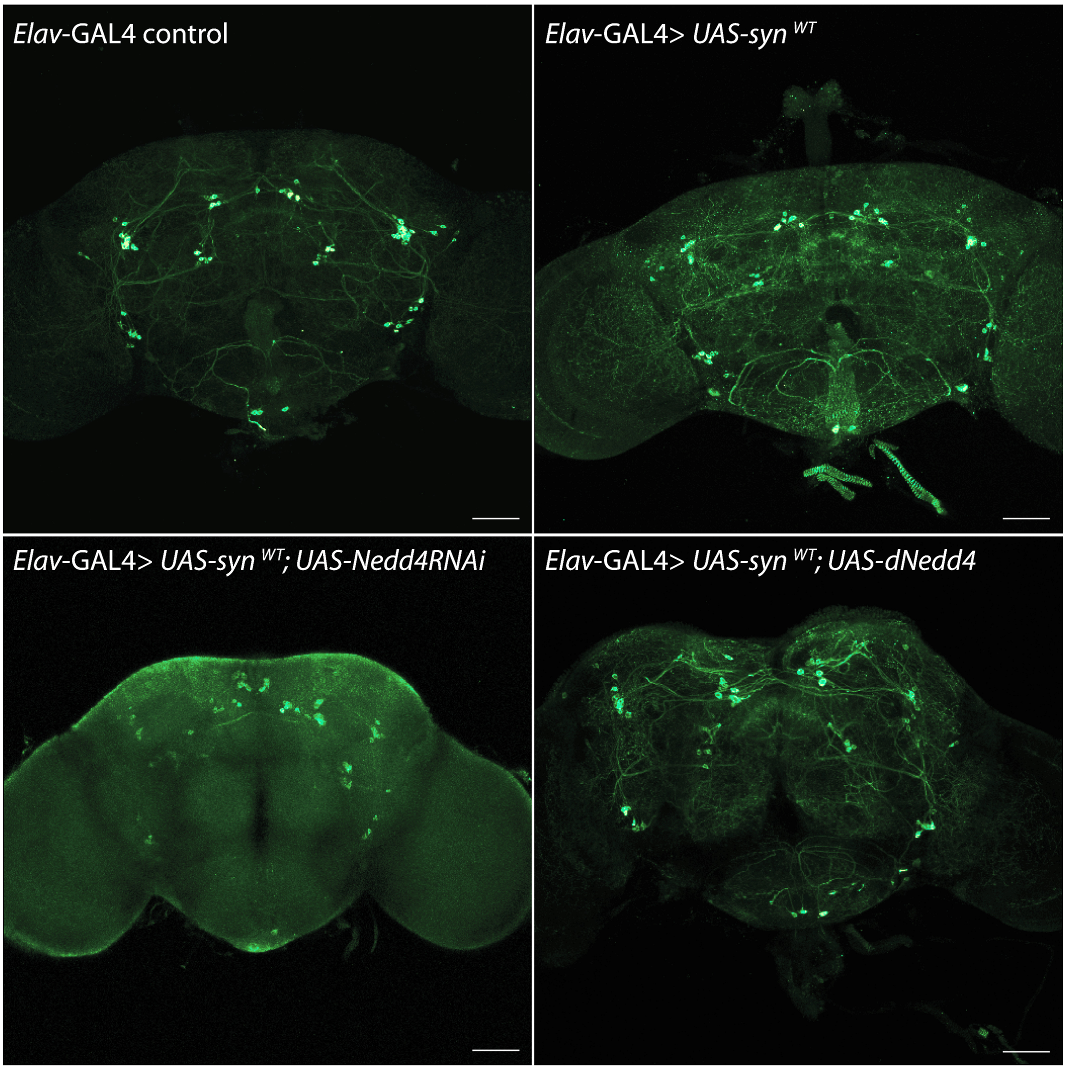


Figure 4.20: Dopaminergic neuron system in pan-neuronally overexpressing α-synuclein lines - Representative images of the dopaminergic neuron system in α-synuclein overexpressing lines at 60 DAE. Dopaminergic neurons were identified by anti-TH staining. Dopaminergic neuron number was found to be largely normal for *Elav*-GAL4/+; *UAS-α-synuclein*^{WT}/+ and *Elav*-GAL4/+; *UAS-α-synuclein*^{WT}/+; *UAS-dNedd4*/+ flies. *Elav*-GAL4/+; *UAS-α-synuclein*^{WT}/+; *UAS-Nedd4-RNAi*/+ flies however, did not always contain the full complement of neurons in each cluster. Control n$\doteq$23, *Elav*-GAL4>α-syn n$\doteq$15, *Elav*-GAL4>α-syn; Nedd4RNAi n$\doteq$10, *Elav*-GAL4>α-syn; Nedd4 n$\doteq$4. Scale bars: 50μm.

pression of the transgenes in these lines. The lack of dopaminergic neuron loss in this system precluded the investigation of dNedd4 as a modifier of this phenotype.

4.3 Discussion

Using a *Drosophila* model of PD we sought to investigate whether alterations in dNedd4 levels can modify α-synuclein-dependent phenotypes.

Firstly, quantification of protein levels in aged flies showed that the pan-neuronal overexpression of dNedd4 resulted in a significant decrease in levels of monomeric α-synuclein compared to α-synuclein only overexpressing flies suggesting increased α-synuclein clearance. Nedd4 RNAi however, did not significantly alter α-synuclein protein levels. This knockdown of Nedd4 levels may not be sufficient effect to α-synuclein protein levels.

The *Drosophila* adult compound eye has been shown to produce a neurodegenerative, 'rough eye' phenotype upon the modulation of some genes involved in neurodegeneration. The suppression or enhancement of this phenotype can be used to identify genetic interactors.

The adult eye is a regular hexagonal pattern of hundreds of individual facets, called ommatidia. Aberrant development or neurodegeneration can result in disruption of this regular pattern. A single ommatidium contains 8 photoreceptor neurons, R1-

R8, and non-neuronal accessory cells. Each of the photoreceptors are associated with a rhabdomere that contain opsin and are surrounded by a number of pigment cells that separate the ommaditia. Along with pigment cells, the ommatidia are associated with mechanosensory bristles, consisting of a neuron, glia and support cells (119). The photoreceptors are covered by 4 cells called cone cells that secrete the external, dioptric features of the ommatidia, the cone and the lens.

The overexpression of α-synuclein specifically in the eye using the post-mitotic driver, *GMR*-GAL4, resulted in normal patterning of the ommatidia in newly eclosed flies suggesting that eye development is not affected in α-synuclein overexpressing flies. The continued overexpression of α-synuclein however, resulted in a mild rough eye phenotype with aberrant patterning of the adult eye. Fused ommatidia were observed as well as increased bristle loss in the α-synuclein overexpressing flies. Mild aberrations were occasionally observed in control flies due to GAL4 toxicity, however the toxicity observed upon α-synuclein overexpression resulted in an appreciably enhanced phenotype. Therefore persistant α-synuclein overexpression in the adult eye results in toxicity. Previously, age-related retinal degeneration was observed upon α-synuclein overexpression in the eye (20), consistent with our findings.

A rough eye phenotype is often associated with neurodegeneration, however this phenotype may be caused by the degeneration of the accessory cells of the ommatidia

(12). It would be of interest to determine, via retinal sectioning, whether there is specific degeneration of the neural photoreceptors of the ommatidia.

The ability of dNedd4 levels to modify this toxic phenotype was investigated. Importantly, alterations in the levels of wild type dNedd4 did not cause toxicity in the eye even at 29°C. The catalytically inactive dNedd4^{C-A} line was originally planned to be used as a control for the ubiquitination activity of dNedd4. Should dNedd4 be modifying α-synuclein-dependent phenotypes via α-synuclein ubiquitination and its consequent clearance, any modification by wild type overexpression should be abrogated upon replacement with dNedd4^{C-A} overexpression. The overexpression of dNedd4^{C-A} alone however resulted in a severe rough eye phenotye with extensive fusing of the ommatidia and bristle loss. Also, loss of pigment was observed. dNedd4^{C-A} could therefore be acting in a dominant negative fashion, disrupting the normal functions of dNedd4 in the eye. This interesting phenotype unfortunately precludes its use as a catalytically inactive dNedd4 control.

The overexpression of dNedd4 was able to significantly improve the appearance of aged adult eyes ectopically expressing α-synuclein. The patterning of the ommatidia was reverted to the appearance of that of the *GMR*-GAL4 control and bristle loss was reduced. Therefore dNedd4 and α-synuclein appear to genetically interact in the *Drosophila* adult eye.

Given, the mild rough eye phenotype occasionally observed in control flies, further experiments should be carried out to quantify this phenotype, including quantification of the loss of photoreceptors in α-synuclein overexpressing flies. Also, although overexpression of the catalytically inactive dNedd4^{C-A} could not be used as a control, the overexpression of the mutant α-synuclein$^{1-120}$ protein should be investigated. α-synuclein$^{1-120}$ is lacking the C-terminal proline-rich motif proposed to bind to Nedd4, therefore toxicity caused by the overexpression of this mutant protein should not be suppressed by dNedd4 overexpression. This experiment would significantly support the proposal that Nedd4 is a modifier of α-synuclein-dependent phenotypes in *Drosophila*

Although Parkinson's disease causes widespread neuronal degeneration, the condition shows a predilection for the loss of dopaminergic neurons resulting in motor dysfunction. Therefore, we wished to translate our findings in the adult eye to the nervous system. It has been previously reported that pan-neuronal overexpression of α-synuclein results in locomotor dysfunction in *Drosophila* correlating with loss of the dorsomedial dopaminergic neurons. We therefore investigated the effect of alterations in dNedd4 levels on α-synclein overexpression phenotypes in the nervous system.

A negative geotaxis assay was performed to analyse locomotor dysfunction in α-synuclein pan-neuronally overexpressing flies. To assess motor function, the percentage of flies able to climb above a particular point was recorded. *Drosophila* adults display a gradual decline in locomotor function as they age, as shown by our *Elav*-GAL4 control. Similarly to previous findings we observed that the rate of locomotor dysfunction was increased in α-synuclein overexpressing flies. The enhanced decline in climbing ability was rescued by the overexpression of Nedd4. However, unexpectedly, Nedd4 RNAi was able to modifiy this phenotype. When average distance was calculated as a measure of climbing ability, enhanced locomotor dysfunction was not observed in the α-synuclein transgenic flies. Therefore further investigation is required to fully understand the phenotypes observed and to confidently assess locomotor dysfunction in α-synuclein transgenic flies. It should be noted decreased climbing ability was not observed in α-synuclein transgenic flies by Auluck et al. (98).

Finally, the effect of α-synuclein expression and Nedd4 modification on dopaminergic neuron degeneration was investigated. DA neurons in *Drosophila* are arranged in distinct clusters and do not degenerate with age (22). Pan-neuronal α-synuclein expression has been previously reported to cause loss of dopaminergic neurons specifically in the dorsomedial cluster. Surprisingly, dopaminergic neuron loss was not observed in α-synuclein transgenics.

Inconsistencies in dopaminergic neuron loss have been observed between studies using this model (120). Degeneration of DA neurons from the dorsomedial clusters was similarly described by Auluck et al. (98) however complete loss of the neurons from this region was not observed, in constrast to previous findings (20).

The method used to image DA neurons may explain the discrepancies observed between studies with whole mount imaging and imaging of paraffin-embedded sections resulting in differing neuron numbers. Studies analysing the effect of α-synuclein overexpression on DA neuron loss using a *TH*-GAL4 or *Elav*-GAL4 driver observed dramatic loss of TH-positive neurons in paraffin embedded sections (98)(20). One of these groups (121) repeated their original findings using whole mount techniques and surprisingly did not observe neuron loss from the dorsomedial clusters, however, observed weakened TH staining that was specific to the dorsomedial clusters. There is some controversy in *Drosophila* PD models as to whether there is true degeneration or changes in TH immunoreactivity which may suggest impaired function rather than cell death.

Interestingly, when α-synuclein is overexpressed along with dNedd4 knockdown, we found that staining became very weak making analysis of these neurons very difficult. This phenomenon was not observed when dNedd4 alone was knocked down. The DA neuron detection technique I have used in this study results in the appropriate complements of DA neurons as described in (22) in control flies. Therefore I believe this method to be reliable for DA neuron analysis. However, the difference in imaging technique, whole mount as opposed to sequential paraffin sections, is likely the cause of the discrepancies between counts observed.

Chapter 5

Discussion

5.1 SMN regulates growth and differentiation in *Drosophila*

In this study I aimed to analyse the role of SMN in growth and development in *Drosophila*. The observation of extra-neuromuscular defects in SMA patients suggests a role for SMN in development (51) (50) . The effect of reduction of SMN levels on muscle development and NMJ structure and function has been extensively studied in *Drosophila* (17) (40) (18), whereas the function of SMN in the development of other tissues has been largely overlooked. More recently *Drosophila* (48) and mouse (49) studies have implicated SMN in growth and differentiation, and growth pathways have been found to modify SMN-dependent phenotypes in *Drosophila* (18)(54) (58). Therefore, I aimed to further this investigation into SMN function in growth and differentiation in *Drosophila* with a focus on the development of the larval CNS, the eye imaginal disc and the female germline.

SMN was found to be highly expressed in the mitotically dividing larval tissues, the CNS and imaginal discs. In the male germline, SMN and the SMN complex members, Gemin3 and Gemin5 are enriched in stem cells and form a concentration gradient that corresponds to the developmental stage. In the female germline, SMN is highly expressed in the developing egg chambers.

Growth defects were observed in smn^B mutants and smn^{73A0} mitotic clones in the diploid tissues. Analysis of smn^B larvae at the wandering third instar stage and at a later time point suggest that growth is delayed in these mutants since the CNS of the larvae reach an appropriate size but after an elongated larval phase. A reduced cell size was shown to contribute to the growth defect observed in the CNS. Interestingly, in smn^B mutant larvae, the outer proliferating centre of the optic lobe was underdeveloped suggesting that low SMN levels are insufficient to support the derivation of this region of optic lobe neuroblasts. pNBs that are 'born' during the embryonic phase however, in which maternal levels of SMN have yet to be depleted, are able to survive. It is plausible that higher levels of SMN are required for neuroblast derivation than maintenance.

Mitotic clonal analysis showed severe proliferation defects in the eye imaginal disc that were cell autonomous. smn^{73A0} clones were outcompeted by their wild-type twins, resulting in no mutant clones or occasionally very small clones in the eye disc. It would be interesting to induce these clones in a *Minute* background, thereby weakening the growth potential of the competing twin spots.

SMN RNAi clones were able to proliferate in the eye imaginal disc. *smn*^{73A0} is a severe loss of function allele, therefore it is unsurprising that these clones produce a more severe defect than a reduction in levels by RNAi in the same tissue. These clones should be used to further understand the underlying mechanism of the growth defect observed. Efforts were made during this study to examine cell cycle phasing and cell size using FACS analysis in RNAi clones, in comparison to their wild type neighbours, however this was technically unsuccessful. This is an essential experiment in the understanding how SMN affects cell growth and proliferation. The relative contributions of apoptosis and defects in proliferation must also be considered in further experiments.

SMN regulates cell differentiation in the larval and adult testis (48) with SMN over-expression resulting in delayed differentiation and mutation causing premature differentiation into mature sperm. I have investigated whether this control of differentiation is unique to the testis or whether SMN serves as a master of differentiation regulation. The developing *Drosophila* eye is an excellent system in which to study differentiation given that cell differentiation occurs in a well characterised sequential fashion and all stages of differentiation can be visualised in each disc. Unlike the situation observed in the testis, differentiation defects were not observed in the developing eye when SMN levels were modified using the same overexpression lines. Similarly, RNAi did not cause differentiation defects in the eye, however this milder reduction in SMN levels could be insufficient to disrupt differentiation.

Interestingly, the cell-fate determinant, Prospero, was found to be mislocalised in *smn*^{B} larval CNS. Rather than being completely excluded to the ganglion mother cells, expression of Prospero protein can be observed in punctate structures in the post-embryonic neuroblasts. The presence of the transcription factor Prospero in the stem cells may have implications for their differentiation and division. The punctate Prospero containing foci can be seen to colocalise with the SMN complex components Gemin 3 and Gemin 5.

A key experiment in our understanding of the role of SMN in growth and differentiation is the investigation of the effect of SMN loss on neural cell lineage and fate in the CNS.

Given the high expression of SMN in the diploid tissues of the larva and the interest in the role of SMN in differentiation, mitotically dividing tissues were predominantly analysed, however whether SMN loss equally effects endocycling larval tissues such as the salivary gland should be considered in further experiments.

However, the endocycling nurse cells of the germline were considered in this study. Germline and mitotic clonal analysis revealed that SMN loss results in a highly penetrant nurse cell chromosome morphology defect. Unlike wild type nurse cell nuclei which have a dispersed organisation from stage 6 of oogenesis onwards, the nurse cell chromosomes of *smn* mutant egg chambers remain in a condensed state having a 'blob-like' appearance. This defect can be rescued upon overexpression of SMN.

The nurse cell chromosome morphology defect phenocopies a number of mutations in endocycle regulators including Cyclin E, APC/C, Dacapo and E2F1. These mutations and that of SMN all fail to appropriately transition from a condensed polytene chromosome structure at endocycle 5 to a dispersed polytene chromosome morphology at endocycle 6. This transition is very tightly linked to the cell cycle. A common feature of these mutations is an increase in heterochromatin due to the absence of a truncated S-phase at endocycle 5. It would be very interested to see if *smn* mutant nurse cells similarly displayed an over-representation of heterochromatin.

Chromatin modifications can dictate chromosome morphology during cell cycle progression. In particular methylation and acetylation of certain histone proteins result in chromosomes having a more condensed or dispersed appearance. Further investigation into whether chromatin modifications influence chromosome morphology in *smn* clones should be carried out.

Finally, SMN-containing U bodies have been shown to physically and genetically interact with P bodies (33). The P body component, Otu, has been proposed to control chromosome dispersal in an RNP complex with Squid and Hrb27C (75). It is suggested that this RNP complex regulates an unknown RNA target that controls chromosome dispersal at endocycle 5. SMN is known to function in the assembly of snRNPs and may function in the assembly of other RNP complexes. A potential hypothesis for the role of SMN in nurse cell chromosome morphology is the assembly of the Squid, Otu, Hrb27C RNP complex. This would be a very interesting line of investigation regarding the role of SMN in the regulation of nurse cell chromosome morphology.

5.2 Nedd4, a putative modifier of a *Drosophila* model of Parkinson's disease

The presence of α-synuclein containing inclusions in the brain is one of the hallmarks of Parkinson's disease and the identification of factors involved in α-synuclein clearance is an essential step towards the development of novel therapies. A recent study showed that the human E3 ubiquitin ligase, Nedd4 and its yeast orthologue, Rsp5p was able to ubiquitinate α-synuclein, thereby targeting it to the endosomal-lysosomal pathway for degradation (84). In this study, I have used a characterised *Drosophila* model of Parkinson's disease to investigate whether Nedd4 affords protection against α-synuclein-dependent phenotypes in the fly.

Overexpression of dNedd4 in an α-synuclein overexpression background resulted in decreased levels of α-synuclein protein in comparison to α-synuclein single transgenic flies. This finding implicates dNedd4 in the promotion of α-synuclein clearance *in* *vivo*. This finding should be further investigated in the fly; it is necessary to show that dNedd4 binds and ubiquitinates α-synuclein in *Drosophila*, paralleling the situation in yeast and cell lines.

The eye specific overexpression of α-synuclein was found to cause an age-related mild rough eye phenotype in *Drosophila* that was ameliorated by the overexpression of dNedd4. This finding suggests that α-synuclein and dNedd4 genetically interact and that dNedd4 can protect against α-synuclein-induced toxicity in the eye. This finding should be furthered by quantification of the phenotype by the analysis of photoreceptor degeneration by retinal sectioning. Also, this finding would be strengthened by the analysis of the α-synuclein$^{1-120}$ line. The α-synuclein$^{1-120}$ transgene lacks the C-terminal proline-rich motif proposed to bind to Nedd4. Therefore the protection afforded by dNedd4 should be abrogated upon replacement of α-synuclein^{WT} with α-synuclein$^{1-120}$.

Given the clinical presentation of the disease, we wished to investigate the effect of dNedd4 levels on a previously reported α-synuclein-induced locomotor dysfunction phenotype in *Drosophila* (20). In preliminary experiments we observed an enhancement of age-related decline in climbing ability in flies overexpressing α-synuclein that was ameliorated by overexpression of dNedd4. This was complicated however, by the finding that knockdown of Nedd4 levels similarly rescued the enhancement of motor dysfunction. It is necessary to repeat these studies to confidently assess the effect of α-synuclein expression on locomotor function and the rescue by dNedd4.

Finally, dopaminergic neurons were analysed in α-synuclein transgenic flies. Dopaminergic neurons in the *Drosophila* adult do not degenerate with age (22) however, in α-synuclein expressing flies, a marked reduction in dopaminergic neurons in the dorsomedial cluster has been reported (20). In our hands, the overexpression of α-synuclein pan-neuronally or specifically in the DA neurons did not cause degeneration of dopaminergic neurons. Inconsistencies in the dopaminergic neuron loss phenotype has been observed in a number of studies (120) and appears to be the result of differences in TH positive neuron detection. Paraffin embedded sections have generally been shown to display neuron loss, whereas whole mount analysis, similar to that performed in this study, does not result in DA neuron loss. It has been suggested than loss of dopaminergic neuron function rather than cell death may occur in *Drosophila* therefore the study of dopaminergic neuron activity as opposed to neuron loss is an important line of future investigation.

The ability of Nedd4 to reduce α-synuclein protein levels, modify α-synuclein-induced toxicity in the eye and in preliminary experiments, suppress the enhancement of decline in climbing ability in α-synuclein transgenic flies, supports the hypothesis that Nedd4 may afford protection against α-synuclein-induced toxicity *in vivo*. It has been proposed that Nedd4 targets α-synuclein for degradation via the ESCRT pathway. The investigation of the effects of other components of this pathway on α-synuclein in *Drosophila* should be examined in the future.

5.3 Concluding statement

In this study I have used *Drosophila* to investigate the function of SMN, the causative gene in SMA, in growth and development. SMN was shown to function in growth in the developing *Drosophila* larvae and in the differentiation of specific tissues. SMN was also found to regulate nurse cell chromosome morphology during oogenesis. I also used a *Drosophila* model of Parkinson's disease to investigate Nedd4, a putative suppressor of α-synuclein-induced toxicity. Although further work is necessary for confirmation, this study provides compelling evidence supporting the hypothesis that Nedd4 affords protection against α-synuclein toxicity *in vivo*.

References

[1] **Global burden of neurological disorders: estimates and projections**. In *Neurological disorders: public health challenges*, chapter 2, pages 26–39. WHO, 1st edition. 1

[2] B a Edgar and C F Lehner. **Developmental control of cell cycle regulators: a fly's perspective.** *Science (New York, N.Y.)*, **274**(5293):1646–52, December 1996. 2

[3] H Stocker and E Hafen. **Genetic control of cell size.** *Current opinion in genetics & development*, **10**(5):529–35, October 2000. 2

[4] Ville Hietakangas and Stephen M Cohen. **Regulation of tissue growth through nutrient sensing.** *Annual review of genetics*, **43**:389–410, January 2009. 2

[5] Chris P Ponting. **The functional repertoires of metazoan genomes.** *Nature reviews. Genetics*, **9**(9):689–98, September 2008. 2

[6] André Pires-daSilva and Ralf J Sommer. **The evolution of signalling pathways in animal development.** *Nature reviews. Genetics*, **4**(1):39–49, January 2003. 2

[7] F Hirth and H Reichert. **Conserved genetic programs in insect and mammalian brain development.** *BioEssays : news and reviews in molecular, cellular and developmental biology*, **21**(8):677–84, August 1999. 2

[8] L T Reiter, L Potocki, S Chien, M Gribskov, and E Bier. **A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster.** *Genome research*, **11**(6):1114–25, June 2001. 2

[9] a H Brand and N Perrimon. **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development (Cambridge, England)*, **118**(2):401–15, June 1993. 3, 4, 115

[10] J Lawrence Marsh and Leslie Michels Thompson. **Drosophila in the study of neurodegenerative disease.** *Neuron*, **52**(1):169–78, October 2006. 5

[11] Joseph B Duffy. **GAL4 system in Drosophila: a fly geneticist's Swiss army knife.** *Genesis (New York, N.Y. : 2000)*, **34**(1-2):1–15, 2002. 5, 10

[12] Frank Hirth. **Drosophila melanogaster in the study of human neurodegeneration.** *CNS & neurological disorders drug targets*, **9**(4):504–23, August 2010. 5, 6, 140

[13] Ruben J Cauchi and Marcel van den Heuvel. **The fly as a model for neurodegenerative diseases: is it worth the jump?** *Neuro-degenerative diseases*, **3**(6):338–56, January 2006. 5

[14] T B Chou and N Perrimon. **The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster.** *Genetics*, **144**(4):1673–9, December 1996. 6, 8, 15, 16

[15] Daniel St Johnston. **The art and design of genetic screens: Drosophila melanogaster.** *Nature reviews. Genetics*, **3**(3):176–88, March 2002. 7, 9

[16] F Pignoni and S L Zipursky. **Induction of Drosophila eye development by decapentaplegic.** *Development (Cambridge, England)*, **124**(2):271–8, January 1997. 10

[17] Y. B. Chan. **Neuromuscular defects in a Drosophila survival motor neuron gene mutant**. *Human Molecular Genetics*, **12**(¿12):1367–1376, June 2003. 13, 28, 30, 31, 40, 91, 145

[18] Howard Chia-Hao Chang, Douglas N Dimlich, Takakazu Yokokura, Ashim Mukherjee, Mark W Kankel, Anindya Sen, Vasanthi Sridhar, Tudor a Fulga, Anne C Hart, David Van Vactor, and Spyros Artavanis-Tsakonas. **Modeling spinal muscular atrophy in Drosophila.** *PloS one*, **3**(9):e3209, January 2008. 13, 28, 30, 31, 60, 91, 145

[19] Ji-Long Liu, Michael Buszczak, and Joseph G Gall. **Nuclear bodies in the Drosophila germinal vesicle.** *Chromosome research*, **14**(4):465–75, January 2006. 13

[20] Mel B Feany and Welcome W Bender. **A Drosophila model of Parkinson ' s disease**. *Nature*, **404**(March), 2000. 13, 96, 98, 112, 113, 114, 115, 122, 130, 133, 134, 139, 143, 151, 152

[21] Anna Myat, Pauline Henry, Veronica Mccabe, Louisa Flintoft, Daniela Rotin, and Guy Tear. **Drosophila Nedd4 , a Ubiquitin Ligase , Is Recruited by Commissureless to Control Cell Surface Levels of the Roundabout Receptor**. *Neuron*, **35**:447–459, 2002. 13, 115, 117, 124

[22] Katherine E White, Dickon M Humphrey, Frank Hirth, Sean T Sweeney, Serge Birman, and Centre National De. **The dopaminergic system in the aging brain of Drosophila**. *Frontiers in neuroscience*, **4**(December):1–12, 2010. 21, 113, 133, 142, 143, 152

[23] S Lefebvre, L Bürglen, S Reboullet, O Clermont, P Burlet, L Viollet, B Benichou, C Cruaud, P Millasseau, and M Zeviani. **Identification and characterization of a spinal muscular atrophy-determining gene.** *Cell*, **80**(1):155–65, January 1995. 23, 24, 90

[24] U R Monani, D D Coovert, and a H Burghes. **Animal models of spinal muscular atrophy.** *Human molecular genetics*, **9**(16):2451–7, October 2000. 23, 24, 26

[25] Umrao R Monani. **Spinal muscular atrophy: a deficiency in a ubiquitous protein; a motor neuron-specific disease.** *Neuron*, **48**(6):885–96, December 2005. 23

[26] Charlotte J Sumner. **Molecular mechanisms of spinal muscular atrophy.** *Journal of child neurology*, **22**(8):979–89, August 2007. 23

[27] Arthur H M Burghes and Christine E Beattie. **Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick?** *Nature reviews. Neuroscience*, **10**(8):597–609, August 2009. 25, 27

[28] Karl B Shpargel and a Gregory Matera. **Gemin proteins are required for efficient assembly of Sm-class ribonucleoproteins.** *Proceedings of the National Academy of Sciences of the United States of America*, **102**(48):17372–7, November 2005. 24

[29] Q Liu, U Fischer, F Wang, and G Dreyfuss. **The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins.** *Cell*, **90**(6):1013–21, September 1997. 24, 26

[30] Stuart J Grice, James N Sleigh, Ji-Long Liu, and David B Sattelle. **Invertebrate models of spinal muscular atrophy: Insights into mechanisms and potential therapeutics.** *BioEssays : news and reviews in molecular, cellular and developmental biology*, **33**(12):956–65, December 2011. 26

[31] Ji-long Liu, Zheng Wu, Zehra Nizami, Svetlana Deryusheva, T K Rajendra, Kelly J Beumer, Hongjuan Gao, A Gregory Matera, Dana Carroll, and Joseph G Gall. **Coilin Is Essential for Cajal Body Organization in Drosophila melanogaster**. *Molecular Biology of the Cell*, **20**:1661–1670, 2009. 26

[32] Alexander-Francisco Bruns, Jeroen van Bergeijk, Christina Lorbeer, Anna Nölle, Julia Jungnickel, Claudia Grothe, and Peter Claus. **Fibroblast growth factor-2 regulates the stability of nuclear bodies.** *Proceedings of the National Academy of Sciences of the United States of America*, **106**(31):12747–52, August 2009. 26

[33] Lin Lee, Siân E Davies, and Ji-Long Liu. **The spinal muscular atrophy protein SMN affects Drosophila germline nuclear organization through the U body-P body pathway.** *Developmental biology*, **332**(1):142–55, August 2009. 26, 33, 68, 73, 88, 89, 149

[34] Ruben J Cauchi, Luis Sanchez-Pulido, and Ji-Long Liu. **Drosophila SMN complex proteins Gemin2, Gemin3, and Gemin5 are components of U bodies.** *Experimental cell research*, **316**(14):2354–64, August 2010. 26, 45

[35] Francesca Gabanella, Claudia Carissimi, Alessandro Usiello, and Livio Pellizzoni. **The activity of the spinal muscular atrophy protein is regulated during development and cellular differentiation.** *Human molecular genetics*, **14**(23):3629–42, December 2005. 28

[36] A Fidziaska, J Rafaowska, and Z Glinka. **Ultrastructural study of moetoneurons in Werdnig-Hoffmann disease**. *Clin Neuropathol.*, **3**(6):260–5, 1984. 28

[37] G Vrbová. **Understanding motoneurone development explains spinal muscular atrophy**. *Arch Ital Biol.*, **145**(3-4):325–35, 2007. 28

[38] Lyndsay M Murray, Laura H Comley, Derek Thomson, Nick Parkinson, Kevin Talbot, and Thomas H Gillingwater. **Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy.** *Human molecular genetics*, **17**(7):949–62, April 2008. 28

[39] Shingo Kariya, Gyu-Hwan Park, Yuka Maeno-Hikichi, Olga Leykekhman, Cathleen Lutz, Marc S Arkovitz, Lynn T Landmesser, and Umrao R Monani. **Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy.** *Human molecular genetics*, **17**(16):2552–69, August 2008. 28

[40] T K Rajendra, Graydon B Gonsalvez, Michael P Walker, Karl B Shpargel, Helen K Salz, and a Gregory Matera. **A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle.** *The Journal of cell biology*, **176**(6):831–41, March 2007. 28, 39, 40, 91, 145

[41] A Fidzianska, H Goebel, and I Warlo. **Acute infantile spinal muscular atrophy. Muscle apoptosis as a proposed pathogenetic mechanism.** *Brain*, **113**(2):433–445, 1990. 28

[42] Rebeca Martínez-Hernández, Carolina Soler-Botija, Eva Also, Laura Alias, Lidia Caselles, Ignasi Gich, Sara Bernal, and Eduardo F Tizzano. **The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation.** *Journal of neuropathology and experimental neurology*, **68**(5):474–81, May 2009. 28

[43] Sibylle Jablonka, Stefan Wiese, and Michael Sendtner. **Axonal defects in mouse models of motoneuron disease.** *Journal of neurobiology*, **58**(2):272–86, February 2004. 28

[44] Wilfried Rossoll, Sibylle Jablonka, Catia Andreassi, Ann-Kathrin Kröning, Kathrin Karle, Umrao R Monani, and Michael Sendtner. **Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons.** *The Journal of cell biology*, **163**(4):801–12, November 2003. 28

[45] M. L. McWhorter, U.R Monani, A.H.M Burghes, and C.E Beattie. **Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding**. *The Journal of Cell Biology*, **162**(5):919–932, September 2003. 28

[46] Christian Eggert, Ashwin Chari, Bernhard Laggerbauer, and Utz Fischer. **Spinal muscular atrophy: the RNP connection.** *Trends in molecular medicine*, **12**(3):113–21, March 2006. 28

[47] M B Lowrie and G Vrbová. **Dependence of postnatal motoneurones on their targets: review and hypothesis.** *Trends in neurosciences*, **15**(3):80–4, March 1992. 28

[48] Stuart J Grice and Ji-Long Liu. **Survival motor neuron protein regulates stem cell division, proliferation, and differentiation in Drosophila.** *PLoS genetics*, **7**(4):e1002030, April 2011. 29, 43, 54, 58, 62, 72, 82, 83, 86, 91, 145, 147

[49] Thomas M Wishart, Jack P-W Huang, Lyndsay M Murray, Douglas J Lamont, Chantal a Mutsaers, Jenny Ross, Pascal Geldsetzer, Olaf Ansorge, Kevin Talbot, Simon H Parson, and Thomas H Gillingwater. **SMN deficiency disrupts brain development in a mouse model of severe spinal muscular atrophy.** *Human molecular genetics*, **19**(21):4216–28, November 2010. 29, 91, 145

[50] S Rudnik-Schöneborn, R Heller, C Berg, C Betzler, T Grimm, T Eggermann, K Eggermann, R Wirth, B Wirth, and K Zerres. **Congenital heart disease is a feature of severe infantile spinal muscular atrophy.** *Journal of medical genetics*, **45**(10):635–8, October 2008. 29, 91, 145

[51] Srinivasan Shanmugarajan, Eichi Tsuruga, Kathryn J Swoboda, Bernard L Maria, and William L Ries. **Bone loss in survival motor neuron (Smn / SMN2) genetic mouse model of spinal muscular atrophy**. *Journal of Pathology*, (April):52–60, 2009. 29, 91, 145

[52] Hermann Aberle, A Pejmun Haghighi, Richard D Fetter, Brian D Mccabe, Tiago R Magalha, and Corey S Goodman. **wishful thinking Encodes a BMP Type II Receptor that Regulates Synaptic Growth in Drosophila**. *Neuron*, **33**:545–558, 2002. 31

[53] Brian D Mccabe, Guillermo Marque, A Pejmun Haghighi, Richard D Fetter, M Lisa Crotty, Theodore E Haerry, Corey S Goodman, Michael B O Connor, South San Francisco, and Sax Marque. **The BMP Homolog Gbb Provides a Retrograde Signal that Regulates Synaptic Growth at the Drosophila Neuromuscular Junction**. *Neuron*, **39**:241–254, 2003. 31

[54] Anindya Sen, Takakazu Yokokura, Mark W Kankel, Douglas N Dimlich, Jan Manent, Subhabrata Sanyal, and Spyros Artavanis-Tsakonas. **Modeling spinal muscular atrophy in Drosophila links Smn to FGF signaling.** *The Journal of cell biology*, **192**(3):481–95, February 2011. 31, 91, 145

[55] Peng Huang and Michael J Stern. **FGF signaling in flies and worms : More and more relevant to vertebrate biology**. *Cytokine and Growth Factor Reviews*, **16**:151–158, 2005. 31

[56] Marion Silies, Yeliz Yuva-aydemir, Sigrídur Rut Franzdóttir, and Christian Klämbt. **The eye imaginal disc as a model to study the coordination of neuronal and glial development**. *Fly*, (March):71–79, 2010. 31, 33

[57] Sigrídur Rut Franzdóttir, Daniel Engelen, Yeliz Yuva-Aydemir, Imke Schmidt, Annukka Aho, and Christian Klämbt. **Switch in FGF signalling initiates glial differentiation in the Drosophila eye.** *Nature*, **460**(7256):758–61, August 2009. 31

[58] Ke Ning, Carsten Drepper, Chiara F Valori, Mansoor Ahsan, Matthew Wyles, Adrian Higginbottom, Thomas Herrmann, Pamela Shaw, Mimoun Azzouz, and Michael Sendtner. **PTEN depletion rescues axonal growth defect and improves survival in SMN-deficient motor neurons.** *Human molecular genetics*, **19**(16):3159–68, August 2010. 32, 91, 145

[59] Barry J Thompson. **Developmental control of cell growth and division in Drosophila.** *Current opinion in cell biology*, **22**(6):788–94, December 2010. 32, 33

[60] B a Edgar and T L Orr-Weaver. **Endoreplication cell cycles: more for less.** *Cell*, **105**(3):297–306, May 2001. 33

[61] Astrid Jeibmann and Werner Paulus. **Drosophila melanogaster as a model organism of brain diseases.** *International journal of molecular sciences*, **10**(2):407–40, February 2009. 34

[62] Jean-Yves Roignant and Jessica E Treisman. **Pattern formation in the Drosophila eye disc.** *The International journal of developmental biology*, **53**(5-6):795–804, January 2009. 36

[63] Rita Sousa-Nunes, Louise Y Cheng, and Alex P Gould. **Regulating neural proliferation in the Drosophila CNS.** *Current opinion in neurobiology*, **20**(1):50–7, February 2010. 35, 37

[64] Boris Egger, James M Chell, and Andrea H Brand. **Insights into neural stem cell biology from flies. Phil. Tran**(February 2007):39–56, 2008. 35

[65] Fengwei Yu, Chay T Kuo, and Yuh Nung Jan. **Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology.** *Neuron*, **51**(1):13–20, July 2006. 38

[66] Jason Q Boone and Chris Q Doe. **Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells.** *Developmental neurobiology*, **68**(9):1185–95, August 2008. 52, 55

[67] Rebecca Bastock and Daniel St Johnston. **Drosophila oogenesis.** *Current biology : CB*, **18**(23):R1082–7, December 2008. 70, 71

[68] Hyun O Lee, Jean M Davidson, and Robert J Duronio. **Endoreplication: polyploidy with purpose.** *Genes & development*, **23**(21):2461–77, November 2009. 70

[69] K J Dej and a C Spradling. **The endocycle controls nurse cell polytene chromosome structure during Drosophila oogenesis.** *Development (Cambridge, England)*, **126**(2):293–303, January 1999. 70, 71

[70] John M Abrams. **Competition and compensation: coupled to death in development and cancer.** *Cell*, **110**(4):403–6, August 2002. 83

[71] M a Lilly and a C Spradling. **The Drosophila endocycle is controlled by Cyclin E and lacks a checkpoint ensuring S-phase completion.** *Genes & Development*, **10**(19):2514–2526, October 1996. 86

[72] Helena Kashevsky, Julie a Wallace, Bruce H Reed, Cary Lai, Aki Hayashi-Hagihara, and Terry L Orr-Weaver. **The anaphase promoting complex/cyclosome is required during development for modified cell cycles.** *Proceedings of the National Academy of Sciences of the United States of America*, **99**(17):11217–22, August 2002. 86

[73] Amy Hong, Karine Narbonne-Reveau, Juan Riesgo-Escovar, Haiqing Fu, Mirit I Aladjem, and Mary a Lilly. **The cyclin-dependent kinase inhibitor Dacapo promotes replication licensing during Drosophila endocycles.** *The EMBO journal*, **26**(8):2071–82, April 2007. 86

[74] Irena Royzman, Aki Hayashi-Hagihara, Kimberley J Dej, Giovanni Bosco, Janice Y Lee, and Terry L Orr-Weaver. **The E2F cell cycle regulator is required for Drosophila nurse cell DNA replication and apoptosis.** *Mechanisms of development*, **119**(2):225–37, December 2002. 86

[75] Jennifer S Goodrich, K Nicole Clouse, and Trudi Schüpbach. **Hrb27C, Sqd and Otu cooperatively regulate gurken RNA localization and mediate nurse cell chromosome dispersion in Drosophila oogenesis.** *Development (Cambridge, England)*, **131**(9):1949–58, May 2004. 88, 89, 149

[76] Linda N Keyes and Allan C Spradling. **pathway required for the structure and function of germline chromosomes**. *Development*, **1431**:1419–1431, 1997. 88

[77] Cheryl Van Buskirk and Trudi Schüpbach. **Half pint regulates alternative splice site selection in Drosophila.** *Developmental cell*, **2**(3):343–53, March 2002. 88

[78] Ana Eulalio, Isabelle Behm-Ansmant, and Elisa Izaurralde. **P bodies: at the crossroads of post-transcriptional pathways.** *Nature reviews. Molecular cell biology*, **8**(1):9–22, January 2007. 88

[79] U Sheth and R Parker. **Decapping and Decay of Messenger RNA Occur in Cytoplasmic Processing Bodies**. *Science*, **300**(5620):805–808, 2003. 88

[80] Ji-Long Liu and Joseph G Gall. **U bodies are cytoplasmic structures that contain uridine-rich small nuclear ribonucleoproteins and associate with P bodies.** *Proceedings of the National Academy of Sciences of the United States of America*, **104**(28):11655–9, July 2007. 88

[81] Emily Clough, Woongjoon Moon, Shengxian Wang, Kathleen Smith, and Tulle Hazelrigg. **Histone methylation is required for oogenesis in Drosophila.** *Development (Cambridge, England)*, **134**(1):157–65, January 2007. 89

[82] Jeremy A Daniel, Marilyn G Pray-grant, and Patrick A Grant. **Effector Proteins for Methylated Histones**. *Cell cycle*, (July):919–926, 2005. 89

[83] Manika Pal-Bhadra, Boris a Leibovitch, Sumit G Gandhi, Madhusudana Rao, Utpal Bhadra, James a Birchler, and Sarah C R Elgin. **Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery.** *Science (New York, N.Y.)*, **303**(5658):669–72, January 2004. 90

[84] George K Tofaris, Hyoung Tae, Raphael Hourez, Jin-woo Jung, Kwang Pyo, and Alfred L Goldberg. **Ubiquitin ligase Nedd4 promotes α-synuclein degradation by the endosomal lysosomal pathway**. *PNAS*, 2011. 93, 108, 110, 111, 115, 150

[85] Sonia Gandhi and Nicholas W Wood. **Molecular pathogenesis of Parkinson's disease**. *Human Molecular Genetics*, **14**(18):2749–2755, 2005. 94, 95, 100

[86] Andrew J Lees, John Hardy, and Tamas Revesz. **Parkinson's disease.** *Lancet*, **373**(9680):2055–66, June 2009. 94, 95

[87] Ian Martin, Valina L Dawson, and Ted M Dawson. **Recent advances in the genetics of Parkinson's disease.** *Annual review of genomics and human genetics*, **12**:301–25, September 2011. 94

[88] Maria Grazia Spillantini, Marie Luise Schmidt, Virginia M Lee, John Q Trojanowski, Ross Jakes, and Michael Goedert. **Alpha-synuclein in Lewy bodies**. *Nature*, **388**:839–840, 1997. 95

[89] William Dauer and Serge Przedborski. **Parkinson's Disease : Mechanisms and Models**. *Neuron*, **39**:889–909, 2003. 95, 96, 101

[90] Serena Bellani, L Sousa, and Jacopo Meldolesi. **The regulation of synaptic function by α-synuclein**. **3**(2):106–109, 2010. 95

[91] Linda Narhi, Stephen J Wood, Shirley Steavenson, Yijia Jiang, Gay May Wu, Dan Anafi, Stephen A Kaufman, Francis Martin, Karen Sitney, Paul Denis, Jean-claude Louis, Jette Wypych, Anja Leona Biere, and Martin Citron. **Both Familial Parkinson s Disease Mutations Accelerate Alpha-Synuclein Aggregation**. **274**(14):9843–9846, 1999. 96

[92] Benoit I Giasson, John E Duda, Ian V J Murray, Qiping Chen, M Souza, Howard I Hurtig, Harry Ischiropoulos, John Q Trojanowski, and Virginia M Lee. **Oxidative Damage Linked to Neurodegeneration by Selective -Synuclein Nitration in Synucleinopathy Lesions**. *Science*, **985**(2000), 2012. 96

[93] Deniz Kirik, Carl Rosenblad, Corinna Burger, Cecilia Lundberg, Teit E Johansen, Nicholas Muzyczka, Ronald J Mandel, and Anders Bjo. **Parkinson-Like Neurodegeneration Induced by Targeted Overexpression of -Synuclein in the Nigrostriatal System**. *Gene Therapy*, **22**(7):2780–2791, 2002. 96, 98

[94] Benoit I Giasson, John E Duda, Shawn M Quinn, Bin Zhang, John Q Trojanowski, and Virginia M Lee. **Neuronal α-Synucleinopathy with Severe Movement Disorder in Mice Expressing A53T Human α-Synuclein**. *Neuron*, **34**:521–533, 2002. 98

[95] Magali Periquet, Tudor Fulga, Liisa Myllykangas, Michael G Schlossmacher, and Mel B Feany. **Aggregated α-Synuclein Mediates Dopaminergic Neurotoxicity In Vivo**. *J. Neurosci.*, **27**(12):3338 –3346, 2007. 98, 99, 133

[96] Li Chen, Magali Periquet, Xu Wang, Alessandro Negro, Pamela J Mclean, Bradley T Hyman, and Mel B Feany. **Tyrosine and serine phosphorylation of α-synuclein have opposing effects on neurotoxicity and soluble oligomer formation**. *The Journal of Clinical Investigation*, **119**(11):3257–3265, 2009. 99, 133

[97] Li Chen and Mel B Feany. **α-Synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease**. *Nature Neuroscience*, **8**(5):657–663, 2005. 99, 133

[98] Pavan K Auluck. **Chaperone Suppression of α-Synuclein Toxicity in a Drosophila Model for Parkinson's Disease**. *Science*, **865**(2002), 2012. 100, 133, 134, 142, 143

[99] Daniela Rotin and Sharad Kumar. **Physiological functions of the HECT family of ubiquitin ligases**. *Nature reviews. Molecular cell biology*, **10**(June):398–409, 2009. 101, 102, 108, 109

[100] Casey Cook, Caroline Stetler, Leonard Petrucelli, Sonja W Scholz, Tim Mhyre, Habtom Ressom, Dennis W Dickson, Melinda A Lynch-day, Kai Mao, Ke Wang, Katerina Venderova, and David S Park. **Disruption of Protein Quality Control in Parkinson ' s Disease**. *Cold Spring Harbor Perspectives in Medicine*, 2012. 101

[101] Esther Wong, Ana Maria Cuervo, Christopher M Dobson, Daryl A Bosco, Matthew J Lavoie, A Gregory, Bryan Chen, Marco Retzlaff, Thomas Roos, Rebecca C Taylor, Andrew Dillin, and Steven Finkbeiner. **Integration of Clearance Mechanisms : The Proteasome and Autophagy Integration of Clearance Mechanisms : The Proteasome and Autophagy**. *Cold Spring Harbor Perspectives in Biology*, 2012. 102, 106

[102] Lynn Bedford, David Hay, Anny Devoy, Simon Paine, Des G Powe, Rashmi Seth, Trevor Gray, Ian Topham, Kevin Fone, Nooshin Rezvani, Maureen Mee, Tim Soane, Robert Layfield, Paul W Sheppard, Ted Ebendal, Dmitry Usoskin, James Lowe, and R John Mayer. **Depletion of 26S proteasomes in mouse brain neurons causes neurodegeneration and Lewy-like inclusions resembling human pale bodies.** *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **28**(33):8189–98, August 2008. 101

[103] Esther Wong and Ana Maria Cuervo. **Autophagy gone awry in neurodegenerative diseases**. *Nature neuroscience*, **13**(7):805–811, 2010. 101, 105

[104] L Stefanis, K E Larsen, H J Rideout, D Sulzer, and L a Greene. **Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death.** *The Journal of neuroscience*, **21**(24):9549–60, December 2001. 101

[105] Julie L Webb, Brinda Ravikumar, Jane Atkins, Jeremy N Skepper, and David C Rubinsztein. **α-Synuclein Is Degraded by Both Autophagy and the Proteasome ***. *Biochemistry*, **278**(27):25009 –25013, 2003. 101, 107, 110

[106] M Catherine Bennett, John F Bishop, Yan Leng, P Boon Chock, Thomas N Chase, and M Maral Mouradian. **Degradation of -Synuclein by Proteasome ***. *The Journal of biological chemistry*, (14):33855–33858, 1999. 101, 110

[107] George K Tofaris, Robert Lay, and Maria Grazia Spillantini. **α-Synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome**. *FEBS*, **509**:22–26, 2001. 103, 110

[108] George K Tofaris, Azam Razzaq, Bernardino Ghetti, Kathryn S Lilley, and Maria Grazia Spillantini. **Ubiquitination of α-Synuclein in Lewy Bodies Is a Pathological Event Not Associated with Impairment of Proteasome Function ***. *The Journal of biological chemistry*, **278**(45):44405–44411, 2003. 103, 110

[109] Camilla Raiborg and Harald Stenmark. **The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins**. *Nature*, **458**(March):445–452, 2009. 105, 107

[110] B Yang and S Kumar. **Nedd4 and Nedd4-2 : closely related ubiquitin-protein ligases with distinct physiological functions**. *Cell Death and Differentiation*, **17**(1):68–77, 2009. 108, 109

[111] Kieran F Harvey and Sharad Kumar. **Nedd4-like proteins : an emerging family of ligases implicated in diverse cellular functions**. *Trends in Cell Biology*, **8924**(99):166–169, 1999. 109

[112] Sally K Mak, Alison L Mccormack, Amy B Manning-bog, Ana Maria Cuervo, and Donato A Di Monte. **Lysosomal Degradation of α-Synuclein in Vivo *** . *Journal of Biological Chemistry*, **285**(18):13621–13629, 2010. 110

[113] Ana Maria Cuervo, Esther S P Wong, and Marta Martinez-vicente. **Protein Degradation , Aggregation , and Misfolding**. *Movement Disorders*, **25**, 2010. 110

[114] Robert G Pendleton, Feroz Parvez, Marwa Sayed, and Ralph Hillman. **Effects of Pharmacological Agents upon a Transgenic Model of Parkinson s Disease in Drosophila melanogaster**. *Pharmacology*, **300**(1):91–96, 2002. 113

[115] Yunan Zhong, Alina Shtineman-kotler, Leo Nguyen, Konstantin G Iliadi, L Gabrielle, and Daniela Rotin. **A Splice Isoform of DNedd4 , DNedd4-Long , Negatively Regulates Neuromuscular Synaptogenesis and Viability in Drosophila**. *PloS one*, **6**(11), 2011. 122, 124

[116] Bryant Ing, Alina Shteiman-kotler, Marylisa Castelli, Pauline Henry, Youngshil Pak, Bryan Stewart, Gabrielle L Boulianne, and Daniela Rotin. **Regulation of Commissureless by the Ubiquitin Ligase DNedd4 Is Required for Neuromuscular Synaptogenesis in**. *Molecular and Cellular Biology*, **27**(2):481–496, 2007. 124

[117] Marian B Wilkin, Ann-marie Carbery, Maggy Fostier, Hanna Aslam, Sabine L Mazaleyrat, Jenny Higgs, Anna Myat, Dana A P Evans, Michael Cornell, and Martin

Baron. **Regulation of Notch Endosomal Sorting and Signaling by Drosophila Nedd4 Family Proteins**. *Current biology : CB*, **14**(dx):2237–2244, 2004. 124

[118] Yufeng Yang, Isao Nishimura, Yuzuru Imai, Ryosuke Takahashi, and Bingwei Lu. **Parkin Suppresses Dopaminergic Neuron-Selective Neurotoxicity Induced by Pael-R in Drosophila**. *Neuron*, **37**:911–924, 2003. 133

[119] Frank Hsiung and Kevin Moses. **Retinal development in Drosophila: specifying the first neuron.** *Human molecular genetics*, **11**(10):1207–14, May 2002. 139

[120] Yakov Pesah, Heather Burgess, Brooke Middlebrooks, Kari Ronningen, Jude Prosser, Vijaya Tirunagaru, John Zysk, and Graeme Mardon. **Whole-Mount Analysis Reveals Normal Numbers of Dopaminergic Neurons Following Misexpression of a-Synuclein in Drosophila**. *Genesis*, **159**:154–159, 2005. 142, 152

[121] Pavan K Auluck, Marc C Meulener, and Nancy M Bonini. **Mechanisms of Suppression of α-Synuclein Neurotoxicity by Geldanamycin in Drosophila.** *The Journal of biological chemistry*, **280**(4):2873–8, January 2005. 143

Statement of Authorship

I can confirm that except where acknowledged, this thesis is wholly my own work. No part of this thesis has been submitted for any other degree in this university or elsewhere.

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