'Non-self' mutation in a Drosophila model of expanded CAG repeat neurodegenerative disease

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Andrew William Scott, BSc. (Hons)

School of Biological Sciences, Department of Molecular and Biomedical Science

The University of Adelaide

Table of Contents

Index of Figures and Tables

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

VI

Declaration

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x

Abbreviations

°C: degrees Celsius

µg: microgram

µL: microlitre

Aβ: amyloid beta

AD: Alzheimer's disease

ADAR: Adenosine deaminase acting on RNA

AGO: Argonaute

AGS: Aicardi-Goutieres syndrome

ALG: astrocyte-like glia

ALS: Amyotrophic lateral sclerosis

AMP: antimicrobial peptide

A-T: Ataxia telangiectasia

ATXN: Ataxin

BAC: bacterial artificial chromosome

BBB: blood-brain barrier

C1q: Complement component 1q

CARD: Caspase recruitment domain

cDNA: complementary DNA

CG: cortex glia

cGAS: Cyclic GMP-AMP synthase

cm: centimetre

CrPV: Cricket paralysis virus

DAMP: damage/danger-associated molecular pattern

Dcr: Dicer

DCV: Drosophila C virus

DM: Myotonic dystrophy

Dome: Domeless

Drp1: Dynamin related protein 1

DRPLA: Dentatorubral-pallidoluysian atrophy

Drpr: Draper

dsRNA: double-stranded RNA

ECM: extracellular matrix

EDTA: ethylene diamine tetra-acetic acid

EG: ensheathing glia

elav: embryonic lethal abnormal vision

ER: endoplasmic reticulum

FTD: Frontotemporal dementia

FXTAS: Fragile X tremor-ataxia syndrome

GFP: Green fluorescent protein

GMR: glass multimer reporter

HD: Huntington's disease

HDL2: Huntington's disease-like-2

Hop: Hopscotch

HTT: Huntington

IAP: Inhibitor of apoptosis

IFIH1: Interferon-induced helicase C domain-containing protein 1

IFN: Interferon

IKK: IκB kinase

IL: Interleukin

IMD: Immune deficiency

IMM: inner mitochondrial membrane

IR: inverted repeat

IRF3: Interferon regulatory factor 3

JAK: Janus kinase

JNK: c-Jun N-terminal kinase

LGB: lateral glioblast

LGP2: Laboratory of Genetics and Physiology 2

Loqs: Loquacious

M: molar

MANF: Mesencephalic astrocyte-derived neurotrophic factor

MAVS: Mitochondrial antiviral-signalling protein

MBNL: Muscleblind-like

Mcr: Macroglobulin complement-related

MDA5: Melanoma differentiation-associated protein 5

mg: milligram

MiC: MANF immunoreactive cell

MiMIC: Minos mediated integration cassette

mL: millilitre

mM: millimolar

mm: millimetre

MQ: MilliQ™ purified water

mRNA: messenger RNA

MS: Multiple sclerosis

mtDNA: mitochondrial DNA

mtROS: mitochondrial ROS

NEG: neuropil ensheathing glia

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

ng: nanogram

NG2: Nerve-glial antigen 2

NLR: NOD-like receptor

nM: nanomolar

NOD: Nucleotide-binding oligomerization domain

NPC: neuroepithelial precursor cell

nrv2: nervana 2

NVU: neurovascular unit

OMM: outer mitochondrial membrane

OPC: oligodendrocyte precursor cell

ORF: open reading frame

PAMP: pathogen-associated molecular pattern

PBac: PiggyBac

PCR: polymerase chain reaction

PD: Parkinson's disease

PG: perineural glia

PINK1: PTEN-induced kinase 1

pmol: picomole

polyQ: polyglutamine

PRR: pattern recognition receptor

qRT-PCR: quantitative real-time polymerase chain reaction

RAN: repeat-associated non-AUG

Ref(2)P: Refractory to sigma P

repo: reversed polarity

RIG-I: Retinoic acid-inducible gene I

RING: rapid iterative negative geotaxis

RISC: RNA-induced silencing complex

RLR: RIG-I-like receptor

RNAi: RNA interference

ROS: reactive oxygen species

Rp49: Ribosomal protein 49

RT-PCR: reverse transcription polymerase chain reaction

SCA: Spinocerebellar ataxia

SG: stress granule

siRNA: small interfering RNA

SJ: septate junction

SMBA: Spinal bulbar muscular atrophy

SOP: sensory organ progenitor

SPG: subperineural glia

SQSTM1: Sequestosome 1

ssRNA: single-stranded RNA

STAT: Signal transducer and activator of transcription

STING: Stimulator of Interferon genes

TAE: tris-acetate EDTA

TDP43: TAR-DNA binding protein

TEG: tract ensheathing glia

TJ: tight junction

TLR: Toll-like receptor

TNF: Tumour necrosis factor

UAS: upstream activation sequence

Upd: Unpaired

UPR: unfolded protein response

UPS: ubiquitin proteasome system

UTR: untranslated region

vDNA: viral DNA

VNC: ventral nerve cord

VSR: viral suppressor of RNAi silencing

w/v: weight/volume

Nomenclature

The *Drosophila* nomenclature used in this thesis is based on conventional notation as stated on the *Drosophila* database, Flybase (www.flybase.org). Genes are shown by italicised text (e.g. *htt*) and proteins are shown by non-italicised text (e.g. htt).

Abstract

Copy number expansion of tandem repeat sequences beyond a pathogenic threshold causes more than twenty neurodegenerative diseases known as dominantly-inherited expanded repeat diseases. The repeat expansions arise in a diverse range of genomic locations within unrelated genes. Repeat-containing RNA represents a product derived from all affected loci and has thus been hypothesised to constitute a plausible common pathogenic agent. Evidence for bi-directional transcription of repeat RNA, the products of which are predicted to form complementary double-stranded RNA (dsRNA), has been observed across all tested expanded repeat disease loci. Previous work has demonstrated that expression of repeat CAG.CUG dsRNA causes eye-specific and neuronal pathology in a *Drosophila* model of expanded repeat disease. Additional work revealed that repeat dsRNA not only induces an inflammatory response, but is dependent upon several components of the innate inflammatory system for the resultant pathology. This thesis uses this established *Drosophila* model of expanded repeat disease to further define the innate inflammatory mechanisms underlying expanded repeat dsRNA pathology at both the cellular and molecular level.

Co-expression of repeat dsRNA and a viral protein that inhibits antiviral pathway activation led to complete suppression of the resultant eye pathology. This suggests that the repeat dsRNA is recognized by the host antiviral machinery as a 'non-self' threat, thus inducing a damaging inflammatory response that causes the subsequent eye pathology. The reduction of key mitophagy components led to an enhancement of the repeat dsRNA eye pathology, indicating that mitochondrial quality control is protective in response to the expression of repeat dsRNA. The tissue-specific expression of the repeat dsRNA in glial cells responsible for the development and maintenance of the *Drosophila* blood-brain barrier led to neurodegeneration and mortality, highlighting these glial cell subtypes as key non-cell autonomous determinants of dsRNA-mediated neuronal dysfunction. The characterisation of pathways and cell types that underlie expanded repeat pathogenesis are critical for defining the molecular and cellular determinants of this novel 'non-self' RNA pathogenesis. The validation of this model as replicating the corresponding human diseases will enable the development of effective therapeutic interventions for this group of diseases.

CHAPTER 1: Introduction

Neurodegenerative disease constitutes an umbrella term describing a number of debilitating conditions characterised by neuronal loss and subsequent inhibition of cognitive and/or motor function. The probability of developing a neurodegenerative disease increases with age (particularly beyond 60 years of age) which, when married with the ageing global population, presents a significant and rising socioeconomic concern for both affected families and the broader public (1). However, despite decades of intense research focus into the mechanisms that underlie neurodegeneration, progress towards effective therapeutic intervention remains limited, serving to highlight the complexity of the causal agents mediating neurodegenerative disease development and progression.

Major neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Typically, two forms of each disease have been identified; inherited familial forms are early-onset and arise through genetic mutation, whilst the more common sporadic late-onset forms are thought to develop through contributions from both genetic risk factors and environmental stressors. The characterisation of causal disease genes and genetic risk factors implicated in neurodegenerative disease has provided clues into the responsible underlying pathways.

Of these diseases, HD distinguishes itself by way of constituting a monogenic disorder, whereas all of AD, PD, ALS and FTD can arise through mutations in a number of separate (but often related) genes (2). Indeed, HD belongs to a distinct group of monogenic disorders, together referred to as expanded repeat neurodegenerative diseases. Investigation into the pathophysiology of HD and related CAG expanded repeat disorders forms the primary focus of this thesis.

1.1 Expanded repeat neurodegenerative disease

The copy number expansion of a repeat sequence beyond a pathogenic threshold gives rise to more than 20 dominantly-inherited neurodegenerative disorders **(Figure 1.1)** (3, 4). The repeat sequences are highly unstable, leading to their expansion via defective DNA mismatch repair (5-7). As such, expanded repeat diseases generally exhibit a molecular phenomenon known as anticipation, whereby further expansion of the repeat length through subsequent generations correlates with more severe clinical symptoms and an earlier age-of-onset (8-10). Despite the individual repeat expansion lesions occurring in unrelated genes **(Figure 1.1)**, there exists substantial overlap between the diseases in terms of neurological symptoms and copy number repeat disease thresholds, suggesting that a common pathogenic pathway may underlie this family of diseases (4). Indeed, the recent discovery of a hexanucleotide GGGGCC repeat constituting the most common cause of familial ALS/FTD (11, 12) has highlighted the idea that other forms of neurodegenerative disease may also share commonality in disease development and progression.

1.1.1 Polyglutamine (PolyQ)

There are several pathogenic products that can be derived from repeat expansion loci, including both RNA and protein species that have been proposed as key toxic agents. Expanded CAG trinucleotide repeat sequences that occur within the coding region of the affected gene are typically translated into corresponding polyglutamine (polyQ) tracts **(Figure 1.1)**, which are thought to constitute the primary pathogenic agent in the so-termed polyglutamine diseases (13-16). Indeed, polyQ toxicity has been demonstrated in a range of animal models, including *C. elegans*, *Drosophila* and mouse (17). The molecular mechanism(s) by which polyQ proteins drive cellular dysfunction are not completely clear; mitochondrial dysfunction, transcriptional dysregulation, disrupted axonal transport and inflammation have all been proposed to contribute to polyQ-mediated pathology (18, 19).

Figure 1.1: Location and composition of repeat sequences giving rise to dominantly-inherited expanded repeat disease. ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; FXTAS, fragile X tremor ataxia syndrome; SCA, spinocerebellar ataxia; SMBA, spinobulbar muscular atrophy; DRPLA, dentatorubral-pallidoluysian atrophy. Figure reproduced from (4)

1.1.2 Repeat-associated non-AUG (RAN) translation

Not all repeat sequences are located within coding regions of the affected gene **(Figure 1.1)**, and thus are not expected to encode a repeat polypeptide within the normal protein. However, recent evidence has demonstrated the production of expanded repeat-derived proteins from hairpin-forming RNA in the absence of a canonical open reading frame (ORF), termed repeat-associated non-AUG translation (RAN translation) (20). Initially discovered in the CUG trinucleotide repeat disorder SCA8, where RAN translation led to the production of repeat proteins in all three reading frames of both the sense (CUG) and antisense (CAG) repeat transcripts (20). This follows on from observations of bi-directional transcription at all expanded repeat loci tested, highlighting the importance of both the sense and antisense strands in expanded repeat disorders (21). RAN translation has since been reported in seven additional repeat expansion disorders, including HD (22), fragile X-associated tremor ataxia syndrome (FXTAS) (23), and forms of ALS/FTD caused by the *C9orf72* repeat expansion (24-

26). How do these non-canonical disease gene products contribute to expanded repeat disease? Experimental work in yeast, *Drosophila* and cell lines investigating RAN translated peptides derived from the *C9orf72* locus have demonstrated that impairment to nucleocytoplasmic transport may underlie neuronal dysfunction in ALS/FTD (27-29), while endoplasmic reticulum (ER) stress and proteasome impairment have also been highlighted as potential pathological mechanisms (30). Indeed, the field of RAN translation is still in its infancy, as such it is likely that the current dedicated research focus will provide a much greater understanding of RAN translation. This includes both the mechanisms surrounding the initiation of RAN translation in a disease context and how the resultant proteins contribute to disease development and progression. Furthermore, how the diverse array of RAN-translated polypeptides derived from 4 and 5 base repeat sequences may contribute to expanded repeat disease is yet to be uncovered.

1.1.3 Single-stranded RNA (ssRNA)

In diseases where either polyQ or RAN translated proteins (or both) are produced, it is likely that they participate in the pathogenesis of the given disease, though the extent of this contribution remains unclear. In addition, disease-specific protein products are yet to be described in all forms of expanded repeat neurodegenerative disease. However, underlying all expanded repeat loci is the production of repeat-containing RNA molecules, making RNA a plausible common pathogenic agent (4, 31). Single stranded RNA (ssRNA) products derived from repeat loci form hairpin structures (32, 33) which have been proposed to cause cellular dysfunction through several mechanisms.

At the forefront is the ability of RNA hairpins to interact with and sequester RNA-binding proteins, thus interrupting their normal function (34). For instance, co-localisation and subsequent dysregulation of the Muscleblind (MBNL) family of splicing factors by repeat RNA foci has been implicated in several expanded repeat diseases, including DM1 and 2 (35, 36), HDL2 (37), SCA8 (38) and FXTAS (39). Loss of MBNL alternative splicing recapitulates many of the clinical symptoms associated with DM (40, 41), suggesting that RNA-mediated sequestration of RNA-binding proteins may underlie key aspects of expanded repeat disease pathogenesis. Of note, antisense repeat transcripts derived from SCA2 (42) and ALS/FTD (43)

repeat loci have also been demonstrated to form RNA foci, suggesting that both strands produced from expanded repeat loci can potentially act to sequester RNA-binding proteins and thus contribute to disease pathogenesis. Of note, a repeat-containing antisense transcript to HTT (denoted as HTTAS_v1) was demonstrated to negatively regulate HTT expression (44). The expression level of HTTAS_v1 was decreased in HD brain tissue, suggesting that the transcript may be protective as opposed to detrimental (44). Thus, the generation of antisense transcripts spanning expanded repeat loci may not be inherently deleterious in the context of disease.

1.1.4 Double-stranded RNA (dsRNA)

In addition to ssRNA, the observation of bi-directional transcription across expanded repeat loci (21) has raised the intriguing possibility of the opposing repeat transcripts forming perfectly double-stranded RNA (dsRNA) molecules. Indeed, work from Lawlor *et al* in a *Drosophila* model of CAG expanded repeat disease demonstrated that expression of 100 copies of either CAG ($rCAG_{100}$) or CUG ($rCUG_{100}$) ssRNA was not detrimental, while co-expression of both transcripts together ($rCAG_{100}.CUG_{100}$) led to striking toxicity when expressed in eye tissue, as well as dysfunction when expressed neuronally (45). The observed pathology was dependent upon Dicer-2 (45), a key component of the RNA interference (RNAi) pathway, which acts to process dsRNA into small 21 nucleotide RNA fragments (21mers) in order to facilitate silencing of homologous transcripts (46, 47). Indeed, an enrichment of CAG_7 21mers was detected in $rCAG_{100}.CUG_{100}$ flies, indicating 1) the activity of the RNAi pathway in response to the presence of expanded repeat RNA, and 2) that the RNAi pathway may underlie the pathology (45). These findings were confirmed in an independent *Drosophila* model of DM1, where co-expression of 250 copies of both the sense CUG and antisense CAG transcripts led to Dicer-2 dependent pathology (48). In contrast to the dsRNA-mediated pathology observed in Lawlor *et al* and Yu *et al*, the expression of ssRNA CGG/CCG repeat sequences in isolation led to toxicity in a *Drosophila* model of fragile x-associated tremor/ataxia syndrome (FXTAS), while co-expression of the repeat sequences ameliorated the toxicity in an RNAi-dependent fashion (49). This work provides evidence that the RNAi pathway can also act protectively in response to repeat dsRNA, more in line with its canonical role in antiviral transcript silencing (50). Interestingly,

CAG$_7$ 21mers have also been detected in human HD brain samples, and the neurotoxicity exerted by these repeat RNAs was dependent upon Ago2, another key component of the RNAi pathway (51). Thus, the RNAi pathway is also active in the vertebrate system in response to expanded repeat RNA, further supporting its participation in disease pathogenesis.

A subsequent microarray analysis conducted on *Drosophila* expressing *rCAG$_{100}$.CUG$_{100}$* dsRNA found that a number of transcripts common to the innate immune response were significantly altered in the presence of repeat dsRNA (52). Further analysis revealed that the Toll immune signalling pathway was required for the dsRNA pathology, while autophagy plays a key role in restricting the observed toxicity (52). Finally, the presence of repeat dsRNA induced an inflammatory response, as measured through the transcriptional upregulation of both *Drosomycin*, a downstream peptide synthesised by the Toll signalling pathway, and *Eiger*, the *Drosophila* orthologue of potent inflammatory cytokine *tumour necrosis factor* (*TNF*) (52). Taken together, the results suggest that expanded repeat dsRNA could represent a foreign or 'non-self' molecule to the host innate immune system, thus invoking an inflammatory response that could be detrimental to cellular function and survival. Of note, the *rCAG$_{100}$.CUG$_{100}$* dsRNA *Drosophila* model described in Lawlor *et al* and Samaraweera *et al* is the model used for the majority of work in this thesis (45, 52).

Intriguingly, Dicer-2 also participates in inflammatory signalling independent of its role in the RNAi pathway. The infection of flies with Drosophila C virus (DCV) leads to the induction of the antiviral peptide *Vago* in a Dicer-2 but not RNAi dependent manner (53), while Dicer-2 can also regulate Toll signalling (and subsequently *Drosomycin* induction) in response to microbial and viral challenge (54). Thus, the upregulation of *Drosomycin* observed in response to expanded repeat dsRNA (52) may also represent Dicer-2 activity separate from the RNAi pathway. Finally, Dicer-2 shares significant domain homology with members of the mammalian RIG-1-like receptor (RLR) family of RNA sensing molecules **(Figure 1.2)** (55, 56), which act to detect viral or other 'non-self' RNA molecules and induce a downstream antiviral signalling response (57). Taken together, these findings strongly point to a role for Dicer-2 in RNA sensing not only in the RNAi pathway, but also as part of the inducible antiviral RNA inflammatory response.

Figure 1.2: Homology between selected DExD/H-box helicase proteins in human and *Drosophila*. Significant helicase domain homology is observed between RIG-I-like receptors IFIH1 (encoding MDA5), RIG-I, human Dicer and *Drosophila* Dicer-1 and Dicer-2. Helicase domain location denoted by yellow box. Alignment performed using the MegAlign Pro program (DNAStar). Figure reproduced from (343) **(Appendix A)**.

Indeed, there is precedence for participation of the inflammatory response in expanded repeat disease pathogenesis. Upregulated inflammatory signalling is detectable in HD gene carriers preceding the onset of clinical manifestations (58, 59), while the activation of microglia, the resident immune cell within the CNS, correlates with neuronal dysfunction in HD gene carriers (60, 61). Thus, the inflammatory response shapes as an excellent candidate pathway to investigate in the context of expanded repeat neurodegenerative disease.

1.2 Innate inflammation

The normal role of the innate inflammatory system is one of protection. It represents the first line of defence against dangerous pathogens and other 'non-self' material that challenge the host (62) and promotes tissue repair following injury or insult (63). A fully functioning innate immune system is of utmost importance to prevent a pathogen invader from successfully replicating within the host body (64). However, failure of the initial acute inflammatory response to degrade/remove the threat and thereby resolve the situation can lead to chronic inflammatory activation, resulting in tissue damage and cellular dysfunction that can give rise to a number of autoimmune and autoinflammatory diseases (65, 66). In addition, a wealth of compelling research now places inflammation as a key underlying pathogenic mechanism in neurodegenerative disease (67). This includes a range of neurological disease-causing mutations in genes that regulate inflammatory signalling through either positive or negative mechanisms (68). In this manner, the innate immune

system represents somewhat of a double-edged sword in organismal health, one that must be tightly-regulated to prevent detrimental outcomes. Thus, a greater understanding of the mechanisms governing inflammatory signalling under both homeostatic and disease conditions is required.

1.2.1 Pattern recognition receptors (PRRs)

In order to initiate inflammatory defence mechanisms against pathogens, they must first be detected through the actions of a group of receptor molecules collectively termed pattern recognition receptors (PRRs). These receptors act to sense a wide range of stimuli that fall into several groups; conserved structures found on microbes known as pathogen-associated molecular patterns (PAMPs), and endogenously derived danger/damage-associated molecular patterns (DAMPs) that are released from dying/dysfunctional cells to indicate homeostatic disruption (69). Following detection, PRRs interact with downstream adaptor molecules to augment distinct inflammatory signalling cascades specified to the threat (70). Many PRR families are conserved through to invertebrates, including the Toll-like receptors (TLR)s, of which Toll was initially discovered in *Drosophila* (71). Major groups of vertebrate PRRs include TLRs, the RNA-sensing RIG-I-like receptors (RLRs), and the inflammasome-forming Nod-like receptors (NLRs) (72).

Of particular interest for the RNA-based model of expanded repeat disease used in this thesis are the RNA-sensing PRRs that predominantly serve antiviral based roles **(Table 1.1)**. A number of human TLR molecules are capable of detecting RNA species; TLR3, which engages viral dsRNA ligands, and TLR7/8, both sensors of viral and bacterial ssRNA (73). Inflammasome sensor NLRP3 can respond to a range of viral/bacterial ssRNA and dsRNA triggers but relies on upstream receptors to initiate signalling (74). Finally, the RLR members RIG-I and MDA5 (encoded by *IFIH1*) act to detect dsRNA viral replication intermediates (57). Less is understood regarding the third RLR member LGP2, though recent evidence has highlighted its importance in augmenting RIG-I/MDA5 antiviral signalling (75). *Drosophila* lacks a characterised orthologue of the receptors RIG-I and MDA5, though Dicer-2 may serve as a functional equivalent in invertebrates through a viral RNA-sensing role independent of its activity in the invertebrate RNAi pathway (53-55).

8

Table 1.1: Cellular localisation of known RNA pattern recognition receptors in vertebrates and their ligands

PRR	Cellular Localisation	Ligands
TLR		
TLR3	Endosome	dsRNA, endogenous mRNA
TLR7	Endosome	ssRNA, 'self' RNA immune complexes
TLR8	Endosome	ssRNA, 'self' RNA immune complexes
RLR		
RIG-I	Cytosol	Short dsRNA
MDA5	Cytosol	Long dsRNA
NLR		
NLRP3 (indirectly)	Cytosol	ssRNA, dsRNA

The PRR-mediated detection of pathogenic or 'non-self' material begins a cascade of signalling that results in the propagation of a stimuli-specific response, which forms the activation step of the innate immune response.

1.2.2 Innate immune signalling

The integration of PRR signalling through to the induction of inflammatory mediators that coordinate pathogen defence and tissue repair is critical to ensure an appropriate response is raised against any offending stimuli. Adaptor molecules, kinases and downstream transcription factors all participate in signal transduction pathways **(Figure 1.3)**. Indeed, further complexity is added to the response by the significant crosstalk that exists between some innate immune pathways, knowledge of which is still relatively limited in many cases. This cross-talk can occur at the stage of ligand recognition, signalling infrastructure or transcription factor(s) (76). This level of functional complexity can aid to produce additional layers of specificity through a synergistic or complementary response, or act as a

Figure 1.3: Diagram of a prototypical innate immune signalling pathway. Pathogens or other 'non-self' molecules are detected by the appropriate PRR (in blue), leading to its interaction with an adaptor molecule (yellow). Typically, a signal transduction complex will form consisting of the adaptor and other components, including (but not limited to) kinase, protease and ligase enzymes (green). This results in the subsequent activation of one or more transcription factors (orange), leading to the downstream induction of target inflammatory effector molecules. Figure adapted from (76).

compensatory mechanism following the inactivation of a particular inflammatory pathway via mutation or pathogen-mediated antagonism (77).

The particular transcriptional program(s) activated are dependent upon the ligand/receptor interaction. Microbial infection typically induces expression of inflammatory cytokines such as *TNF* and members of the interleukin (IL) family, whereas viral RNA ligands lead to the transcription of interferon stimulated genes (ISGs), including cytokines that specifically augment an antiviral response (69, 70). Cytokines (along with other induced molecules such as chemokines) are pleiotropic mediators of inflammation that communicate signals within and between cells to coordinate the recruitment of immune cells to the site of infection/injury and elimination of the pathogen/infected cells through cell death mechanisms (69, 78).

Pathogen infection and other insults can also activate mechanisms tied to host stress responses. For example, a number of stressors such as hypoxia, heat shock, mitochondrial dysfunction and viral challenge induce the formation of RNA stress granules (SGs) (79). RNA

binding proteins (RBPs) located within SGs act to harbour non-essential mRNA transcripts to prevent their translation, thus relieving a portion of translational demand on the cell upon stress (80). Stalled mRNAs can then either be released through dissociation of the RBP complexes if the stress is resolved, or shuttled to processing bodies (P-bodies) for degradation (81, 82). In addition, several dedicated degradative mechanisms can also be upregulated as a means to remove the potentially threatening material.

1.2.3 Degradative pathways

In order to prevent the chronic activation of inflammatory signalling, cellular clearance mechanisms operate to degrade trigger molecules (including PAMPs and DAMPs) and damaged organelles that are capable of propagating further inflammation. A range of specialised clearance pathways exist; the ubiquitin proteasome system (UPS) and unfolded protein response (UPR) mediate protein degradation (83, 84), while RNA exosomes and stress granules (SGs) target RNA species (85, 86).

At the forefront of cellular degradation is autophagy, a process by which cytoplasmic molecules and damaged organelles are targeted for recycling/removal. This occurs first through the incorporation of targeted cargo into a double-membrane vesicle known as an autophagosome, followed by transportation to and fusion with a lysosome to facilitate degradation **(Figure 1.4)** (87). Notably, autophagy plays an important role in degrading the disease-associated misfolded proteins characteristic of several neurodegenerative diseases; including polyQ repeat-containing mutant Htt in HD (88), Aβ42 in AD (89), mutant α-Syn in PD (90) and mutant TDP-43 in ALS/FTD (91). Indeed, specialised forms of autophagy exist based on target substrates; notable forms include xenophagy, which degrades bacterial and viral pathogens, and mitophagy, a critical process for mitochondrial quality control via the removal of damaged/dysfunctional mitochondria (92).

In addition to maintaining a healthy pool of mitochondria to provide the energy required by the cell, the removal of damaged mitochondria is critical for upholding homeostasis. Under stress, mitochondria can release a range of DAMPs to further stimulate an inflammatory response. Mitochondrial DNA (mtDNA), ATP and mitochondrial ROS (mtROS) derived from
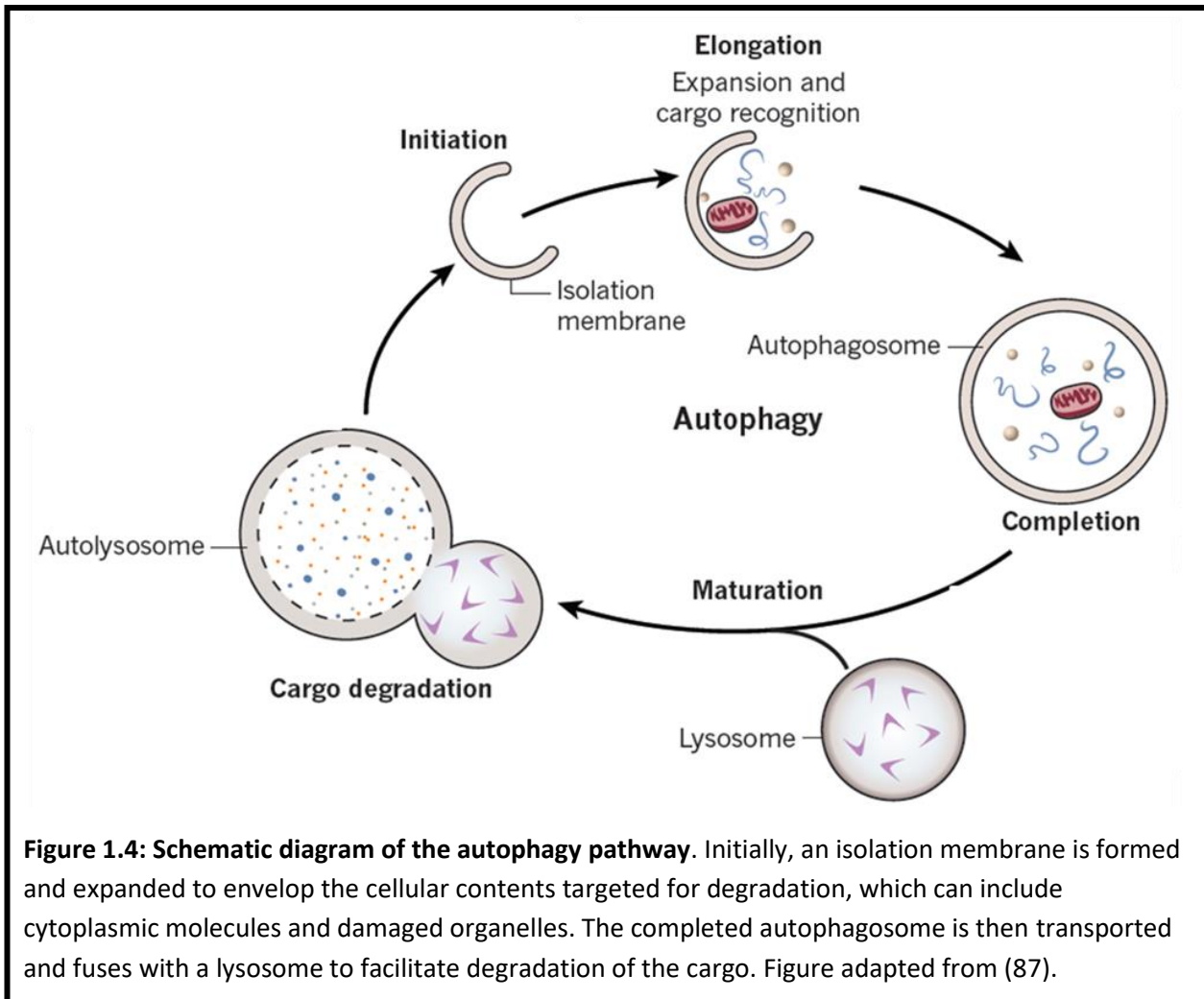
Figure 1.4: Schematic diagram of the autophagy pathway. Initially, an isolation membrane is formed and expanded to envelop the cellular contents targeted for degradation, which can include cytoplasmic molecules and damaged organelles. The completed autophagosome is then transported and fuses with a lysosome to facilitate degradation of the cargo. Figure adapted from (87).

damaged mitochondria are all potent activators of the NLRP3 inflammasome (93-95), while mtDNA can also induce Type I interferon signalling by binding TLR9 (96) and the cGAS/STING DNA sensing antiviral pathway (97, 98). Thus, the efficient removal of mitochondrial trigger molecules through mitophagy is essential for preventing inappropriate inflammatory signalling. Notably, mutations in key mitophagy regulators *PINK1* or *Parkin* both lead to autosomal recessive familial PD (99, 100), highlighting the importance of mitophagy in neurodegenerative disease.

1.2.4 Mitochondria as an antiviral hub

Mitochondria themselves are also key hubs of the innate inflammatory response, in particular as a platform to launch antiviral RNA signalling. In addition to the previously noted

range of mitochondrially-derived DAMPs that drive NLRP3 inflammasome activation in response to metabolic stress (101), healthy mitochondria can also serve as a platform for antiviral RNA signalling.

Upon the sensing of cytosolic viral 'non-self' RNA products, receptors RIG-I and MDA5 interact via their respective caspase activation and recruitment (CARD) domains with the adaptor molecule mitochondrial antiviral signalling protein (MAVS), which is anchored the outer mitochondrial membrane (OMM) (102). This interaction facilitates the recruitment of adaptor molecules and kinases to MAVS, ultimately resulting in NF-κB and interferon regulatory factor 3 (IRF3) mediated transcription of pro-inflammatory cytokines and interferon stimulated genes (ISGs) respectively **(Figure 1.5)** (102, 103). In addition, MAVS promotes inflammasome signalling through its function as an adaptor molecule (104, 105) and can also trigger inflammasome activation via membrane permeabilization (106). Finally, MAVS (and thus the RLR antiviral RNA pathway) can initiate antiviral cell death in response to viral challenge, including both apoptosis (107-109) and necroptosis (110, 111). Indeed, several viral pathogens encode inhibitors of MAVS-mediated cell death (108), highlighting its importance in restricting the spread and replication of viruses.

Intriguingly, recent *in vitro* evidence has shown that both RIG-I and MDA5 oligomerize on their respective dsRNA targets to form filaments (112-115). This RLR oligomerization is important for the nucleation and formation of MAVS aggregates, the functional signalling complexes that drive downstream activation of NF-κB and IRF3 (116-118). Recent evidence has demonstrated that MAVS aggregation is inhibited through physical interactions with N-terminal truncated MAVS isoforms and PINK1/Parkin-mediated mitophagy (119). Furthermore, scaffold protein FAF1 can form aggregates that bind to and prevent MAVS aggregation under homeostatic conditions (120). Indeed, a number of other protein-protein interactions regulate MAVS activation (both positively and negatively) at the mitochondrial level (121), demonstrating that RLR/MAVS-mediated signalling is tightly controlled to ensure that antiviral signalling occurs at the appropriate time and magnitude in response to pathogenic or sterile challenge.

Figure 1.5: Overview of RLR-MAVS-mediated antiviral signalling in vertebrates. Viral or other 'non-self' dsRNA is detected by the cytoplasmic RNA sensors RIG-I and MDA5. RIG-I recognizes short dsRNA, whereas MDA5 recognizes long dsRNA. Both RIG-I and MDA5 form a repeating filament structure upon the dsRNA that interacts with mitochondrial adaptor protein MAVS via their CARD domains. MAVS molecules form a functional signalling complex that recruits kinases and other adaptor molecules, ultimately leading to NF-κB and IRF3-mediated induction of pro-inflammatory cytokines and IFN stimulated genes respectively. Additionally, RLR-MAVS signalling can also promote antiviral cell death as a mechanism to restrict the spread of viral material.

Thus, mitochondria serve not only as a metabolic hub within the cell, but also as a platform to initiate and augment innate inflammatory signalling and augment inflammation through the release of trigger molecules. Indeed, the propagation of inflammation can lead to cell death and dysfunction, and not surprisingly has been implicated in systemic and CNS disease.

1.2.5 Inflammation and neurodegeneration

Genetic lesions affecting inflammatory components give rise to a number of monogenic autoinflammatory diseases, either through gain of inflammatory signalling or loss of negative regulation (66). What is now becoming increasingly clear is that mutations leading to inflammatory dysregulation also underlie major neurodegenerative diseases, including AD, PD and ALS/FTD (68).

Genetic variants in the innate immune genes *TREM2, PLCG2* and *ABI3* are all risk factors in late-onset AD (LOAD) (122, 123). In addition, *APOE*, a major susceptibility gene for LOAD, drives immune dysregulation through a TREM2-mediated pathway (124). Indeed, amyloid-β induces inflammation (125-127), though recent work has demonstrated that it also functions protectively as an antimicrobial peptide (128-130). Thus, the role of amyloid-β in neuroinflammation appears multi-faceted and is in need of further definition.

Many of the genes responsible for familial PD are critical for mitochondrial dynamics and quality control, in particular *PINK1* and *Parkin* (131) but also including *LRRK2* (132), *DJ-1* (133, 134) and *VPS35* (135). Furthermore, the accumulation of α-synuclein (derived from the *SNCA* gene, mutated in autosomal-dominant PD) impairs mitochondrial dynamics (136) and can also trigger inflammation directly (137, 138). Failure to remove damaged mitochondria leads to the release of trigger molecules including mtDNA and ROS that propagate inflammation (139, 140), thus highlighting mitochondrial-mediated inflammation as a key contributor to PD pathogenesis.

Several familial genes tied to ALS/FTD are involved in stress responses. ALS-linked mutations in the autophagy adaptor proteins p62/sequestosome 1 and optineurin lead to impaired autophagy and cellular trafficking (141, 142), while other ALS and FTD-linked genes also function in autophagic processes (143). Additionally, *TARDBP* and *FUS* both encode RNA-binding proteins (TDP-43 and FUS respectively) that associate with stress granules (80, 144). Both TDP-43 and FUS also regulate RNA splicing (145, 146), implicating RNA metabolism in the pathogenesis of ALS and FTD. Finally, the most frequent cause of inherited ALS and FTD, an expanded hexanucleotide GGGGCC repeat within the *C9orf72* gene, impairs autophagy

(147) and dysregulates immune signalling (148, 149). Thus, dysfunctional degradative machinery appears a key underlying contributor to both ALS and FTD.

As previously discussed, inflammatory activation precedes the onset of clinical symptoms in HD gene carriers (58, 59). In addition, known HD disease gene products (polyQ and RNA) have been shown to induce inflammatory activation (52, 150). This raises the possibility that the products, once expanded beyond a pathogenic threshold, are detected by the innate immune system as foreign or 'non-self', leading to a damaging inflammatory response within the host. Indeed, endogenous repeat dsRNA molecules (such as Alu repeats) are typically subject to post-transcriptional modifications such as adenosine-to-inosine (A-to-I) editing to mark them as 'self' molecules and prevent the activation of RNA sensing machinery (151, 152). Mutations in the A-to-I editing enzyme *adenosine deaminase acting on RNA 1* (*ADAR1*) lead to aberrant Type I interferon signalling and the development of the auto-inflammatory neurological disorder Aicardi-Goutieres syndrome (AGS) (153, 154). Furthermore, augmented Type I interferon activity through gain-of-function mutations in *IFIH1* (encoding RNA sensor MDA5) also gives rise to AGS (153, 155). Intriguingly, genetic deletion of either MDA5 or its downstream antiviral adaptor MAVS rescues embryonic lethality and aberrant inflammatory signalling observed in *Adar1* null mice (152, 156), thus highlighting 'non-self' sensing of dsRNA as a key driver of inflammatory dysfunction in neurological disease (157). Finally, the requirement of RLR-like sensor Dicer-2 in driving dsRNA-mediated toxicity in *Drosophila* (45), and the ability of repeat dsRNA to induce an inflammatory response (52) raise the possibility that 'non-self' recognition of expanded repeat dsRNA may comprise a key pathogenic factor in repeat expansion disorders.

In addition, given the role of non-cell autonomous pathways in the inflammatory response, the distinct cell types within the CNS are important to consider when assessing the role of inflammation in neurodegenerative disease. Historically, approaches have taken a neuron-centric view, though in recent years the focus has turned to the non-neuronal glial cells that habituate the CNS, and as such their functional diversity and importance in neurodegeneration is beginning to emerge.

1.3 Glial cells and other non-neuronal cells of the nervous system

Long thought of as passive neuronal support cells, the currently identified roles of glial cells within the nervous system are diverse and of critical importance to neuronal development, health and function (158). Thus, it is not surprising that glial cells have been heavily implicated in the development and progression of neurodegenerative disease through both helpful and harmful mechanisms (159). In line with the diverse roles they perform within the nervous system, a range of glial cell types have been described based on distinct morphological and functional characteristics **(Figure 1.6 A)**. While the majority of *in vitro* and *in vivo* analyses into glial form and function has been in a mammalian context, at least some of these features are evolutionarily conserved through to invertebrates such as *Drosophila* and *C. elegans*, where the utility of a simpler model system has been extremely useful in unravelling key questions in cell biology (160, 161). Indeed, even within specific glial subtypes there exists remarkable heterogeneity that has only recently become clear (162-165), highlighting that there is likely still much to learn regarding glial function in both the healthy and compromised CNS.

1.3.1 The development and normal functions of glia

Microglia comprise approximately 10-20% of the total glial population in mammals (159, 166) and act primarily as the resident immune cells of the CNS. They originate from mesodermal progenitors in the yolk-sac during embryogenesis, then subsequently invade the developing CNS and begin to proliferate rapidly **(Figure 1.6 B)** (167, 168). Under homeostatic conditions, microglia constantly scan the CNS environment for pathogens and other homeostatic disruptions through the expression of a cluster of receptors (169). In addition, they participate in synapse remodelling (170, 171), neurogenesis through the removal of apoptotic neuronal bodies (172) and neuronal support through the secretion of neurotrophic factors (173, 174). Upon detection of CNS injury or insults such as brain injury, pathogens or other 'non-self' molecules, microglia undergo "reactive gliosis"; altering their morphology and transcriptional signature in a threat-dependent manner (175). These responses can be of a helpful manner through controlled removal of the threat/danger signal, followed by tissue repair and remodelling (176). However, dysfunctional microglia

and/or the persistence of an insult/injury can result in the escalation of pro-inflammatory signalling, cell death and tissue damage (177, 178). Thus, microglial function within the CNS needs to be remarkably fine-tuned to ensure appropriate responses to CNS stimuli.

Astrocytes represent the most abundant cell type within the vertebrate CNS, encompassing approximately 30% of the total glial cell population (179). At least two classes of astrocytes are present: fibrous astrocytes inhabit white matter and comprise cylindrical processes reminiscent of long fibres, while protoplasmic astrocytes are located in grey matter and are characterised by finely branching processes (180, 181). Both astrocytes and oligodendrocytes (collectively termed macroglia) originate from neuroepithelial cells based in the neural tube and forebrain (158) that then form radial glia during approximately embryonic day 9-10 (E9-10) in mice **(Figure 1.6 B)** (182). Radial glial cells initially give rise only to neuronal cells before switching to produce glial precursors after neurogenesis (183, 184). Following this, a number of secreted molecules including sonic hedgehog (SHH), fibroblast growth factors (FBFs) and cytokines provide positional cues to the precursor cells, specifying differentiation into either astrocytes or oligodendrocyte precursor cells (OPCs) (158, 182).

Astrocytes form distinct tiling across the entire CNS (181), connected by gap junctions spanning the entire network that allow efficient cell-cell communication (185, 186). Under normal conditions, astrocytes play a central role in neuronal circuit plasticity, regulating both the formation and pruning of synapses during development via the release of molecular signals (187-189). Through their close association with synapses, astrocytes are also responsible for facilitating a homeostatic environment for proper neuronal communication, including the uptake and clearance of neurotransmitters to prevent excitotoxicity (190, 191). Similar to microglia, astrocytes can transition to a "reactive" state upon sensing CNS injury/insult, facilitating a set of functional changes that can either promote CNS repair or exacerbate tissue damage depending on the stimuli (192, 193).

Oligodendrocytes comprise the second distinct macroglial cell type and are responsible for myelination of axonal fibres within the CNS (194). Myelination provides faster nerve impulse conduction along axons, important for maintaining neural connectivity in larger vertebrate

Figure 1.6: Vertebrate glial cells and their developmental origins. **A)** The neurovascular unit (NVU) is comprised of glial and other non-neuronal cell types. Vascular cells (pericytes and endothelial cells) enclose blood capillaries in order to form the blood-brain barrier (BBB) in conjunction with secreted structural proteins that form the basement membrane. Astrocyte end feet make contact with the vasculature, facilitating rapid communication between neurons and the BBB to fine-tune nutrient supply. Oligodendrocytes myelinate neuronal projections, while microglia scan the CNS microenvironment for homeostatic disruptions. Diagram reproduced with modifications from (270). **B)** Lineage diagram of vertebrate glial cells. Neural crest cells give rise to Schwann cell precursors, which then migrate to the PNS to generate both myelinating and non-myelinating Schwann cells. Neuroepithelial progenitor cells (NPCs) produce radial glial cells, which then generate neurons and, following a 'gliogenic switch', both oligodendrocyte precursor cells (OPCs) and astrocytes. OPCs subsequently differentiate into mature oligodendrocytes. Mesodermally-derived primitive macrophages migrate to the CNS during embryonic development, where they form the mature microglial population. Corresponding cell types (if featured in both diagrams) are colour-coded.

brains (195). As with astrocytes, oligodendrocyte precursor cells (OPCs) derive from radial glial cells, migrating from the ventricular zone of the embryonic spinal cord to the CNS during early development and give rise to mature oligodendrocytes of the adult brain **(Figure 1.6 B)** (196, 197). The proliferation of OPCs continues into adulthood in order to replace dying oligodendrocytes and thus maintain myelin coverage (198). The ensheathment of vertebrate peripheral nerves is a role carried out by neural crest-derived Schwann cells, which can differentiate into both myelinating and non-myelinating forms **(Figure 1.6 B)** (199). Of note, OPCs are often referred to as NG2 (Nerve-glial antigen 2) glial progenitor cells based on their stable expression of the proteoglycan NG2 (200). Intriguingly, the self-renewing NG2 cells have been proposed to also generate astrocytes (201) and even neurons, though this ability remains controversial (202-204). Aside from their role in remyelinating nerves following oligodendrocyte death/CNS injury, little more is known about putative NG2 cell functionality (205). Therefore, NG2 cells will be grouped with oligodendrocytes in future discussion.

1.3.2 The neurovascular unit (NVU)

Composed of neurons, glial cells and other non-neuronal cell types, the neurovascular unit (NVU) acts as a means to connect the energy-intensive brain with appropriate blood supply (206). The NVU represents a specialized complex that encompasses components of the vertebrate blood-brain barrier (BBB) and the surrounding neurovasculature (207). Neurons, microglia and astrocytes are present, as well as vascular cells including pericytes and endothelial cells **(Figure 1.6 A)**. Endothelial cells form the core structure of the BBB, acting as a physical barrier separating the tightly regulated CNS environment and the systemic circulatory system (208). Connecting endothelial cells are diffusive molecular complexes known as tight junctions (TJs) that allow selective transport of molecules and ions across the BBB (209, 210). Surrounding the endothelium is a basement membrane comprised of secreted structural proteins that form a distinct extracellular matrix (ECM). The ECM provides additional regulation of BBB permeability, while also acting as a scaffold for growth factors and other structural support molecules (211, 212). The final specialised NVU cell type, pericytes are situated between the endothelium, astrocytes and neurons **(Figure 1.6 A)**. Similar to astrocytes, they communicate with neighbouring cells through gap junctions

(213) and regulate a number of vital BBB processes including the regulation of endothelial cells and tight junctions to control permeability (214), clearance of foreign material (215) and immune signalling (216). Interestingly, pericytes also display multipotent stem cell activity following ischemic injury, suggesting that they take on new functionality following CNS insult (217, 218).

In an NVU context, astrocytes extend processes (also known as astrocyte endfeet) to cover blood vessels **(Figure 1.6 A)** where they aid in the development of tight junctions (219) and maintain endothelium integrity through bi-directional signalling (220, 221). Maintaining intimate physical contact with both neurons and the CNS vasculature allows astrocytes to act as a conduit to receive and relay messages between neurons and the vasculature to fine-tune metabolic homeostasis (208, 222). Microglia maintain their role as resident CNS sentinels, scanning for signs of neurovascular damage (223).

The NVU is of vital importance for coupling the brain with blood flow in order to meet the high metabolic demand of neural activity. In addition, the BBB protects the strictly homeostatic environment within the CNS from systemic microbes and immune cells that can instigate neuronal damage and death (224). Thus, the NVU further illustrates the essential role glia and other non-neuronal cells play in preserving proper neuronal function.

1.3.3 Glial cells in neurodegenerative disease

While glial cells are now recognized as indispensable for CNS function, their dysregulation not only promotes neurodegeneration but even appears to be a proximal cause in some cases (225). Factors such as ageing and genetic lesions can cause dysfunctional microglia and astrocytes that react inappropriately to CNS insults such as disease gene products, leading to heightened inflammatory signalling and neuronal cell death (2). As such, it is not surprising that maladaptive glial cells have now been highlighted as potential therapeutic targets to ameliorate neurodegenerative disease (179, 226, 227).

As the resident immune cells of the CNS, microglia have garnered much of the research focus on the contribution of glial cells to neurodegeneration. Genome-wide association studies have recently identified coding variants in the microglial genes *TREM2*, *PLCG2* and

ABI3 as genetic risk factors for AD (122, 123), implicating microglia as a causal contributor in the disease. Further supporting a causal role, reactive microglia have been observed preceding the onset of clinical symptoms in HD (61, 228) and ALS (229). In addition, pro-inflammatory microglia drive disease progression through interactions with disease-specific gene products including Aβ (126, 230) and Tau in AD (231), α-synuclein in PD (232, 233), mHTT in HD (150) and mSOD1 in ALS (234, 235). Failure of microglia to effectively remove disease products instead facilitates their redistribution and further propagation throughout the CNS (236-238), a phenomenon remarkably conserved through to phagocytic glia in *Drosophila* (239). Thus, the efficient degradation of disease gene products through microglia is critical to prevent neuroinflammation.

Indeed, through normal ageing microglia progressively lose their homeostatic functionality and take on a primed, pro-inflammatory phenotype that can exacerbate neuronal damage through an exaggerated inflammatory response to CNS insult/injury (177, 240, 241). The removal of genetically-induced senescent microglia and astrocyte cells prevents cognitive decline in a mouse model of tau-dependent neurodegeneration (242), highlighting ageing microglia as a potential therapeutic target. Inflammation stemming from sepsis increases the risk of developing neurodegeneration in later life (243), suggesting that systemic molecules may also contribute to the sensitization of microglia within the CNS and thus drive an exaggerated neuroinflammatory response to a subsequent CNS challenge.

Like microglia, astrocytes also display reactivity states in response to CNS stimuli that can have important implications for CNS disease progression (181). Disease lesions disrupt normal astrocyte function and result in non-cell autonomous neuronal dysfunction and death (244-246). Recent work from Liddelow and colleagues has demonstrated that LPS-induced reactive microglia can induce a toxic astrocytic state through the release of TNF, IL-1α and C1q (247). These reactive astrocytes (termed A1 astrocytes following pro-inflammatory macrophage and microglial nomenclature) are potent promotors of neuronal and oligodendrocyte cell death *in vivo* and, importantly, were observed in post-mortem CNS tissue derived from AD, PD, HD and ALS patients, indicating that disease-specific CNS insults drive a similar transition (247). In addition, reactive astrocytes transition to a microglial-induced pro-inflammatory A1 phenotype through normal ageing, suggesting that A1

astrocytes may also underlie age-related cognitive decline (248). Pharmacological inhibition of A1 astrocyte conversion ameliorates neurodegeneration in mouse models of PD (249), further highlighting the therapeutic potential in targeting astrocyte reactivity.

Interestingly, reactive astrocytes induced through ischemic injury display a neuroprotective phenotype (termed A2) (193), demonstrating that astrocytes can be helpful or harmful depending upon the form of environmental challenge. Indeed, reactive A2 astrocytes function to promote wound repair and axonal regeneration following spinal cord injury (250-252). In addition to reactive gliosis, astrocytic degeneration is a common feature in neurodegenerative disease (253-256), indicating that astrocytic loss of function may also contribute to neuronal dysfunction. Astrocytes are capable of degrading Aβ plaques via lysosomal activity (257-259) and the close association of reactive pro-inflammatory astrocytes and microglia with amyloid deposits in AD (260) may reflect the loss of this phagocytic capability. Taken together, these results highlight the importance of astrocytes in non-cell autonomous neuronal decline and support the participation of multiple cell types in the pathogenesis of neurodegenerative disease.

While oligodendrocytes lack the defined reactivity of microglia and astrocytes in response to stimuli, their dysfunction and loss contributes to a number of CNS disorders. Most notably, oligodendrocyte loss and the subsequent demyelination of nerves in the CNS are hallmarks of multiple sclerosis (MS), an autoimmune inflammatory disorder that results in axonal degradation and neuronal dysfunction (261). Whether the autoimmune response precedes oligodendrocyte and myelin loss or vice versa is still unclear (262), though recent work has shown that oligodendrocyte death triggers an autoimmune response directed against myelin that results in neurological dysfunction (263), providing evidence that oligodendrocyte loss may represent the initial trigger for demyelinating diseases.

However, evidence of oligodendrocyte dysfunction in neurodegenerative disease is not limited to MS. Oligodendrocyte degeneration precedes disease onset in mouse models of ALS (264, 265) and AD (266), while demyelination is a recurring feature in the motor cortex and spinal cord regions of ALS patients (264, 265) and in pre-symptomatic HD patients (267). Additionally, oligodendrocyte-specific expression of an expanded mHTT protein leads to

progressive demyelination and neurodegeneration in mice (268). Glial-secreted TNF causes selective cell death of oligodendrocytes (269), therefore it is tempting to speculate that inflammatory signalling upon CNS insult may lead to oligodendrocyte death as part of a non-cell autonomous response, thus further exacerbating inflammation and neurodegeneration in CNS disorders.

Disruption of the neurovascular unit is another structural feature frequently observed in neurodegenerative disease (270, 271). Permeabilization of the blood-brain barrier allows the infiltration of blood-derived neurotoxic molecules such as fibrinogen, a coagulation factor that promotes neuroinflammation in MS and AD via engagement with microglia and Aβ respectively (272, 273). Systemic immune cells including neutrophils, phagocytes and lymphocytes can also enter the CNS following BBB disruption and drive neuronal tissue damage (274, 275). BBB leakage is an early pathogenic event in both mouse and fly models of HD (276, 277), suggesting that it may prove a promising therapeutic target for intervention. Notably, the BBB impairment observed in *Drosophila* was also replicated through expression of the mutant expanded Ataxin 3 protein, the genetic lesion responsible for the expanded repeat disease spinocerebellar ataxia 3 (SCA3), the most prevalent cause of dominantly inherited ataxia (277). As such, the BBB may be extremely sensitive to the presence of repeat-containing disease gene products, a hallmark of the expanded repeat family of diseases.

An appreciation for the diversity of essential roles that glial cells play under both normal and disease conditions is fast emerging. However, with this increased research focus has come the realisation that glia are far more complex and heterogeneous than first thought (158). As such, the conserved diversity of glial cells through to invertebrates, along with a powerful genetic toolkit means that *Drosophila* provides an excellent *in vivo* model system to further our understanding of glial cell function and their contribution to disease pathology.

1.3.4 Glial cells in *Drosophila*

Despite representing the most abundant cell type within the CNS, our understanding of glial biology during both healthy and diseased CNS states remains limited, in part due to the

complexity of glial cells and their functions in vertebrates. In this regard, simpler yet evolutionarily conserved model organisms such as *Drosophila* are remarkably useful in providing insights into basic cell biology (160, 278). Indeed, similar to vertebrates, in *Drosophila* there exist a number of distinct glial cell types based on morphology, localisation and function **(Figure 1.7)**.

Surface glia groups ***perineural glia*** and ***subperineural glia***, both of which cover the entire brain and ventral nerve cord in two adjacent single-cell layers (279). Together, they comprise the *Drosophila* blood-brain barrier which separates the CNS and circulating hemolymph (279, 280). In addition, they also ensheath peripheral nerves that project into and out of the CNS (280). Within the larval brain, surface glial cells derive from four mapped clusters of currently unidentified glia progenitors that spread over the entire brain hemisphere (281). Within the ventral nerve cord, perineural glial cells derive from two CNS neuro-glioblasts; NB2-5 exclusively and NB5-6 which also generates subperineural glial cells **(Figure 1.7 C)** (282). Additionally, three sensory organ progenitors (SOPs) within the PNS give rise to perineural glia **(Figure 1.7 C)** (283). Post-embryonically, perineural glia undergo extensive proliferation which likely produces the perineural glial cells of the adult brain (284). Subperineural cells are generated embryonically within the ventral nerve cord where they stem from five distinct but spatially unrelated neuro-glioblasts (NB1-1, NB2-2, NB5-6, NB1-3 & NB7-4, some of which also give rise to other glial subtypes including cortex glia) **(Figure 1.7 C)** (281, 285). In addition, subperineural glia of the PNS are generated from both a single CNS-derived neuro-glioblast (NB1-3) and two PNS-derived SOPs, one of which is a shared progenitor with peripheral wrapping glia (283). During development, subperineural glia do not proliferate but instead expand greatly in size and connect to one another by forming septate junctions (280). Individual cells are connected to one another through septate junctions that tightly control paracellular transport, reminiscent of tight junctions that link vertebrate endothelial cells (286). Thus, surface glial cells share many characteristics with the mammalian BBB and are likely to be equally important in preserving the tightly-regulated CNS environment.

Underneath surface glial cells in the peripheral nervous system (PNS) lie ***wrapping glia***, which act to wrap individual axonal fibres in a strikingly similar fashion to non-myelinating
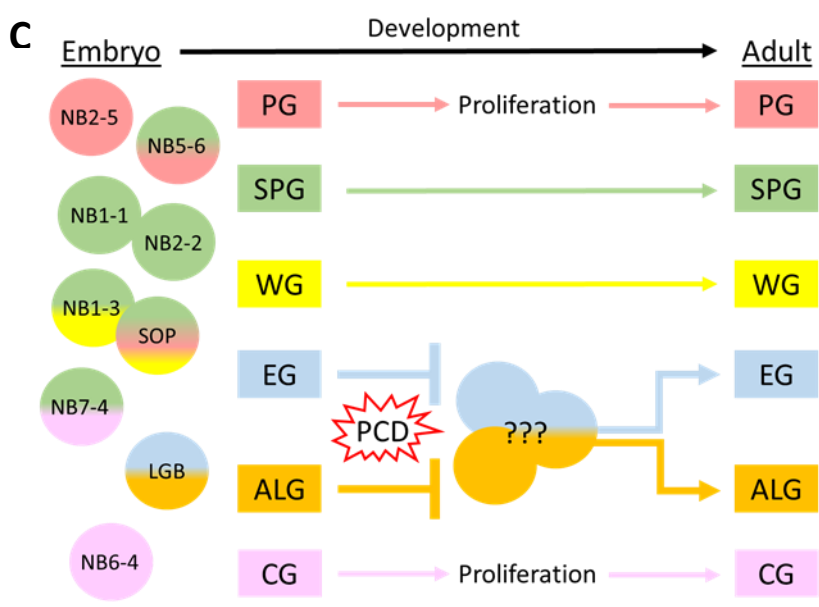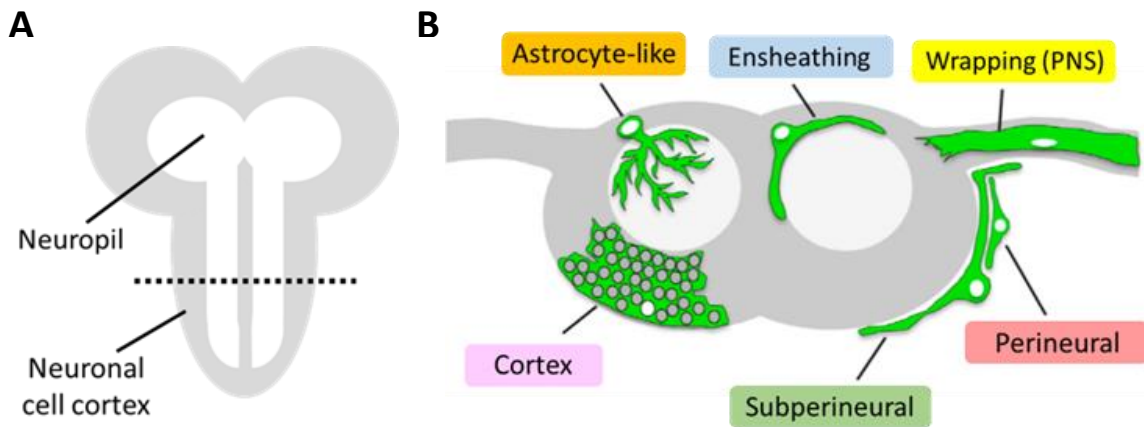
Figure 1.7: The glial cells of *Drosophila melanogaster*. A) Overview of the larval *Drosophila* CNS. The neuronal cortex region (gray) contains neuronal cell bodies and most glial cell bodies. The neuropil (white) is composed of axonal and dendritic projections together with glial cell processes to form a synaptically dense region. The dotted line represents the region shown in **B)** Cross-section of the larval ventral nerve cord displaying glial subtypes. Morphological organisation is conserved in the adult brain. Perineural and subperineural glia enclose the entire CNS and PNS to form the *Drosophila* blood-brain barrier. Ensheathing glia border neuropil regions to separate them into distinct compartments in addition to wrapping axonal tracts, while astrocyte-like glia extend fine processes into the neuropil. Cortex glia form a honeycomb-like structure around neuronal cell bodies. Wrapping glia ensheath peripheral nerves. Image reproduced with modifications from (278). **C)** Origin of glial cells within the ventral nerve cord. Details of glial cell development in *Drosophila* are located within the text. Corresponding cell types in diagrams **B-C** are colour-coded.

Schwann cells in the vertebrate periphery (287, 288). Wrapping glia stem from progenitors together with subperineural glial cells within the ventral nerve cord; the neuro-glioblast NB1-3 and a ventral SOP **(Figure 1.7 C)** (283). Like subperineural glia they do not proliferate, but rather extend in length to span peripheral nerves (283). Recent work has also demonstrated the importance of wrapping glia-derived signalling in the development of the peripheral BBB (289), suggesting that they can also regulate peripheral nerve insulation via non-cell autonomous mechanisms. Additionally, wrapping glial cell differentiation is reliant upon EGF receptor activation (289), a mechanism shared with the formation of myelin in vertebrates (290). This suggests that, despite the lack of myelin in *Drosophila*, wrapping glia share commonalities with both myelinating and non-myelinating peripheral cells.

Two distinct glial subsets populate the neuropil region of the *Drosophila* CNS, ***ensheathing glia*** and ***astrocyte-like glia***, collectively termed neuropil glia **(Figure 1.7 B)**. Neuropil glia share common progenitors in both the larval brain and ventral nerve cord. Within the larval brain, they are derived from a single neuro-glioblast cluster known as basal procephalic neuropile glia (BPLG), whereas the lateral glioblast (LGB) generates the neuropile glia of the ventral nerve cord **(Figure 1.7 C)** (285). However, these embryonically derived neuropile glia (also termed primary glia) undergo programmed cell death during metamorphosis which is followed by a second wave of gliogenesis that produces the adult (secondary) neuropile glia **(Figure 1.7 C)** (291). The secondary neuropile glia are produced from multipotent neuro-glioblast precursors within the brain and a set of unidentified progenitors in the ventral nerve cord (282, 291).

Ensheathing glia extend across the edge of the synapse-rich neuropil to form boundaries separating the distinct lobes (279). In addition, a morphologically-distinct subset of ensheathing glia associate closely with axon tracts that connect the neuropil compartments (292), thus showing similarity to oligodendrocytes in this regard. Ensheathing glia function as the resident phagocytes of the brain to clear axonal debris following injury (293, 294) and in this manner share functional similarity with mammalian microglia. Indeed, via signalling from the glial engulfment receptor Draper, ensheathing glia can modulate the magnitude of the phagocytic response to scale with injury severity, providing evidence that *Drosophila* glial cells may also display reactivity in an injury-dependent manner (295). Furthermore,

ensheathing glia show an age-dependent decline in phagocytic ability via reduced expression of Draper (296) that can lead to neurodegeneration (297), highlighting glial senescence as an evolutionarily conserved process that contributes to neurological decline.

Astrocyte-like glia infiltrate the neuropil regions with elaborate branching processes (279); a single astrocyte is capable of extending processes into multiple distinct neuropil compartments (292). Their densely ramified morphology bears striking resemblance to protoplasmic astrocytes of the mammalian brain (298). Indeed, *Drosophila* astrocytes display functional conservation as well; they retain important roles in neurotransmitter homeostasis (299, 300) and synapse remodelling (301, 302). Whether they exhibit reactivity in response to stimuli in *Drosophila* is unknown, though they do show tiling behaviour similar to vertebrate astrocytes (298), suggesting that they may at least be capable of cell-cell communication. This is supported by the observation that focal axonal injury leads to widespread glial activation within the *Drosophila* CNS (295), which may rely on glial coupling to transmit a distant injury signal.

Though ensheathing and astrocyte-like glia show a number of functional similarities with vertebrate microglia and astrocytes respectively, they have not yet been demonstrated to display the potent immune signalling that is characteristic of reactive glial cells upon CNS insult (175). However, recent work has uncovered a novel microglial-like cell type that infiltrates the *Drosophila* CNS transiently during development (303). The cells, termed MANF immunoreactive Cells (MiCs), appear in the pupal brain following distinct disruptions to homeostasis; the triggering of either inflammation or autophagy, or the silencing of the neurotrophic factor MANF (303). MiCs display motility and express both Draper and the immune transcription factor Relish, suggesting that they are potentially capable of performing at least two microglial functions within the developing CNS; phagocytosis and inflammatory signalling (303). The understanding of this cryptic cell type is very limited, leaving several important questions unresolved. Are there other methods to induce their appearance? Are they derived from a dedicated precursor or do they differentiate from another cell type following homeostatic disruption? Do they interact with other cell types within the CNS? Further investigation of these cells may provide insights into how the

28

Drosophila CNS reacts under conditions of stress and the if MiCs constitute "reactive" glial cells in invertebrates.

The final glial subtype in *Drosophila*, **cortex glia**, populate the cortical region of the CNS between surface glia and the neuropil **(Figure 1.7 B)**. Here, they wrap individual neuronal cell bodies and forming a distinct honeycomb-like structure; a single cortex glial cell can ensheath approximately 100 neuronal bodies (279). This spongiform morphology bears resemblance to protoplasmic astrocytes that associate closely with neuronal cell bodies (304, 305). Within the larval brain they are derived from the same set of unidentified neuro-glioblast clusters as surface glia (282, 285). In the ventral nerve cord they are generated from two defined progenitors; NB6-4 which represents a dedicated cortex glia progenitor, whilst NB7-4 which gives rise to both cortex and subperineural glial cells **(Figure 1.7 C)** (285).

Surprisingly little is known about the functions carried out by cortex glia, though recent work suggests that they play an important role in regulating neuronal excitability; defective cortex glial cells lead to a pronounced susceptibility to epilepsy-like seizures following environmental stressors (306, 307). Thus, in controlling neuronal firing, cortex glia also share functionality with mammalian astrocytes (305). In addition, cortex glia appear vital in clearing apoptotic neuronal corpses during development through expression of *Draper* (308), showing that a number of *Drosophila* glial subtypes can act as semi-professional macrophages. Whether this functional redundancy allows different glial subtypes to compensate for one another following injury/insult is not clear. However, disruption of cortex glia morphogenesis leads to the resultant invasion of astrocyte-like glia into the cortex region and vice-versa (309), indicating a level of communication between these glial subtypes.

Indeed, glial cells appear to be central players in *Drosophila* models of neurodegeneration in both helpful and harmful capacities (310). Glial-mediated inflammatory signalling is necessary for neurological decline in a *Drosophila* model of ataxia-telangiectasia (A-T) (311, 312) and through normal ageing (313), suggesting that invertebrate glia are capable of launching inflammatory responses. Notably, these findings were observed through the targeting of all *Drosophila* glial cells. As such, determining the individual glial subtype(s) regulating inflammation will be an important area of future research. In this regard, genetic

tools designed to manipulate and observe individual glial subtypes are now widely available (160, 292), further positioning *Drosophila* as an excellent animal model with which to interrogate glial cell biology.

1.4 *Drosophila* as a model organism in immunity and neurodegeneration

A fast generation time, well annotated genome and widely available genetic toolkit means that *Drosophila* has proven a powerful model to dissect the molecular underpinnings of important biological processes. As part of the toolkit comes the GAL4/UAS bipartite expression system, which facilitates the specific expression of candidate transgenes under control of an upstream activation sequence (UAS) by the GAL4 protein (314). GAL4, an exogenous transcriptional activator derived from yeast, acts under the control of a spatial and/or temporal promoter known as a 'driver'. Upon GAL4 binding to UAS sites, the candidate transgene is expressed in a specific spatiotemporal manner, allowing the analysis of candidate gene function in targeted tissue-types. Adding to the utility of the system, transgenic RNAi libraries are widely available for conditional target gene inactivation (315, 316). Thus, the GAL4/UAS system represents a powerful and efficient method for targeted gene manipulation (317) and has been extremely beneficial for *Drosophila*-based research into the main themes of this thesis, namely innate inflammation (including glial cells) (160, 318, 319) and human neurodegenerative disease (320, 321).

A number of key innate inflammatory pathways are conserved from human to fly, including NF-κB signalling and the Toll pathway, initially discovered in *Drosophila* (71). In addition, the more compact genome of *Drosophila* offers less redundancy, an advantage when dissecting the complex pathways that shape innate immunity and inflammation (318). This degree of conservation is still being uncovered – only recently has a *Drosophila* orthologue of key antiviral protein Stimulator of Interferon Genes (STING) been characterised (322, 323), which functions in the antimicrobial response despite the lack of an interferon-based pathway in the fly. Indeed, there are other differences to consider; an antibody-mediated adaptive immune system is absent in *Drosophila*, though recent evidence suggests that immunological memory may still occur via an RNAi-mediated mechanism (324). Furthermore, while lacking antiviral RNA sensors RIG-I and MDA5 as well as their adaptor

molecule MAVS, the *Drosophila* Dicer proteins share significant homology with both RLRs **(Figure 1.2)** and Dicer-2 appears to act as an RNA sensor as part of the inducible antiviral response in *Drosophila* (53-55). Overall, the advantages of *Drosophila* as a *in vivo* model far outweigh its limitations in the field of immunity and inflammation.

Despite superficial differences, the *Drosophila* brain shows remarkable conservation with its vertebrate counterparts in terms of CNS development (325), signalling and regulatory mechanisms (326), as well as behavioural outputs such as sleep and memory (327). As such, the fly has proven a fruitful model for tackling the pathogenic mechanisms underlying human neurodegenerative disease (321). *Drosophila* models have been generated for AD (328-330), PD (331-334) and ALS/FTD (335-338). Additionally, expanded repeat neurodegenerative diseases have also been extensively modelled (339, 340), including the $rCAG_{\sim100}.CUG_{\sim100}$ dsRNA model utilized in this research (45, 52). This model was developed to investigate the role of bi-directionally transcribed repeat dsRNA in expanded repeat neurodegenerative disease, following data highlighting the occurrence of bi-directional transcription at many expanded repeat loci (21). The GAL4/UAS system is used to express constructs containing the untranslated repeat sequences, which are predicted to form either hairpin structures in the case of expression of $rCAG_{\sim100}$ or $rCUG_{\sim100}$ alone, or perfectly double-stranded RNA when both are expressed in conjunction **(Figure 1.8)** (45, 341). As previously discussed, expression of either the $rCAG_{\sim100}$ or $rCUG_{\sim100}$ repeat constructs alone does not cause pathology **(Figure 1.8)** (45, 342), whereas both constructs together ($rCAG_{\sim100}.CUG_{\sim100}$) lead to pathology when expressed in the *Drosophila* eye **(Figure 1.8)**, and neuronal dysfunction when expressed either neuronally or in glial cells (45, 52). Of particular note, the high level of neuronal dysfunction observed via glial expression of the repeat dsRNA indicates that non-cell autonomous mechanisms contribute to the pathology **(Figure 1.9)** (52).

Further dissection of the pathogenic mechanisms underlying the dsRNA eye pathology demonstrated that the repeat dsRNA not only induces an inflammatory response, but that inflammation is also required for the pathology (52). RNAi pathway components Dicer-2 and Ago2 are required for the resultant pathology **(Figure 1.9)** (45, 343) **(Appendix A)**,

Figure 1.8: Expression of CAG.CUG repeat constructs using the GAL4/UAS system. Under the control of a tissue-specific promoter, the GAL4 transcription factor binds to UAS sites in order to drive expression of **A)** *rCAG~100* or **B)** *rCUG~100*, each predicted to form hairpin RNA and **C)** *rCAG~100* and *rCUG~100* in conjunction, predicted to form perfectly double-stranded RNA. **D-E)** Expression of either *rCAG~100* or *rCUG~100* alone does not cause disruptions to the eye, while **F)** co-expression of *rCAG~100* and *rCUG~100* in conjunction leads to a disruptive eye phenotype and areas of necrosis. Figures **A-C** reproduced from (341). Figures **D-F** reproduced from (45).

implicating the RNAi response in the pathogenesis. However, over-expression of essential RNAi pathway cofactors *R2D2* and *Loquacious* rescued the eye pathology **(Figure 1.9)** (343) **(Appendix A)**. These findings suggest that 1) the RNAi pathway is in fact a protective mechanism in response to the expression of repeat dsRNA, and therefore 2) a distinct antiviral RNA response underlies the dsRNA pathology **(Figure 1.9)**. Indeed, Dicer-2 has been demonstrated to act as an RNA sensor to induce antiviral signalling (53, 54) and shares significant domain homology with known mammalian RNA sensors RIG-I and MDA5 **(Figure 1.2)** (55, 343) **(Appendix A)**. This highlights the possibility that Dicer-2 detects the repeat dsRNA as a 'non-self' or viral entity. Supporting this, ectopic expression of human *adenosine deaminase acting on RNA 1* (*ADAR1*), which acts to edit endogenous RNA transcripts to prevent their 'non-self' recognition by RNA sensors, also rescued the repeat dsRNA eye pathology **(Figure 1.9)** (343) **(Appendix A)**. Taken together, these findings suggest that expression of the dsRNA induces a damaging antiviral inflammatory response **(Figure 1.9)**.

The experiments in this study focus the role of the innate inflammatory system in driving repeat dsRNA-mediated pathology in the previously established $rCAG_{\sim100}.CUG_{\sim100}$ *Drosophila* model (45, 52). This includes investigating how the dsRNA is recognised by pattern recognition machinery **(Chapter 3)** and further examination of innate immune pathways that contribute to the dsRNA pathology **(Chapter 4)**. Finally, individual glial cell types are tested to determine their non-cell autonomous contribution to the strong dsRNA-mediated neuronal dysfunction observed via pan-glial expression of repeat dsRNA **(Chapter 5)**.
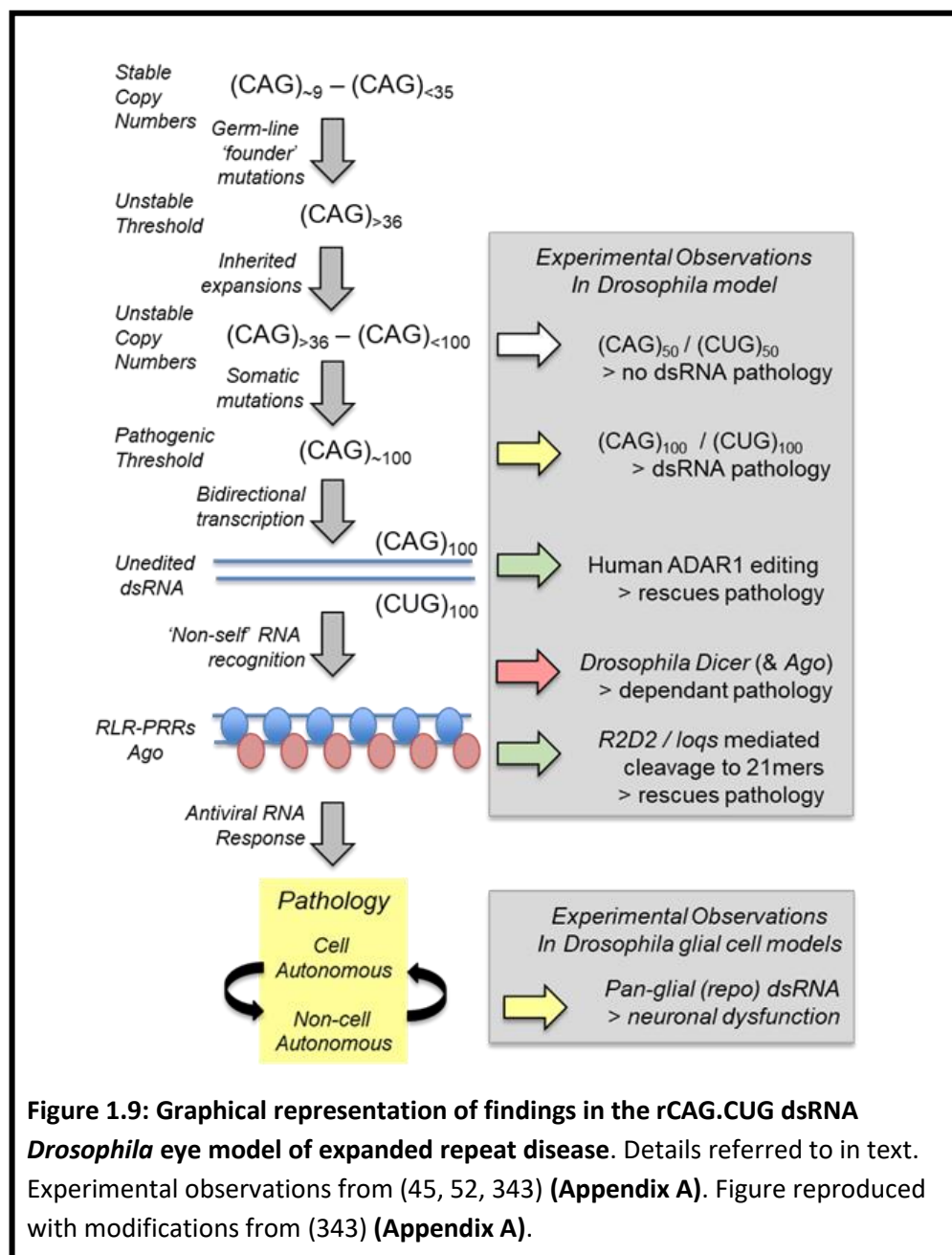
Figure 1.9: Graphical representation of findings in the rCAG.CUG dsRNA *Drosophila* eye model of expanded repeat disease. Details referred to in text. Experimental observations from (45, 52, 343) **(Appendix A)**. Figure reproduced with modifications from (343) **(Appendix A)**.

CHAPTER 2: Materials & Methods

2.1 Materials

Enzymes

Power SYBR® Green PCR master mix – Applied Biosystems

GoTaq® Flexi DNA Polymerase – Promega

DNAse I – Ambion

Kits

RNeasy® mini kit – Qiagen

Wizard® SV Gel and PCR Clean-Up System – Promega

High Capacity cDNA Reverse Transcription kit – Applied Biosystems

Other reagents

Deoxyribonucleoside triphosphate set (PCR grade) - Promega

1kb Plus DNA ladder – Invitrogen

TRIzol™ - Invitrogen

Oligonucleotides

All oligonucleotides are standard PCR and were obtained from Sigma-Aldrich unless specified. Sequences are present 5' to 3'.

General oligos – obtained from Promega

Random hexamers

Primers for PCR and sequencing

UASp-Fwd: GGCAAGGGTCGAGTCGATAG

UASp-Rev: AGGTTTAACCAGGGGATGCT

UASp-Seq-Fwd: CAAGGGTCGAGTCGATAG

UASp-Seq-Rev: TAACAAGTATGAATGTCAGGTT

R2D2-Rev: GGACGCAGTAGTCACGCAG

dADAR-Rev: AGAACTGCGCCATCCTTAAACTG

hADAR1-Rev: GCCATTGTAATGAACAGGTGGTT

hADAR2-Rev: CCCACGTAAAAGGGAGGCTC

Primers for qRT-PCR

Vago-Fwd: TGCAACTCTGGGAGGATAGC

Vago-Rev: AATTGCCCTGCGTCAGTTT

rp49-Fwd: GACGCTTCAAGGGACAGTATCTG

rp49-Rev: AAACGCGGTTCTGCATGAG

Nazo-Fwd: GCTGATCGGAGGACTACTGC

Nazo-Rev: TTGAAATTTCCCTCCGTAAGTC

STING-Fwd: CCGGTGTCTATCGTCCTTTC

STING-Rev: CGCTTTAGTTCCTGCATCTG

CG33926-Fwd: GCGACCGTCATTGGATTGG

CG33926-Rev: TGATGGTCCCGTTGATAGCC

Charon-Fwd: TCTCCAATCACGGTAAACAATG

Charon-Rev: GAACTTTGGTCGGATCTA CTGG

Drosomycin-Fwd: CGTGAGAACCTTTTCCAATATGATG

Drosomycin-Rev: TCCCAGGACCACCAGCAT

The following primers for qRT-PCR were obtained from Geneworks

Eiger-Fwd: CTGCTCGTGAATGCGATTCAT

Eiger-Rev: TGCAGTATGCACGATTCCGA

Buffers and solutions

Agarose gel loading dye (6X): 30% glycerol, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol.

TAE buffer: 40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA (pH 8.2).

Squishing buffer: 10mM Tris-HCl (pH 8.2), 1mM EDTA, 25mM NaCl

Drosophila media

Drosophila stocks were maintained on fortified (F1) medium composed of 1% (w/v) agar, 18.75% compressed yeast, 10% treacle, 10% polenta, 1.5% acid mix (47% propionic acid, 4.7% orthophosphoric acid) and 2.5% tegosept (10% para-hydroxybenzoate in ethanol).

Drosophila stocks

Transgenic repeat lines
The following recombinant dsRNA lines were used and have been described previously (45, 341).

$rCAG_{\sim100}.rCUG_{\sim100}^{S1}$ - $rCUG_{\sim100}$ [H,I]; $rCAG_{\sim100}$ [G,I]

$rCAG_{\sim100}.rCUG_{\sim100}^{S2}$ - $rCAG_{\sim100}$ [A,E]; $rCUG_{\sim100}$ [E,F]

$rCAG_{\sim100}.rCUG_{\sim100}^{M2}$ - $rCAG_{\sim100}$ [J,K]; $rCUG_{\sim100}$ [J,G]

$rCAG_{\sim100}.rCUG_{\sim100}^{M3}$ - $rCAG_{\sim100}$ [J,K]; $rCUG_{\sim100}$ [E,F]

$rCAG_{\sim100}.rCUG_{\sim100}^{W2}$ - $rCUG_{\sim100}$ [D]; $rCAG_{\sim100}$ [G]

Controls
4xUAS – 4 transgenes containing UAS sites without an insert in the attB system (344) [22A, 58A, 68E, 96E]

2xUAS – 2 transgenes containing UAS sites without an insert in the attB system (344) [51C, 68E]

UAS – 1 transgene containing UAS sites without an insert in the attB system (344) [51C]

Stocks obtained from Bloomington *Drosophila* Stock Centre (BDSC) are denoted with their corresponding Bloomington stock number (BL).

GAL4 driver lines

GMR-GAL4 – BL 1104 (345)

elavII-GAL4 – BL 8765 (346)

Repo-GAL4 – BL 7415 (347)

nrv-GAL4 – BL 6799 (348)

R54C07-GAL4 (referred to as *SPG-GAL4*) – BL 50472 (349)

R75H03-GAL4 (referred to as *TEG-GAL4*) – BL 39908 (349)

R56F03-GAL4 (referred to as *NEG-GAL4*) – BL 39157 (349)

R86E01-GAL4 (referred to as *ALG-GAL4*) – BL 45914 (349)

R54H02-GAL4 (referred to as *CG-GAL4*) – BL 45784 (349)

Mutant insertion alleles

upd1^{YM55} – BL 4767

upd3^{d11639} – BL 19355

drpr^{MI07659} – BL 43909

Mcr^{EY07421} – BL 15997

ECSIT^{PL00455} – BL 19508

Over-expression

UAS-CrPV1A 148 #1 was obtained from Christophe Antoniewski (350)

UAS-CrPV1A 148 #2 was obtained from Christophe Antoniewski (350)

UAS-CrPV1A 108 #1 was obtained from Christophe Antoniewski (350)

UAS-CrPV1A 108 #2 was obtained from Christophe Antoniewski (350)

PBac(STAT92E-GFP.FLAG) – BL 38670

UAS-Draper (Isoform I) – BL 67035 (351)

UAS-Draper (Isoform II) – BL 67036 (351)

UAS-Draper (Isoform III) – BL 67037 (351)

UAS-parkin – BL 51651

UAS-Ref(2)P-GFP – (352)

RNAi lines

P(UAS-Stat92E.RNAi)1 – BL 26899

The following lines are from the Vienna *Drosophila* Research Centre (VDRC) collection and were obtained from the VDRC stock centre.

upd1^{v3282}

upd3^{v106869}

drpr^{v27086}

Mcr^{v100197}

PINK1^{v21860}

PINK1^{v109614}

parkin^{v47636}

Ref(2)P^{v105338}

ECSIT^{v31287}

ECSIT^{v106141}

2.2 Methods

DNA manipulation

Agarose gel electrophoresis

Gel electrophoresis was performed using 1% agarose in 1xTAE buffer supplemented with ethidium bromide (EtBr) at a final concentration of 0.5µg/mL. DNA fragments were separated by submerging the gel in a tank containing 1xTAE buffer and applying 100V to the tank. DNA fragments were visualised by UV light exposure.

Gel purification

DNA bands were excised from agarose gels under UV light and purified using the Wizard® SV Gel and PCR Clean-Up System kit according to manufacturer's instructions (Promega).

Drosophila genomic DNA preparation

1 whole adult fly per genotype was frozen on dry ice in a 0.5mL tube. The fly was then mashed in 20µL of squishing buffer **(see section 2.1)** mixed with 5µL 1g/µL Proteinase K (for a final concentration of 200µg/mL) for 20-30 seconds before incubation at 55°C for 3 hours.

Proteinase K was inactivated via incubation at 95°C for 2 minutes, followed by storage at 4°C.

PCR amplification of *Drosophila* genomic DNA

PCR amplification was performed using a Bio-Rad DNAEngine Peltier Thermal Cycler.

Genomic DNA was amplified using GoTaq® Flexi DNA Polymerase reagents with GoTaq® Green Reaction Buffer according to the manufacturer's guidelines (Promega). The cycling conditions were 95°C for 2 minutes, then 35 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, followed by a final 72°C for 5 minutes.

Drosophila husbandry

Flies were raised at 18°C or 25°C with 70% humidity on F1 medium. Crosses were performed at 25°C unless detailed otherwise.

RNA purification

RNA isolation
30 heads from 1-day old adult female flies were collected per genotype for RNA extractions. The heads were snap frozen in liquid nitrogen and stored at -80°C. The tissue samples were homogenised in 1mL TRIzol™ with a plastic pestle and further homogenised with a 20-gauge needle. The homogenate was then pelleted by spinning at 13000 rpm for 10 minutes at 4°C. The resulting supernatant was transferred to fresh RNAse free tubes and 200µL chloroform was added and mixed via shaking vigorously. The samples were then spun at 13000 rpm for 15 minutes at 4°C and the upper aqueous phase was transferred to fresh RNAse free tubes. An equal volume of 100% ethanol was added to the sample and mixed briefly via vortexing. The sample mixtures were then loaded into RNeasy® columns and the remaining purification steps were carried out according to the instructions of the Rneasy® mini kit. The RNA was eluted in 30µL Nuclease-Free water and stored at -80°C until further use.

Quantitative real-time PCR (qRT-PCR)

Reverse transcription

800ng of RNA was DNAse treated using DNAse I according to the manufacturer's instructions (Ambion) and then reverse transcribed using the High Capacity cDNA Reverse Transcription kit to produce cDNA according to manufacturer's instructions (Applied Biosystems). Reverse transcription reactions were performed at 25°C for 10 minutes, 37°C for 2 hours and then 85°C for 5 minutes.

qRT-PCR reactions

Reverse transcription reactions were diluted 1/5 in Nuclease-free water and 2μL was used as the template in a final reaction volume of 10μL. Triplicate reactions were performed for each template with 1.26pmol of each primer together with 1X *Power* SYBR® Green PCR master mix in a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). The cycling conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A standard curve was generated for each primer pair by including serial dilutions from cDNA (neat, 1/2, 1/5, 1/10). A dissociation curve was produced for each primer pair to confirm that a single product was amplified in each reaction. The relative amount of cDNA was quantified using the relative standard curve method and the results were subsequently exported to Microsoft Excel. The quantity of amplified cDNA product for each primer pair was normalised against the quantity of cDNA produced with primers for the housekeeping gene *Ribosomal protein 49* (*Rp49*) for the corresponding sample.

Rapid Iterative Negative Geotaxis (RING) assay

0-1 day old adult male flies were collected in sets of between 10-20 flies and allowed to recover from anaesthesia overnight at 25°C. A minimum of 40 flies were collected per genotype when possible. Flies were transferred to standard vials and into the RING apparatus as described in Gargano *et al.,* (353). The apparatus was tapped several times to transfer the flies to the bottom of the tube, and then imaged after 5 seconds (Google Pixel camera). Experimental flies and their appropriate control were imaged side-by-side. Five consecutive trials were performed per set of flies per timepoint, with the flies allowed to

recover for 1 minute between each trial. The images were then transferred into the Fiji Is Just ImageJ (FIJI) image analysis program and a line was drawn to represent the halfway mark of each vial. Flies were then scored as either "successful" if they passed the halfway mark, or "unsuccessful" if they failed to pass the halfway mark. The percentage of "successful" flies per trial was recorded as "% climbing ability". The "% climbing ability" for each trial formed a single data point, with the total number of trials for each genotype plotted as a scatter graph displaying the average mean and standard deviation using GraphPad Prism 7. The average "% climbing ability" was then calculated for each genotype and compared to appropriate age-matched controls. When comparing two datasets, a 2-tailed Student's t-test was performed to determine significance, while a one-way ANOVA and Dunnett's test were performed to determine significance when comparing more than two datasets. The significance threshold was set at $p<0.05$. The same sets of flies were aged by transferring to fresh food every 2-3 days. RING assays were performed at timepoints of 1, 8 and 15 days of age. Set of flies that fell below 10 individuals during ageing were omitted from further analysis.

Survival assay

The same flies analysed in RING assays were also assayed for their survival, with the number of individuals per genotype recorded per week at 1, 8, 15, 22 and 29 days of age. The number of individuals counted at 1 day of age was designated as 100% survival for each genotype, and subsequent counts were divided by this total count to represent the percentage of individuals remaining.

Eye photography

Eyes of at least 20 one day old female flies were viewed per genotype if possible, with 3 representative eyes photographed per genotype. Eyes were imaged using an Olympus SZX7 microscope fitted with an SZX-AS aperture along with an Olympus ColourView IIIU Soft Imaging System and ToupView image acquisition software. Adobe Photoshop CS was used for image preparation. Anterior is to the left in all presented images.

CHAPTER 3: 'Non-self' recognition of repeat dsRNA

The innate immune system acts as a protective first-line defence against invading pathogens and other 'non-self' material. Pattern recognition receptors (PRRs) act as sensors of a wide range of 'non-self' stimuli, including conserved structures on bacteria and viruses, as well as endogenous molecules released under conditions of stress/injury that constitute danger signals (69). Detection of a threat is followed by the induction of an inflammatory response, resulting in removal of the offending molecules through degradation and/or cell death signalling (354). However, persistence of the threat through genetic or environmental disruption of inflammatory pathways can lead to elevated cell death and tissue damage. Indeed, genetic lesions in a number of genes involved in inflammatory processes have been identified as causal or risk factors in neurodegenerative disease (68). Additionally, elevated cytokine levels are consistently observed in Huntington's Disease (HD) gene carriers and precede the onset of clinical symptoms (58, 59), indicating that inflammation likely represents a proximal causal factor rather than consequence of disease pathology.

The relative conservation of mammalian and fly innate immune pathways allows the use of *Drosophila* to genetically dissect the role of inflammation under conditions of homeostasis and disease (318, 355). Previous work in the *Drosophila* eye tissue model of dsRNA-mediated pathology utilized here has demonstrated the requirement for inflammation in the development of pathology, including the activity of the Toll-like receptor (TLR) innate immune pathway (52) and Dicer-2, which shares homology with the RIG-I-like receptor (RLR) family of antiviral RNA sensors in vertebrates (45). Additionally, expression of the repeat dsRNA induced upregulation of the antimicrobial peptide *Drosomycin* and the potent cytokine/cell death *Eiger*, the orthologue of mammalian TNF (52). Taken together, this evidence led to the hypothesis that repeat dsRNA is recognized as a 'non-self' molecule by the innate immune system, thus provoking a damaging cascade of inflammatory signalling that manifests as pathology (4, 52).

Dicer-2 plays a key role in the invertebrate RNA interference (RNAi) pathway; where it acts to detect viral dsRNA products and process them into 21-nucleotide small-interfering RNAs (siRNAs) (356, 357). The processing step also involves the essential co-factor R2D2, which

forms a complex with Dicer-2 to interact with RNA duplexes (358, 359). Following processing, the siRNAs are loaded into the Argonaute-2 (Ago2) mediated RNA-induced silencing complex (RISC), where it cleaves the siRNA and utilizes the guide strand to locate complementary viral mRNA in order to facilitate their subsequent silencing and/or degradation (360-362). Indeed, Ago2 also contributes to the pathology observed in the repeat dsRNA eye pathology, though not to the extent of Dicer-2 (341). Intriguingly however, over-expression of R2D2 was demonstrated to potently suppress the repeat dsRNA pathology while loss-of-function mutations enhanced the pathology (343) **(Appendix A)**. These findings led to the hypothesis that the RNAi pathway may not drive dsRNA pathogenesis, but in fact act as a competing pathway to restrict the influence of the repeat dsRNA.

Notably, Dicer-2 also displays potent antiviral activity independently of the RNAi pathway; Drosophila C virus (DCV) infection leads to Dicer-2 dependent upregulation of the antiviral peptide *Vago* and the control of viral load through an unknown signal transduction pathway (53). Both Ago2 and R2D2 were dispensable for the pathway, thus uncoupling Dicer-2 from its previously established role in RNAi processing (53). Though not strictly independent of the RNAi pathway, Dicer-2 also regulates the production of reverse-transcribed viral DNA (vDNA) that serves to amplify the antiviral response in a manner distinct from siRNA production but dependent upon Ago2 (363). Taken together, Dicer-2 (and Ago2 to an extent) play important roles in a number of antiviral mechanisms, and these functions can be either helpful or harmful depending upon stimuli context.

Indeed, RNAi machinery displays a high level of adaptive evolution across a number of invertebrate species (364), highlighting the importance and considerable selection pressure placed upon antiviral genes. However, viruses themselves also display adaptation to the antiviral defence system in *Drosophila* as part of the ongoing arms race waged between host and virus. A number of insect viruses encode viral suppressors of RNAi silencing (VSRs) that allow them to inhibit RNAi machinery in order to maximise their replication and spread (50). Interestingly, VSRs display diversity in the steps at which they inhibit the RNAi pathway; Drosophila C virus encodes a VSR (DCV-1A) containing a dsRNA binding domain that allows it to block Dicer-2 from cleaving viral dsRNA (350), while the cricket paralysis virus (CrPV)

encodes CrPV1A, a VSR that interacts with Ago2 to prevent its endonuclease activity downstream of siRNA production (350). Additionally, CrPV1A can also promote the ubiquitination of Ago2, leading to its degradation through an E3-ubiquitin ligase complex (365). Finally, CrPV1A can inhibit host transcription and disrupt the assembly of RNA stress granules independently of its RNAi silencing function, demonstrating that CrPV1A acts to silence multiple antiviral pathways upon infection (366). Indeed, CrPV1A may also act to suppress other uncharacterised antiviral mechanisms based on its diverse range of interactions with host machinery.

The RNAi pathway acts to promote degradation of viral material, thus promoting cell survival. However, focal cell death is also a critical factor in antiviral defence, serving as a potent means to restrict the spreading of a virus (and indeed all pathogens) to adjacent uninfected cells (367). When dsRNA arises endogenously, RNA-editing enzymes act to catalyse the conversion of these repeat sequences to molecules that are recognized as 'self' by pattern recognition machinery. In vertebrates, ADAR1 converts adenosine (A) to inosine (I) in endogenous dsRNA molecules including *Alu* RNA repeats, masking their presence to dsRNA sensor MDA5 (encoded by *IFIH1*) and thus preventing inflammation and cell death (152, 156, 368, 369). Loss of editing or increased receptor sensitivity (through mutations in *ADAR1* and *IFIH1* respectively) are both proximal causes of the auto-inflammatory disease Aicardi-Goutieres Syndrome (AGS) (154, 155), highlighting the importance of 'non-self' to 'self' conversion as a regulatory mechanism. In line with this, ectopic expression of human *ADAR1* rescues repeat dsRNA-mediated eye pathology in *Drosophila* (343) **(Appendix A)**. The bi-directionally transcribed CAG.CUG repeat used in this model provides an ideal target for human ADAR1, which preferentially edits repetitive RNA sequences (370). Thus, masking the dsRNA through A-to-I conversion (CAG-to-CIG) appears to prevent its detection by *Drosophila* inflammatory machinery.

Therefore, several of lines of evidence point towards repeat dsRNA acting as a ligand to drive antiviral inflammation in *Drosophila*. The work described in this chapter further investigates the hypothesis that expanded repeat dsRNA is recognized by host antiviral pathways as a 'non-self' or viral-like molecule, thus leading to a damaging inflammatory response that is responsible for the observed pathology.

3.2 The viral suppressor protein CrPV1A rescues repeat dsRNA-mediated pathology

The cricket paralysis virus (CrPV) infects a broad range of insect species (371) and causes lethality in *Drosophila* (372). In order to avoid antiviral silencing through the RNAi pathway, the virus encodes the CrPV1A protein, a potent viral suppressor that targets and inhibits the activity of the Ago2 protein; both through a direct interaction that blocks the Ago2 endonuclease function (350) and the recruitment of ubiquitination machinery to degrade Ago2 (365). In this manner, CrPV1A blocks Ago2 activity downstream of the Dicer-2/R2D2 processing complex, and thus does not affect the cleavage of viral dsRNA into siRNAs (350).

The requirement for both Dicer-2 and Ago2 in repeat dsRNA eye pathology has led to the suggestion that inflammatory signalling stemming from viral-like recognition of the repeats is a key driver of the cellular dysfunction (45, 341). In order to further pursue this hypothesis, the *CrPV1A* viral suppressor was ectopically expressed in conjunction with the repeat dsRNA specifically within the *Drosophila* eye using the *GMR-GAL4* driver. For this purpose, two distinct forms of CrPV1A were examined; the full-length (148 amino acid) protein is an efficient silencer of Ago2 (350), whereas the truncated (108 amino acid) protein lacks key residues that facilitate interactions with Ago2 and is thus unable to interfere with antiviral signalling (350, 365). The identities of the CrPV1A full-length and mutant constructs were confirmed through sequencing of one each of the full-length and mutant lines obtained (343) **(Appendix A)**.

Ectopic expression of two independent full-length *CrPV1A* constructs alone did not cause disruptions to the eye **(Figure 3.1 B & C)** and a similar lack of phenotype was observed with each of two independent truncated *CrPV1A* mutant constructs **(Figure 3.1 D & E)**. As previously established, expression of repeat dsRNA within the eye caused loss of pigmentation and structural patterning as well as the development of black necrotic spots on the posterior side of the eye **(Figure 3.1 A')**. Strikingly however, co-expression of the dsRNA with full length *CrPV1A* completely rescued all aspects of the dsRNA-mediated pathology in both lines **(Figure 3.1 B' & C')**, indicating that CrPV1A-mediated antagonism of the antiviral response may mask the repeat dsRNA from RNA pattern recognition machinery.

46

Figure 3.1: Ectopic expression of *CrPV1A* completely rescues dsRNA-mediated pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert as a control eye. **B-C)** Expression of two independent full-length (FL) *UAS-CrPV1A* constructs alone. **D-E)** Expression of two independent truncated *UAS-CrPV1A* mutant constructs alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B'-C')** Co-expression of repeat dsRNA together with two independent full-length (FL) *UAS-CrPV1A* constructs. **D'-E')** Co-expression of repeat dsRNA together with two independent truncated *UAS-CrPV1A* mutant constructs. The full length *CrPV1A* encodes a 148 amino acid long protein. The truncated *CrPV1A* encodes a 108 amino acid long protein. The experiment was performed at 25°C using the CAG.CUG$_{\sim100}$^{M3} dsRNA line.

Furthermore, both truncated mutant constructs failed to rescue the dsRNA pathology **(Figure 3.1 D' & E')**, thus demonstrating the requirement of CrPV1A-mediated antiviral silencing activity for the suppression.

The ability of the CrPV1A viral suppressor protein to completely rescue the phenotype supports the hypothesis that expanded repeat dsRNA is recognized as 'non-self' in a manner similar to viral material. Since CrPV1A does not inhibit dsRNA processing but rather the catalytic activity of Ago2 (350, 365), and that the canonical RNAi pathway appears to act as a competing pathway in restricting the dsRNA pathology (343) **(Appendix A)**, it is plausible

Figure 3.2: Summary diagram regarding CrPV1A-mediated suppression of expanded repeat dsRNA eye pathology. While RNAi components Dicer-2 and Ago2 are required for the dsRNA pathology (red), key RNAi binding cofactor R2D2 acts to suppress the pathology (green), suggesting that the RNAi antiviral mechanism constitutes a protective pathway in response to expression of repeat dsRNA. Thus, Dicer-2 appears to act in its established role as an RNA sensor, likely together with Ago2, to drive the dsRNA eye pathology through an antiviral cell death mechanism. CrPV1A antagonizes Ago2 to block the RNAi pathway, but also appears to ablate the antiviral inflammatory mechanism underlying the dsRNA eye pathology.

that Dicer-2 and Ago2 act together in a manner distinct from their roles in RNAi to drive inflammation **(Figure 3.2)**. Indeed, *Drosophila Dicer-2* is related to RIG-I-like receptors *RIG-I* and *IFIH1* (MDA5) through shared homology in their DExD/H helicase domains (55). The helicase domain is critical for the observed RNAi-independent antiviral functions of Dicer-2, including *Vago* induction (53) and viral DNA (vDNA)-mediated amplification of antiviral activity (363). Ago2 is not required for *Vago* induction (53) but is essential for vDNA production (363), suggesting that this pathway may represent another target of the CrPV1A protein.

3.3 Transcript analysis of the inflammatory response to expanded repeat dsRNA

Previous work has demonstrated that the inflammatory response is not only required for the repeat dsRNA pathology but is also triggered by the presence of the 'non-self' dsRNA itself (45, 52). Thus, qRT-PCR was performed to investigate A) if components of other inflammatory pathways are upregulated in response to repeat dsRNA, and B) whether co-expression of *CrPV1A* can ablate any observed induction, thus indicating potential targets for further investigation. The *elavII-GAL4* driver was utilized to express the relevant constructs in all neuronal cells, where the expression of repeat dsRNA causes age-dependent neurodegeneration (45). Neuronal tissue samples were taken from one day old flies in order to maintain a temporal window consistent with previous transcript analysis (52). Additionally, as the neurodegeneration observed in *elavII*>dsRNA flies is age-dependent (45), this timepoint minimises both the possibility of transcript loss due to dead/dying cells within the CNS and the dilution of altered RNA levels by transcripts from unaffected (in this case non-*elavII* expressing) cells in within the sample tissue (*Drosophila* heads).

An important final note; lack of significant alteration to gene expression in response to the repeat dsRNA does not rule out the given gene in participating in the dsRNA pathology. Modest or even no change in transcript levels may be adequate to surpass a functional threshold and thus contribute to the pathology. In addition, post-transcriptional/post-translational regulation of components are both common features in innate immune signalling pathways (373, 374), both of which will not be reflected in this analysis. Nevertheless, qRT-PCR can provide an effective insight into dsRNA-triggered pathway activation via the transcription of downstream response genes.

Drosomycin is an antimicrobial peptide (AMP) synthesised as a result of signalling from the Toll inflammatory pathway (375) and has previously been shown to be upregulated in the presence of repeat dsRNA (52). Therefore, *Drosomycin* transcript levels were analysed to confirm the upregulation and to determine if co-expression of CrPV1A blocks this response. Indeed, *Drosomycin* was significantly upregulated in *elavII*>dsRNA flies **(Figure 3.3)**.

Figure 3.3: The antimicrobial peptide *Drosomycin* is significantly upregulated in response to repeat dsRNA. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. Expression of repeat dsRNA alone leads to a significant upregulation of *Drosomycin* compared to the UAS empty transgene control (p=0.0077), as did co-expression of repeat dsRNA and *CrPV1A* compared to the control (p=0.0360). Co-expression of *CrPV1A* with repeat dsRNA does not significantly suppress the induction of *Drosomycin* compared to repeat dsRNA expression alone (p=0.4240). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ** p<0.01, * p<0.05, ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49.* Each dataset represents 3 biological replicates plated in triplicate. The experiment was performed using the CAG.CUG$_{\sim100}$^{S1} dsRNA and CrPV1A FL #1 lines.

Introduction of CrPV1A did not significantly suppress *Drosomycin* upregulation when compared to *elavII*>dsRNA, though a small trend was observed **(Figure 3.3)**. Thus, *Drosomycin* induction (and Toll pathway activity) is clearly observable in response to dsRNA expression but does not appear to be a target of the CrPV1A viral suppressor.

Overexpression of *Drosomycin* alone (or indeed several other AMPs alone) in neurons or glia leads to age-dependent neurodegeneration in flies, indicating that AMPs can become neurotoxic at high levels (376). The flies used in this qRT-PCR analysis were newly-eclosed, and thus it is likely that *Drosomycin* does not accumulate at the level required for neurotoxicity at the early time point tested in this study. The *Drosophila* Toll pathway is canonically triggered after detection of gram-positive bacteria and fungi as opposed to viral RNA intermediates (377), though Toll pathway mutants exhibit increased susceptibility to Drosophila X virus (DXV) (378) and oral infection of Drosophila C virus (DCV) (379). In addition, recent evidence has demonstrated that Dicer-2 can bind Toll directly and drive induction of *Drosomycin* upon microbial challenge (54), thus providing a plausible explanation for the *Drosomycin* upregulation observed here. Alternatively (though potentially not mutually exclusive), the upregulation of *Drosomycin* may represent a non-cell

autonomous Toll-mediated response to damage/danger associated molecular patterns (DAMPs) and other trigger molecules, released from cells that are damaged/killed by the focal dsRNA-induced inflammatory response.

Given the likely role of cell death signalling in dsRNA pathology, the expression level of potent cytokine and cell death signalling molecule *Eiger* was also investigated. Eiger is the sole *Drosophila* orthologue of tumour necrosis factor (TNF), a subject of intensive research for its key roles in inflammation and programmed cell death (380). Indeed, these key roles are conserved through to Eiger, which interacts with its receptors Wengen and Grindelwald (orthologues of mammalian TNF receptors) to activate the c-Jun N-terminal kinase (JNK) pathway-dependent cell death (381-383). While *Eiger* mutants are rendered susceptible to pathogen challenge and tumour progression (384, 385), overexpression of *Eiger* leads to developmental defects through heightened cell death signalling (381, 383). Elevated levels of mammalian *TNF* is a constantly observed feature in neurodegenerative disease (386, 387), and in pre-symptomatic HD disease gene carriers (58, 59). Finally, upregulation of *Eiger* transcript levels has previously been observed in the repeat dsRNA model used in this study (52). Therefore, *Eiger* transcript levels were assessed to determine if CrPV1A-driven antagonism of the antiviral response affects *Eiger* induction.

Eiger was not significantly altered in *elavII*>dsRNA flies, while also remaining unchanged when *CrPV1A* was co-expressed with the dsRNA **(Figure 3.4)**. Given the potent cell death signalling initiated through *Eiger*, this lack of alteration may represent the possibility that the cells containing elevated *Eiger* are undergoing cell death and thus losing *Eiger* signal. Indeed, Eiger is a highly pleiotropic inflammatory cytokine with a number of physiological roles (388). As such, the focus was shifted to investigate inducible antiviral pathways, which are more likely to respond to the presence of dsRNA given its 'non-self'/viral recognition through Dicer-2. The RNAi response is an important mechanism for combating viral infection, achieving this via the production of siRNAs as opposed to inducing the expression of antiviral peptides (389). However, aside from the RNAi response, the inducible *Drosophila* antiviral response remains poorly understood (390). Thus, peptides that are synthesised through the *Drosophila* antiviral response were investigated as readouts of antiviral pathway activity. Recent work has demonstrated that the RNAi response confers resistance to a

Figure 3.4: *Drosophila TNF* orthologue *Eiger* **is not significantly altered in response to repeat dsRNA**. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. *Eiger* transcript levels were not significantly impacted by the presence of repeat dsRNA alone (p=0.2807) or through co-expression of *CrPV1A* and the dsRNA (p=0.6927) when compared to the UAS empty transgene control. *Eiger* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.4057). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49*. Each dataset represents 3 biological replicates plated in triplicate. The experiment was performed using the CAG.CUG$_{\sim 100}^{S1}$ dsRNA and CrPV1A FL #1 lines.

broad range of viruses, while antiviral activity through the evolutionarily conserved JAK-STAT pathway is restricted to viruses from the *Dicistoviridae* family; DCV and CrPV (391). Additionally, Dicer-2 can respond to DCV independently of its role in RNAi in order to upregulate the antiviral peptide Vago and control infection (53), a process that is evolutionarily conserved in the mosquito *Culex quinquefasciatus* (392). Finally, recent evidence has uncovered co-operation between IMD pathway components Relish and dIKKβ with the *Drosophila* STING orthologue to induce the expression of a battery of antiviral response genes in response to DCV and CrPV (393). This included the novel antiviral peptide Nazo, which strongly attenuates DCV and CrPV infection when overexpressed (393).

To determine whether the antiviral pathways regulating *Vago* and *Nazo* are active in response to the presence of repeat dsRNA, the expression levels of both antiviral peptides were analysed through qRT-PCR. Neither *Vago* nor *Nazo* were significantly altered in response to expression of repeat dsRNA, while co-expression of *CrPV1A* with the dsRNA also did not affect expression levels of the antiviral peptides **(Figure 3.5 A & B)**. Given that expression of either *Vago* or *Nazo* is not elevated in response to repeat dsRNA, any potential antagonism of the antiviral response through CrPV1A is likely masked. Based on the

52
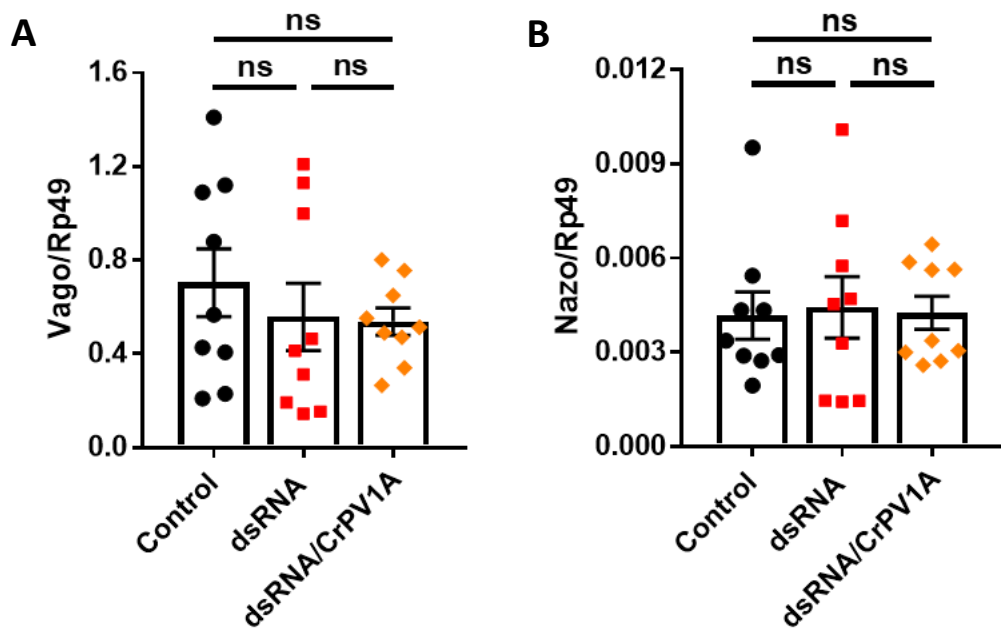
Figure 3.5: Expression levels of the antiviral peptides *Vago* and *Nazo* are not significantly altered in response to repeat dsRNA. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. **A)** *Vago* transcript levels were not significantly impacted by the presence of repeat dsRNA alone (p=0.4843) or through co-expression of *CrPV1A* and the dsRNA (p=0.3030) when compared to the UAS empty transgene control. *Vago* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.9008). **B)** *Nazo* transcript levels were not significantly impacted by the presence of repeat dsRNA alone (p=0.8324) or through co-expression of *CrPV1A* and the dsRNA (p=0.9225) when compared to the UAS empty transgene control. *Nazo* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.8769). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49*. Each dataset represents 3 biological replicates plated in triplicate. The experiments were performed using the CAG.CUG$_{\sim 100}$^{S1} dsRNA and CrPV1A FL #1 lines.

observation that Ago2 is dispensable for the induction of *Vago* (53), it is unlikely that CrPV1A targets this pathway, though this cannot be completely ruled out. *Nazo* is upregulated following CrPV infection (393), though whether CrPV1A targets this pathway is also unknown. However, since Ago2 was shown to be dispensable for *Nazo* induction, the authors hypothesised that this novel pathway may constitute an additional layer of antiviral defence independent of DCV/CrPV-derived VSRs (393).

The observation that expression of dsRNA does not induce *Vago* suggests that the role of Dicer-2 in regulating this pathway is independent of the mechanism(s) responsible for the pathology. Indeed, knowledge of the pathway from Dicer-2 to Vago remains limited, including whether other antiviral peptides are induced through this mechanism. Thus, identification of the essential participants in this pathway (both interactors and induced genes) is required before a definitive assessment of its role in mediating dsRNA pathology can be undertaken. *Nazo* has only recently been characterised as antiviral effector molecule that controls CrPV replication (393), and as such the antiviral mode of action of Nazo remains unknown. Regardless, it would be of interest to determine if *Nazo* can modify the dsRNA eye phenotype, given that its overexpression potently restricts both DCV and CrPV replication (393).

Figure 3.6: The STING/Relish/dIKKβ-mediated antiviral pathway in Drosophila. Current pathway proposed from experimental data obtained by Goto *et al.* Drosophila C virus (DCV) is detected by an unknown viral sensor(s), leading to downstream signalling through STING, dIKKβ and NF-κB transcription factor Relish. Relish (possibly in conjunction with other transcription factors) then induces the expression of a range of antiviral effector genes including *STING* itself, *Nazo*, *Charon* and the uncharacterised *CG33926*. The regulatory protein Kenny (dIKKγ) inhibits this antiviral signalling pathway, potentially through its role in mediating autophagic degradation of the IKK complex. Genes in this pathway tested via qRT-PCR in this study are *STING*, *Relish*, *Nazo*, *Charon* and *CG33926*. Figure adapted from (393).

In addition to *Nazo*, nine other transcripts were also induced via the STING/Relish/dIKKβ-dependent antiviral pathway in response to DCV in both flies and *Drosophila* S2 cells, including a number of uncharacterised genes **(Figure 3.6)** (393). One of these, *CG33926*, was induced potently by all three of Relish, STING and dIKKβ (393). Given its strong induction following DCV infection, *CG33926* was investigated in the context of repeat dsRNA

Figure 3.7: Expression of *CG33926* is significantly downregulated in response to repeat dsRNA. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. *CG33926* transcript levels were significantly reduced in the presence of repeat dsRNA alone (p=0.0099) and through co-expression of *CrPV1A* and the dsRNA (p=0.0126) when compared to the UAS empty transgene control. *CG33926* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.6087). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ** p<0.01, * p<0.05, ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49*. Each dataset represents 3 biological replicates plated in triplicate. The experiment was performed using the CAG.CUG$_{\sim100}$^{S1} dsRNA and CrPV1A FL #1 lines.

expression. Expression of the dsRNA alone led to a striking reduction in *CG33926* expression when compared to the UAS control, a finding also observed when *CrPV1A* was co-expressed with the dsRNA **(Figure 3.7)**. No significant difference was observed when comparing *elavII*>dsRNA and *elavII*>dsRNA/CrPV1A flies **(Figure 3.7)**. Therefore, the expression of repeat dsRNA appears to potently inhibit the induction of *CG33926*.

Unfortunately, the lack of information regarding CG33926 function limits the interpretation of this result. Indeed, RNAi knockdown of *CG33926* promotes DCV replication, but not to the magnitude achieved via knockdown of *dIKKβ*, *Relish*, *STING* or *Nazo* (393), and thus it potentially shares functional redundancy with other genes that were also induced. Of note, *CG33926* is also upregulated following infection from the DNA virus Kallithea virus (394), further strengthening its proposed role as an antiviral effector. Interesting however, is that the presence of dsRNA inhibits *CG33926* expression as opposed to triggering its induction **(Figure 3.7)**. One plausible explanation is that expression of the dsRNA triggers the upregulation of *Kenny* (also known as *dIKKγ*), a regulatory protein that promotes autophagic degradation of the IκB signalling complex to prevent constitutive IMD pathway activity (395). In support of this, Kenny restricts the expression of *CG33926* (in addition to *Nazo* and

STING) following DCV infection, likely through inhibition of the STING/Relish/dIKKβ signalling pathway (393).

This raises the question as to why Kenny downregulates *CG33926* but not *Nazo* in response to repeat dsRNA, given that both genes appear to rely on dIKKβ signalling for induction (393). It is plausible that this reflects the tailoring of antiviral gene transcription in a threat-specific manner; the set of antiviral genes induced through challenge with DCV is likely to display both overlap and distinct differences with the corresponding set upregulated in response to repeat dsRNA. As such, it would be of interest to determine which of the 10 elevated transcripts identified in Goto *et al.,* as DCV-responsive are also induced following expression of repeat dsRNA. Indeed, *CG33926* was induced following DCV infection (393) but strongly repressed in response to the dsRNA **(Figure 3.7)**, which serves to highlight the complexity of the *Drosophila* antiviral response.

Among the other candidates, the IκB gene *Charon* is one of the few that has been previously characterised (393). Charon (also known as Pickle) is vital for Relish-dependent transcription of antimicrobial genes (396), but also inhibits the formation of Relish-Relish homodimers to prevent aberrant IMD signalling following pathogenic challenge (397). Interestingly, neurodegeneration in a *Drosophila* model of Ataxia-Telangiectasia (A-T) is dependent upon Relish-mediated transcription of innate immune genes (311), and as such regulation of Relish by Charon may be vital in the context of neurodegenerative disease. Thus, expression levels of *Charon* were assessed in the presence of dsRNA.

Expression of repeat dsRNA led to a reduction in *Charon* transcript levels when compared to the UAS control, though this was not significant **(Figure 3.8)**. Indeed, a similar decrease in *Charon* was observed compared to the control when *CrPV1A* was co-expressed with the dsRNA, though again significance was not reached **(Figure 3.8)**. Finally, levels of *Charon* were almost identical between *elavII*>dsRNA and *elavII*>dsRNA/CrPV1A flies **(Figure 3.8)**. Given the trend observed, it would be of interest to gain a greater sample size to determine whether or not the reduction is a true biological effect as opposed to variation between samples.

Figure 3.8: Expression of IκB gene *Charon* is not significantly altered in response to repeat dsRNA. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. *Charon* transcript levels were not significantly impacted by the presence of repeat dsRNA alone (p=0.1923) or through co-expression of *CrPV1A* and the dsRNA (p=0.090) when compared to the UAS empty transgene control. *Charon* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.8462). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49*. Each dataset represents 3 biological replicates plated in triplicate. The experiment was performed using the CAG.CUG$_{\sim 100}{}^{S1}$ dsRNA and CrPV1A FL #1 lines.

Like *CG33926*, *Charon* is also negatively regulated by Kenny (dIKKγ) (393). Under homeostatic conditions, Kenny is selectively targeted to autophagosomes for degradation, whereas in response to commensal bacteria it interacts with dIKKβ and instead targets the IKK signalling complex for degradation, thus terminating the immune response (395). As such, a possible scenario to explain *CG33926* and *Charon* downregulation is that expression of repeat dsRNA induces *Kenny*, which then acts to negatively regulate dIKKβ-dependent signalling through degradation of the IKK complex. Co-expression of *CrPV1A* with the dsRNA failed to restore both *CG33926* and *Charon* levels to that of the corresponding UAS controls **(Figures 3.7 & 3.8)**, providing evidence that CrPV1A does not target Kenny. In line with this, RNAi knockdown of *Kenny* attenuates viral replication (393), and thus targeting it would be detrimental to the virus. In line with this, the transcript levels of two of the core regulators of this novel antiviral pathway, STING and Relish, were also monitored in response to the repeat dsRNA.

The innate immune effector STING was first discovered in mammals as a potent regulator of type-I interferon (IFN) production in response to DNA-based pathogens (398, 399). Only recently has a conserved invertebrate STING orthologue been uncovered; responding to

Figure 3.9: Antiviral effector molecule *STING* is not significantly altered in response to repeat dsRNA. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. *STING* transcript levels were not significantly impacted by the presence of repeat dsRNA alone (p=0.5771) or through co-expression of *CrPV1A* and the dsRNA (p=0.5738) when compared to the UAS empty transgene control. *STING* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.9398). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49*. Each dataset represents 3 biological replicates plated in triplicate. The experiment was performed using the CAG.CUG$_{\sim100}$^{S1} dsRNA and CrPV1A FL #1 lines.

both microbial (323) and viral pathogens (322, 393). In *Drosophila*, STING appears to function as an upstream regulator of the NF-κB transcription factor Relish and thus the IMD arm of the innate immune response (323, 393). However, in response to Zika virus infection these roles are reversed; Relish induces the expression of STING, which subsequently promotes autophagy-mediated restriction of the virus (322). Indeed, knowledge regarding the functionality of the invertebrate STING is still in its infancy, whether it directly senses viral nucleic acids or requires an upstream PRR is a topic that warrants further study. Nevertheless, STING is vital for controlling the RNA-based viruses DCV and CrPV (393), and therefore was examined via qRT-PCR to determine whether it responds to repeat dsRNA.

STING expression was not induced following expression of dsRNA or co-expression with *CrPV1A* when compared to the UAS control **(Figure 3.9)**. Additionally, co-expression of *CrPV1A* and dsRNA also did not alter *STING* levels compared to dsRNA alone, suggesting that CrPV1A does not antagonize STING upon infection **(Figure 3.9)**. Taken together, these findings suggest that STING does not participate in the response to repeat dsRNA.

Notably, Ago2 is dispensable for the STING/dIKKβ/Relish-mediated induction of antiviral genes (393), suggesting that this pathway is independent of RNAi and thus not a known

CrPV1A target. Indeed, this does not rule out the possibility that STING participates in the dsRNA pathology, though how STING interacts with viral RNA is not known. One possible scenario could be that Dicer-2 and STING co-operate in antiviral detection and signalling. Dicer-2 acts as an RNA sensor through its helicase domain, which is conserved with mammalian RNA receptors RIG-I and MDA5 (53). Therefore, it would be of interest to determine if Dicer-2 participates in this response as a means to sense viral infection and activate STING.

The NF-κB transcription factor Relish regulates antiviral activity as part of the IMD pathway (372, 400) and as part of the STING/dIKKβ/Relish-mediated response (393). Additionally, Relish is a regulator of antimicrobial gene expression through the IMD pathway in response to bacterial pathogens (401, 402). Relish can homodimerize with itself or form heterodimers with other NF-κB factors DIF and Dorsal to fine tune the battery of genes that are induced following pathogenic challenge (403). Intriguingly, only recently has Relish also been demonstrated to control autophagy in both development (404) and following Zika virus infection in co-operation with STING (322). Conversely, Relish activity is required for neurodegeneration (311, 376) and has been implicated in age-dependent neurological decline (313). Given that its activation can be either of a beneficial or harmful nature in a context-dependent manner, *Relish* transcript levels were measured in response to expression of the repeat dsRNA.

Similar to *STING*, expression of *Relish* was not significantly altered in either *elavII*>dsRNA or *elavII*>dsRNA/CrPV1A flies when compared to the UAS control **(Figure 3.10)**. Additionally, co-expression of *CrPV1A* with the dsRNA did not affect *Relish* transcription when compared to expression of the dsRNA alone **(Figure 3.10)**. Taken together, these results indicate that *Relish* expression is not upregulated in response to the presence of repeat dsRNA. However, it must be taken into consideration that Relish is regulated post-translationally; only upon proteolytic cleavage and removal of its inhibitory IκB domain does Relish translocate to the nucleus to drive transcriptional activation of NF-κB responsive genes (405, 406). Furthermore, the transcriptional program initiated through Relish is dependent upon its dimer configuration; either Toll or IMD downstream genes can be induced, or both (403).

Figure 3.10: NF-κB transcription factor *Relish* is not significantly altered in response to repeat dsRNA. In each case expression is driven in all neuronal cells using the *elavII-GAL4* driver. *Relish* transcript levels were not significantly impacted by the presence of repeat dsRNA alone (p=0.8293) or through co-expression of *CrPV1A* and the dsRNA (p=0.4873) when compared to the UAS empty transgene control. *Relish* levels were not significantly different between dsRNA alone and together with *CrPV1A* (p=0.5384). Depicted for each dataset are the mean and standard error of the mean (SEM). 2-tailed Student's t-tests were used to determine significance; ns p>0.05. Expression levels are normalized to the housekeeping gene *Rp49*. Each dataset represents 3 biological replicates plated in triplicate. The experiment was performed using the CAG.CUG$_{\sim100}$^{S1} dsRNA and CrPV1A FL #1 lines.

Indeed, the dimer configuration partner of Relish for antiviral gene induction is unknown; though *Charon*, a negative regulator of Relish-Relish homodimerization (397), is one of the induced antiviral genes following DCV infection (393). In this manner it may promote heterodimerization of Relish with an unknown antiviral transcription factor to bolster the antiviral response. Thus, a closer investigation of how Relish protein function (or lack of through use of mutant lines) is regulated in the presence of repeat dsRNA may prove more informative to this end.

3.3 Chapter Discussion

The ability for cells to distinguish between 'self' molecules and foreign or abnormal 'non-self' molecules that likely constitute danger is critical not only for host defence but to protect essential cellular components from self-inflicted inflammatory damage (407). Indeed, a number of endogenously-produced molecules require modification from cellular machinery in order to gain 'self' status and thus avoid PRR detection (68). Double-stranded *Alu* RNA repeats require modification from ADAR1 in the form adenosine to inosine (A-I) editing in order to prevent their detection as 'non-self' by the RNA sensor MDA5 (368). Loss of *ADAR1* leads to aberrant inflammatory signalling and lethality in mice, which can be

rescued through the genetic deletion of either the RNA sensor MDA5 (152) or downstream antiviral signal adaptor MAVS (156). *ADAR1* loss-of-function or *IFIH1* (which encodes MDA5) gain-of-function mutations are both associated with the autoinflammatory neurological disorder Aicardi-Goutieres syndrome (AGS) (154, 155, 408), highlighting the importance of 'non-self' to 'self' editing as an immunomodulatory mechanism in human disease.

Indeed, repeat RNA and its subsequent bi-directional transcription are both features common to expanded repeat disorders (21), and as such it has been hypothesised that dsRNA formed in this manner may represent a molecular trigger for inflammation (4). In a *Drosophila* model of expanded repeat disease, pathology caused via the expression of expanded repeat CAG.CUG dsRNA is dependent upon Dicer-2 and Ago-2 **(Figure 3.2)** (45, 341). However, the pathology is suppressed by R2D2, an essential co-factor of Dicer-2 in the RNAi pathway, which suggests that Dicer-2 and Ago2 drive the dsRNA-mediated toxicity via the alternate antiviral RNA pathway, given that the RNAi pathway appears protective (343) **(Appendix A)**. Indeed, Dicer-2 shares homology with the mammalian RNA sensors RIG-I and MDA5 (55) and appears to act in this manner upon viral infection in *Drosophila* (53). Additionally, the pathology is completely suppressed through co-expression of the human *ADAR1* gene (343) **(Appendix A)**, suggesting that conferring 'self' status to the dsRNA masks its presence from antiviral machinery. Taken together, these results support the theory that repeat dsRNA molecules derived from expanded repeat loci are detected as 'non-self' in a manner similar to viral material, leading to cellular damage via inflammatory signalling.

To further challenge this hypothesis, a viral suppressor encoded by the cricket paralysis virus (CrPV) was utilized. The protein, known as CrPV1A, antagonises Ago2 activity without inhibiting Dicer2/R2D2-mediated processing of dsRNA (350), allowing the virus to escape RNAi silencing and successfully replicate. Strikingly, co-expression of the full-length *CrPV1A* viral suppressor with repeat dsRNA leads to a complete suppression of eye pathology. Conversely, a truncated form of CrPV1A unable to inhibit the antiviral response fails to suppress the dsRNA pathology. This finding suggests that repeat dsRNA is recognised as a 'non-self' entity by the host antiviral system, and that CrPV1A ablates the pathway(s) responsible for the subsequent damaging inflammatory response.

Following this, qRT-PCR was performed on flies expressing dsRNA alone and in conjunction with *CrPV1A* in an attempt to uncover pathways that 1) are upregulated in response to repeat dsRNA, and 2) are attenuated by CrPV1A, thus highlighting them as candidates to genetically dissect using the established dsRNA eye model. The neuronal driver *elavII>GAL4* was chosen to drive expression of repeat dsRNA and *CrPV1A*, while the timepoint tested was 0-1 days of age, both to remain consistent with previous transcript analysis on the dsRNA model (52).

STING and *Relish*, regulators of a recently described antiviral pathway (393), were not significantly altered following expression of the dsRNA, thus making any attenuation of this pathway by CrPV1A difficult to discern. Indeed, this novel antiviral pathway is critical for the control of both DCV and CrPV infection and was therefore proposed to act as a secondary inducible defence mechanism should the respective viral suppressors antagonize the RNAi response (393). It is important to note that absence of *STING* or *Relish* upregulation does not rule out their respective participation in the dsRNA-mediated pathology. Relish in particular is subject to critical post-translational regulation, it requires separation from an inhibitory domain in order to operate as a transcription factor (405, 406). In addition, Relish can homodimerize to induce target genes of the IMD pathway, or form heterodimers with another NF-κB transcription factor, DIF, to activate signalling from both the Toll and IMD pathways (403). As such, transcript levels may not accurately portray Relish signalling in response to dsRNA.

Knowledge of the *Drosophila* STING orthologue is still limited and the mechanisms controlling its activation and regulation need to be further investigated. In vertebrates the cytosolic cDNA sensor cyclic-GAMP synthase (cGAS) produces cGAMP which binds and activates STING (409, 410), leading to a potent type-I IFN response against viral pathogens (398, 399). *Drosophila* lack a functional cGAS ortholog able to bind DNA and elicit an interferon transcriptional response (323), suggesting that it may serve to activate a different subset of inflammatory genes in insects. Indeed, *Drosophila* STING co-operates with Relish to induce antimicrobial peptides from the IMD pathway (323) and to upregulate antiviral autophagy following Zika virus infection (322). Taken together, both Relish and STING regulate inflammation as effectors of multiple distinct but often linked pathways. Therefore,

quantitating the downstream target transcripts of these pathways may prove more informative when assessing their responsiveness (if any) to repeat dsRNA.

Thus, in addition to STING and Relish, the transcript levels of several genes that were induced by the STING/dIKKβ/Relish signalling complex upon viral infection (393) were assessed quantitatively as readouts of pathway activity. Namely, the newly-characterised antiviral peptide *Nazo*, the IκB gene *Charon* and the uncharacterised *CG33926* were all chosen for their potent upregulation following DCV infection and their reliance upon the signalling complex for induction (393). Both *Nazo* and *Charon* transcript levels were not significantly altered in the presence of repeat dsRNA or through co-expression of *CrPV1A* with the dsRNA, though *Charon* was modestly reduced in both experimental lines. Conversely, *CG33926* was potently downregulated in response to dsRNA alone and dsRNA/CrPV1A co-expression. The differential expression of *Nazo*, *Charon* and *CG33926* when comparing observations in *Goto et al.,* to the results obtained in this chapter serve to highlight the complexity of the *Drosophila* antiviral response to 'non-self' stimuli. Indeed, while the RNAi response provides comprehensive host protection against a range of viruses (391), inducible antiviral pathways appear to respond in a virus-specific manner (53, 391, 393). The inducible pathways have been hypothesised to act as secondary antiviral defence mechanisms; upregulated should viruses antagonise the RNAi pathway or cell death machinery through the activity of VSRs (393, 411, 412). Thus, the observation that CrPV1A does not alter expression of genes within the STING/dIKKβ/Relish antiviral pathway suggests that this pathway may be induced as a secondary response to compensate for DCV/CrPV-mediated inhibition of the RNAi pathway.

The modest reduction of *Charon* and significant downregulation of *CG33926* raise the possibility of negative regulation within this pathway. Indeed Kenny, the *Drosophila* orthologue of IKK complex regulator NEMO (also known as IKKγ), was demonstrated as a negative regulator of dIKKβ antiviral signalling (393). Thus, it is plausible that Kenny is upregulated in response to the repeat dsRNA, thus inhibiting the STING/dIKKβ/Relish induction of antiviral target genes. Kenny prevents constitutive activation of the IMD pathway by regulating autophagic degradation of the IKK complex (395) and may perform a similar function upon detection of the repeat dsRNA. The fact that *Nazo* is not also

downregulated likely reflects the specificity in antiviral transcriptional programs, that DCV and dsRNA induce different target genes.

Another facet to investigate is the function(s) of *CG33926*, of which current knowledge is scarce aside from its upregulation following viral challenge (393, 394). One can speculate that it may encode an antiviral peptide similar to *Nazo*, or perhaps form part of a degradative complex designed to remove viral material. Indeed, a number of other uncharacterised genes are induced by the STING/dIKKβ/Relish complex following DCV infection (393). Thus, given the potent downregulation of *CG33926* in response to the dsRNA, it would be of interest to determine if these genes are also differentially regulated when comparing DCV and repeat dsRNA challenge.

Another antiviral peptide induced through DCV infection is *Vago*, though it differs to *Nazo* in that its activation is Dicer-2-dependent rather than reliant upon the STING/dIKKβ/Relish complex (53). However, similar to *Nazo*, *Vago* expression was not significantly altered in response to repeat dsRNA, nor when *CrPV1A* was co-expressed. Thus, this Dicer-2 dependent axis of antiviral activity appears unresponsive to repeat dsRNA, suggesting that Dicer-2 has additional antiviral functionality independent of both this pathway and its role in RNA interference. Notably, the VSR encoded by DCV (DCV-1A) inhibits the RNAi pathway through its binding to dsRNA, preventing Dicer-2 from accessing the dsRNA to direct its processing (350, 362). As such, the observation that *Vago* is induced in response to DCV (53, 393) suggests that Dicer-2 can still contribute to inducible antiviral defence through its helicase domain, which is remarkably conserved with the corresponding domain in mammalian RNA sensors RIG-I and MDA5 (55). Thus, while Vago-mediated antiviral activity does not appear to participate to the dsRNA-induced response, it is highly likely that *Vago* is not the only downstream target of Dicer-2. Uncovering the components and targets of Dicer-2 mediated antiviral signalling may provide a number of novel candidate genes to investigate in the context of repeat dsRNA pathogenesis.

In agreement with previous results (52), the Toll pathway-induced antimicrobial peptide *Drosomycin* was significantly elevated in response to dsRNA, but this induction was not blocked through the expression of *CrPV1A*. Given that the *Drosophila* Toll pathway functions

primarily in antibacterial and antifungal defence (413) it is perhaps not surprising that CrPV1A does not inhibit Toll signalling. However, a number of *Drosophila* Toll receptors contribute to dsRNA pathology (52) and Toll pathway mutants are rendered more susceptible to *Drosophila* X virus (DXV) (378) and oral DCV infection (379). Furthermore, Dicer-2 can modulate Toll post-transcriptionally to induce *Drosomycin* in response to viral challenge (54), highlighting the potential for crosstalk between inflammatory pathways. Indeed, Toll proteins in *Drosophila* do not act as PRRs *per se* but instead rely on upstream receptors for pathogen detection (413). In this regard, Dicer-2 may again function as an RNA sensor to activate antiviral Toll signalling independently of RNAi. Whether Dicer-2 mediated activation of the Toll pathway induces canonical target genes (other than *Drosomycin*) or represents a distinct but overlapping transcriptional program tailored to antiviral function remains poorly understood but could be investigated by performing qRT-PCR analysis on other canonical Toll targets.

Lastly, the TNF orthologue *Eiger* was not significantly altered following expression of the dsRNA. Indeed, *Eiger* has previously been shown to be upregulated in this dsRNA model (52), and so this discrepancy may represent biological variation between the samples. Complicating matters further, Eiger is a positive regulator of cell death (388), and so any cells containing elevated *Eiger* may be undergoing cell death and thus weakening the total transcript levels detectable. Blocking Eiger-mediated cell death downstream of Eiger itself may prove useful in preventing this in future analysis.

Therefore, from the results obtained in this study, the repeat dsRNA does not appear to induce the newly characterised STING/dIKKβ/Relish signalling pathway, nor Dicer-2 mediated upregulation of *Vago*. In addition, the co-expression of *CrPV1A* with the dsRNA did not significantly alter the expression of any of the genes tested, suggesting that the VSR encoded by CrPV does not target either of these pathways. However, because none of the target genes tested (excluding *Drosomycin*) were induced in response to the dsRNA, any potential effect of CrPV1A may be masked. Thus, it cannot be completely ruled out that CrPV1A antagonises one or both of these pathways. Indeed, *CG33926* was in fact potently downregulated (and *Charon* to a lesser extent, though this was not significant) in response to the dsRNA, suggesting instead that regulatory components of the STING/dIKKβ/Relish

may be activated. Kenny (also known as dIKKγ) negatively regulates the IκB kinase signalling complex through autophagic degradation (395) and thus presents as another candidate to test via qRT-PCR. Of note, the temporal window used for transcript analysis in this study was restricted to newly-eclosed flies. Given that the neuronal dysfunction observed in *elavII*>dsRNA flies is age-dependent (45), future studies could incorporate a longer time course for transcript analysis. Additionally, determining the degree that CrPV1A functionally rescues dsRNA-mediated neuronal dysfunction over time should also be considered.

As such, the precise pathway that CrPV1A blocks to potently suppress the repeat dsRNA pathology is still unknown. CrPV1A employs several known methods to ensure a favourable environment for viral replication; inhibiting the key RNAi protein Ago2 (350, 365), initiating host transcriptional shutoff and disrupting RNA stress granule formation (366, 414). However, the RNAi pathway acts to restrict the dsRNA pathology (343) **(Appendix A)**, suggesting that CrPV1A-mediated inhibition of this pathway would likely further enhance the pathology as opposed to the observed suppression. The potential role of stress granules in the dsRNA pathology is intriguing; they are vitally important for preventing the translation of abnormal proteins following stress (415), while antiviral stress granules (termed avSGs) act to isolate viral RNA together with 'non-self' RNA sensors in order to drive RLR-MAVS mediated inflammatory signalling (416). This raises the possibility that avSGs could function similarly in *Drosophila* to isolate Dicer-2 and the repeat dsRNA, leading to heightened antiviral signalling that may cause tissue damage and the subsequent pathology observed. Thus, determining whether Dicer-2 colocalizes with stress granule machinery may provide insight into whether this RNA sensor-avSG interaction is conserved in *Drosophila*.

An alternate (but not mutually exclusive) possibility is that CrPV1A ablates an antiviral cell death response. Focal, non-lytic programmed cell death in virus-infected cells is important for restricting the replication and spread of viral material (367). As such, it is not surprising that a number of mammalian and insect viruses deploy inhibitors of apoptosis (IAPs) to avoid the activation of cell death machinery (411). Conversely, viral proteins can also act to induce lytic cell death as a means to lyse infected cells and promote dissemination of viral replicates to neighbouring uninfected cells (417). Thus, antiviral cell death must be tightly regulated to avoid the destruction of essential tissues and facilitate the spreading of viral

material (367). Indeed, this form of focal cell death is effective for stopping the spread of extrinsic threats such as viruses, but would likely cause tissue damage when responding to the expanded repeat dsRNA, which represents inherited 'non-self' material and is thus expressed in every cell.

What form of cell death could participate in the dsRNA pathology? In vertebrates, antiviral cell death can occur in several forms. Apoptosis is regarded as "clean" non-lytic cell death for its ability to contain the contents of the dying cell, preventing the escape of danger signals and subsequent inflammation (418). Necroptosis, a regulated form of necrosis, favours permeabilization of cellular membranes and thus facilitates the release of factors that promote further inflammation (419). Necroptosis also participates in the antiviral response (420, 421) and is targeted by viral cell death suppressors, highlighting its importance in this role (422, 423). The inflammatory caspase-mediated pyroptosis also favours the release of inflammatory factors from dying cells and is thus also lytic in nature, but achieves this through the formation of a membrane pore facilitated via Gasdermin D (424). Information regarding the antiviral actions of pyroptosis are limited, though it has been demonstrated to be induced following infection from several human viruses (425). No known viral suppressors have been demonstrated to antagonise pyroptosis directly (426). Given the inflammatory activation observed in response to repeat dsRNA (45), it would suggest that a lytic, inflammatory form of cell death is most likely to mediate the dsRNA pathology. The appearance of necrotic black spots on the *Drosophila* eye following repeat dsRNA expression further support the idea that a form of necrosis is involved. However, core necroptotic and pyroptotic components lack characterised orthologues in *Drosophila*, though a spreading form of necrotic cell death has recently been described (427). Therefore, characterisation of the potentially lytic cell death occurring in the *Drosophila* expanded repeat disease model used in this study should form part of future work.

The most compelling result described in this chapter provides evidence that expanded CAG.CUG repeat dsRNA is detected as 'non-self' similar to a viral pathogen. The expression of the viral suppressor protein CrPV1A completely rescues the dsRNA eye pathology, suggesting that the antiviral response directed against the dsRNA is a key driver of the pathology. Expansion of the repeat beyond a pathogenic threshold likely renders the RNA as

'non-self', which is supported by the observation that 50 copies of the CAG.CUG dsRNA repeat is not sufficient to cause pathology (343) **(Appendix A)**, whereas expression of 100 copies leads to the established phenotype (45). Alas, the precise mechanism ablated by CrPV1A that leads to the rescued pathology remains unknown, but would likely comprise the key next step in our understanding of the mechanisms that underlie dsRNA-mediated dysfunction.

CHAPTER 4: Innate inflammatory pathways in the mediation of dsRNA pathology

The current understanding regarding the role of innate inflammation in neurodegenerative disease is one of duality. On one hand, it represents the critical first line of defence against invading pathogens, injury and other danger signals (428). It can promote the degradation of disease gene products through the upregulation of appropriate quality control pathways (429, 430) and drive tissue repair following neuronal injury (431, 432). On the other hand, the dysregulation of immune pathways through genetic lesions, ageing and epigenetic changes can lead to a chronic neuroinflammatory response within the CNS that is highly detrimental to neuronal function and survival (2). In line with this, mutations in a number of inflammatory components are causal or risk factors for neurodegenerative disease; each lesion reflecting either the loss of homeostatic functionality and/or the gain of deleterious pro-inflammatory signalling (68).

In Huntington's Disease (HD), heightened microglial and cytokine activation precedes the onset of clinical symptoms in HD gene carriers (58, 59), while microglial activation correlates with the onset and progression of neurological decline in HD (59, 61, 433). A similar correlation has also been observed in a knock-in mouse model of spinocerebellar ataxia type 1 (SCA1) (434), suggesting that inflammation may underlie the pathogenesis of CAG repeat expansion disorders more generally. Indeed, the established *Drosophila* model of CAG.CUG repeat expansion used in this study and described previously (45, 52) has provided several lines of evidence that support the participation of inflammatory pathways in CAG repeat disorders. Firstly, work has demonstrated that several inflammatory components are required for the observed pathological features, namely the antiviral protein Dicer-2 (45) and several members of the Toll family of pattern recognition receptors (PRRs) (52). Secondly, neuronal expression of the repeat dsRNA induces an inflammatory response as observed by the significant upregulation of the Toll pathway-induced *Drosomycin* and potent inflammatory cell death mediator *Eiger*, the *Drosophila* orthologue of *tumour necrosis factor* (*TNF*) (52). Finally, autophagy restricts the dsRNA-mediated pathology, suggesting that degradation of the dsRNA is neuroprotective (52). Thus, it appears that
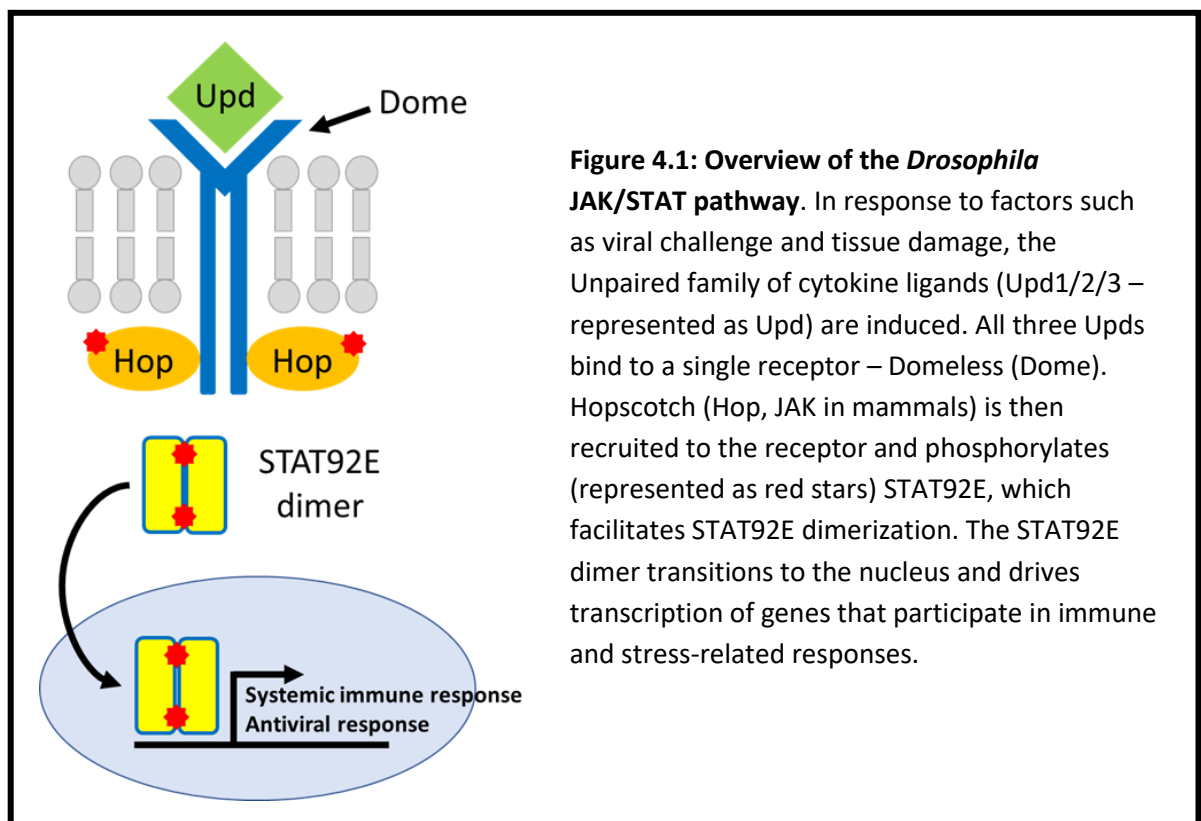
multiple inflammatory pathways underlie the repeat dsRNA pathogenesis, both in restricting and driving the observed pathology.

The *Drosophila* eye has proven a popular tissue model to perform functional genetic screens (435); the precise structural arrangement of the ommatidia means that disruptions to eye development often manifest as easily detectable phenotypes (436). Additionally, the eye is a non-essential tissue, therefore strong levels of toxicity can be tolerated without causing lethality to the fly. The work in this chapter uses the established repeat dsRNA *Drosophila* eye model (45, 52) in order to investigate whether other components/mechanisms involved in the broader *Drosophila* immune response contribute to the pathology.

One final note surrounds the methods used to reduce expression of candidate genes in this chapter. Given the role of Dicer-2 and other components of the RNAi pathway in mediating dsRNA-pathology (45, 343) **(Appendix A)** , the use of RNAi to knockdown gene expression was avoided when possible in order to prevent the potential of the RNAi machinery interfering with any changes to the dsRNA pathology. However, endogenous mutant lines were not available for all candidate genes tested, and so RNAi knockdown was the only obtainable method for reducing expression. Additionally, in several cases (mainly where endogenous mutants were unavailable) mutant lines containing transposon insertions into the target gene were utilised. While these insertions often disrupt expression of the endogenous target gene, many of the insertions contain flanking UAS sites designed to drive misexpression of adjacent genes when combined with a GAL4 driver, potentially including the target gene itself (437). Thus, where insertional mutants have been used, consideration has been made that the target gene may be subject to disruption and/or misexpression when combined with the *GMR-GAL4* driver. Regardless, these lines are informative as part of a preliminary investigation into the contribution of the candidate gene in the dsRNA-mediated eye pathology; an alteration to the dsRNA pathology indicates that the gene contributes and thus warrants future investigation of a more in-depth nature (if the appropriate tools are available).

4.1 The JAK/STAT pathway contributes modestly to dsRNA pathology

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is an important regulator of innate immunity, haematopoiesis and embryonic development, roles that are conserved from vertebrates through to *Drosophila* (438-440). The mammalian JAK/STAT pathway is highly complex - there are four JAKs (JAK1/2/3/TYK4) and seven STATs (STAT1/2/3/4/5a/5b/6), while more than 30 cytokine/receptor combinations can activate the pathway (including members of the interleukin and interferon families) (439). In *Drosophila* there are only single JAK (Hopscotch) and STAT (STAT92E) orthologues, however the signalling cascade remains highly conserved **(Figure 4.1)**, thus allowing a simpler *in vivo* dissection of pathway function and interactions (441, 442).

Figure 4.1: Overview of the *Drosophila* JAK/STAT pathway. In response to factors such as viral challenge and tissue damage, the Unpaired family of cytokine ligands (Upd1/2/3 – represented as Upd) are induced. All three Upds bind to a single receptor – Domeless (Dome). Hopscotch (Hop, JAK in mammals) is then recruited to the receptor and phosphorylates (represented as red stars) STAT92E, which facilitates STAT92E dimerization. The STAT92E dimer transitions to the nucleus and drives transcription of genes that participate in immune and stress-related responses.

There are three related ligands that initiate the *Drosophila* JAK/STAT pathway, the *Unpaired* family of cytokines (*Upd1/2/3*). The diverse range of vertebrate JAK/STAT transcriptional responses is due in part to the large number of different ligand/receptor combinations that can initiate the pathway (439, 443). While this diversity is vastly reduced in the *Drosophila* (3

ligands compared to >30 in vertebrates) there still exists some specificity. All three ligands are induced through tissue damage (444, 445), whilst *Upd2* and *Upd3* are also upregulated following bacterial or viral challenge (391, 446). Once induced, the *Unpaired* ligands engage the *Domeless* (*Dome*) receptor, which bears similarity with the IL-3 and IL-6 receptor families (447). This ligand-receptor interaction recruits *Hopscotch*, which is subsequently phosphorylated along with the cytoplasmic section of the receptor (440). This allows the recruitment and phosphorylation of *STAT92E*, facilitating its dimerization and translocation into the nucleus where it binds to promoters to activate transcription of target genes (448). Target genes include *turandotA* (*totA*) and *turandotM* (*totM*), humoral factors secreted from the *Drosophila* fat body following systemic stressors such as septic injury (446, 449, 450). Of particular interest, JAK/STAT signalling also forms part of the antiviral response directed against both Drosophila C virus (DCV) and cricket paralysis virus (CrPV) (391, 451). Thus, several components of the *Drosophila* JAK/STAT pathway, namely *Upd1*, *Upd3* and *STAT92E*, were investigated to assess if the pathway participates in the dsRNA-mediated pathology.

In order to test for the participation of *Upd1* in dsRNA pathology, *GMR-GAL4* was utilized to co-express both the dsRNA in conjunction with two independent forms of *upd1* reduction **(Figure 4.2)**. Compared to control flies **(Figure 4.2 A)**, the reduction of *upd1* alone through either the introduction of a heterozygous endogenous point mutation **(Figure 4.2 B)** or RNAi knockdown **(Figure 4.2 C)** does not lead to disruption within the eye. As previously demonstrated, eye-specific expression of repeat dsRNA (*GMR*>dsRNA) leads to a pathogenic phenotype, most notably a loss of pigmentation and ommatidial patterning compared to control flies **(Figure 4.2 A & A')**. Reduction of *upd1* in a *GMR*>dsRNA background **(Figure 4.2 B' & C')** did not appear to modify the phenotype, suggesting that *Upd1* is not rate-limiting for the dsRNA pathology.

Like both *Hopscotch* and *STAT92E*, *Upd1* was first characterised for its role in embryonic patterning, where its embryonic induction and secretion activates the JAK/STAT pathway and ultimately results in the expression of the segmentation genes *even-skipped* (*eve*), *fushi tarazu* (*ftz*) and *runt* (452). However, *Upd1* is not required for the induction of stress-related or antiviral genes, which is in contrast to *Upd2* and *Upd3* (391, 446). Thus, any potential inflammatory antiviral activity mediated by the JAK/STAT pathway to remove the 'non-self'
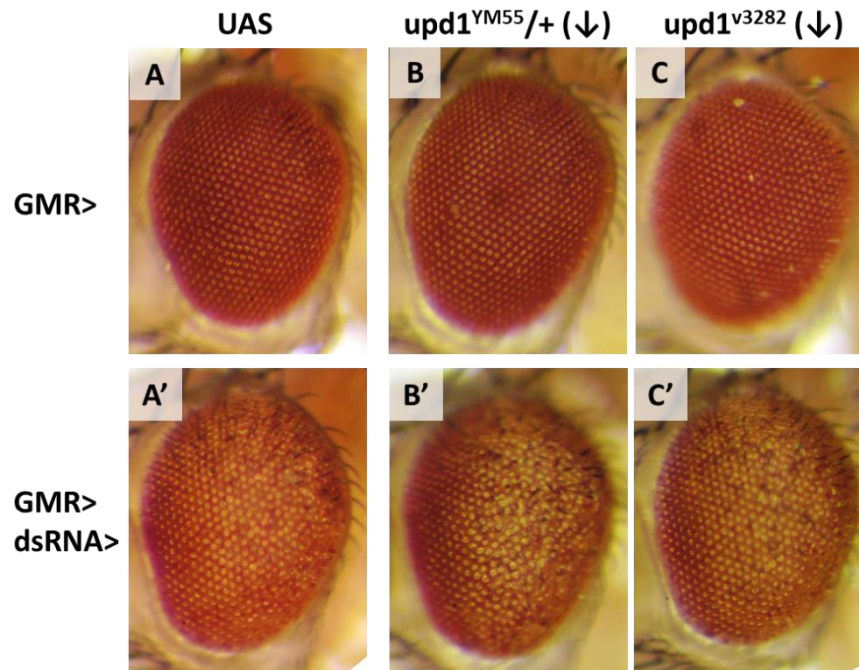
72

Figure 4.2: Reduction of upd1 makes no obvious contribution to repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** A heterozygous *upd1* loss-of-function allele alone. **C)** Expression of a *upd1* RNAi construct alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a heterozygous *upd1* loss-of-function allele. **C')** Co-expression of repeat dsRNA together with a *upd1* RNAi construct. The experiment was performed at 25°C using the CAG.CUG$_{\sim 100}$^{M3} dsRNA line.

dsRNA may require ligand/receptor interactions through *Upd2* and/or *Upd3*. To determine if antiviral activity through the JAK/STAT pathway could play a role in the dsRNA pathology, *Upd3* was manipulated in the *GMR*>dsRNA background **(Figure 4.3)**.

Compared to the control **(Figure 4.3 A)**, alteration of *upd3* through a transgenic P-element insertional mutation alone led to ommatidial disruption, smaller eye size and the presence of black necrotic spots **(Figure 4.3 B)**. These phenotypic characteristics were fully penetrant for this genotype (data not shown). Knockdown of *upd3* via the expression of an RNAi construct did not cause any noticeable eye phenotype alone **(Figure 4.3 C)**. Of note, the transgenic insertion used (*upd3^{d11639}*) contains flanking UAS sites that can cause misexpression of adjacent genes, possibly including *upd3* itself (453). Thus, the observed phenotype **(Figure 4.3 B)** may be caused through the misexpression of endogenous genes
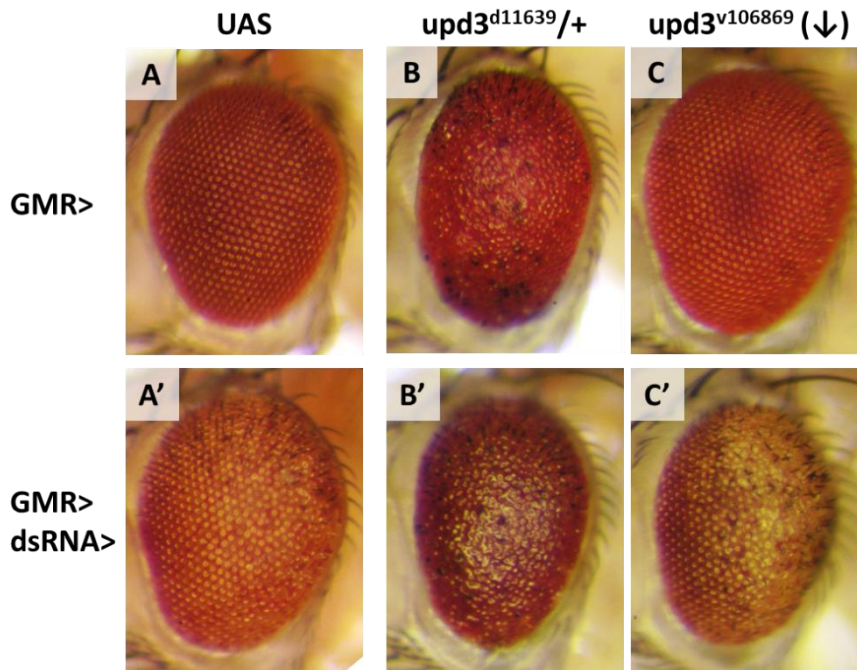
Figure 4.3: Upd3 contributes modestly to repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** A heterozygous *upd3* transgenic P-element insertion alone. **C)** Expression of a *upd3* RNAi knockdown construct alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a heterozygous *upd3* transgenic P-element insertion. **C')** Co-expression of repeat dsRNA together with a *upd3* RNAi knockdown construct. The experiment was performed at 25°C using the CAG.CUG$_{\sim 100}$^{M3} dsRNA line.

flanking the insertion. The use of an endogenous *upd3* mutation lacking UAS sites could be used to test this hypothesis. Indeed, other *upd3* mutations also display reductions in eye size (454), suggesting that *Upd3* plays an important role in eye development, most likely through JAK/STAT signalling.

The presence of the insertional *upd3* mutation in conjunction with *GMR*>dsRNA caused a severe eye phenotype **(Figure 4.3 B')** compared to the control dsRNA expressed alone, though was reminiscent of the *upd3* mutation alone **(Figure 4.3 B)** in phenotype. RNAi knockdown of *upd3* in a *GMR*>dsRNA background **(Figure 4.3 C')** led to a reduction in eye size compared to *GMR*>dsRNA alone **(Figure 4.3 A')** that was consistent among eyes scored. Because either the *upd3* mutation or dsRNA alone both lead to eye phenotypes **(Figure 4.3 B**

& A') it is likely that the phenotype observed when combined **(Figure 4.3 B')** is additive rather than synergistic in nature. Nevertheless, the smaller eye observed when *upd3* is knocked down with *GMR*>dsRNA **(Figure 4.3 C')** suggests that *Upd3* contributes in restricting dsRNA eye pathology.

The observation that *Upd3*, but not *Upd1*, contributes to dsRNA pathogenesis could represent a role for *Upd3* in JAK/STAT-mediated antiviral activity as demonstrated by others (391). In contrast, *Upd1* may remain more strictly dedicated to developmental processes (452). *Upd2* was not tested due to a lack of suitable lines available, but is also upregulated in response to DCV and CrPV (391). As such it would be of interest to investigate the effect of *Upd2* reduction upon the dsRNA pathology and whether a genetic interaction as seen in **Figure 4.3 C'** is observed. The upregulation of *Upd2* and *Upd3* in response to the sensing of CrPV is particularly relevant given the role of the CrPV viral suppressor in ameliorating the dsRNA pathology **(see Chapter 3)**. As such, monitoring the expression levels of the *Unpaired* genes in the presence of dsRNA may aid in determining their level of participation in any potential JAK/STAT response to repeat dsRNA.

While the *Unpaired* ligands display partial redundancy within the JAK/STAT pathway (442, 455), STAT92E is the sole downstream transcription factor of the pathway, and thus participates in all aspects of JAK/STAT function. Interestingly, STAT92E can also be non-canonically activated through a Draper signalling cascade following axonal injury, which leads to a feed-forward loop of *draper* transcription to increase phagocytic capacity and confer neuroprotection (295). Additionally, west nile virus (WNV) infection in a mosquito cell line (*Culex quinquefasciatus*) leads to STAT92E activation through Dicer-2 mediated induction of the antiviral peptide Vago (392). The canonical JAK/STAT receptor Domeless was not required for this activation, suggesting that another unknown receptor participates in this aspect of STAT92E signalling (392). Notably, Dicer-2 and Vago are both required for an analogous antiviral response in *Drosophila* (53), though whether the role of STAT92E is conserved was not explored. Thus, STAT92E is a vital transcriptional factor in the context of innate immunity following exposure to a range of stimuli, including bacterial and viral pathogens.
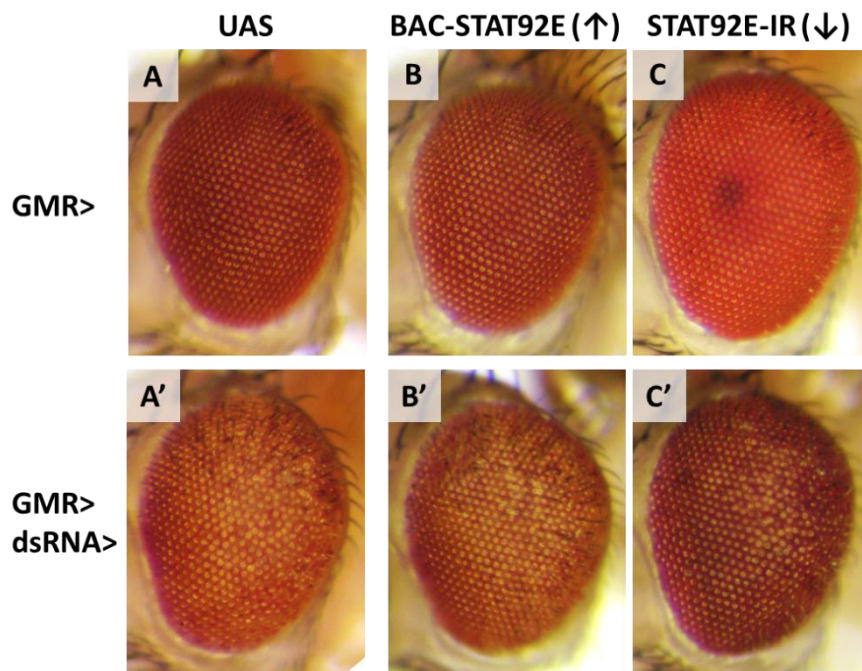
Figure 4.4: Alteration of STAT92E has limited effect on repeat dsRNA-mediated eye pathology.
In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** A transgenic BAC insertion containing the *STAT92E* gene. **C)** Expression of an inverted repeat construct targeting *STAT92E* alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a transgenic BAC insertion containing the *STAT92E* gene. **C')** Co-expression of repeat dsRNA together with an inverted repeat construct targeting *STAT92E*. The experiment was performed at 25°C using the CAG.CUG$_{\sim 100}^{M3}$ dsRNA line.

In order to investigate if STAT92E contributes to the repeat dsRNA pathology, separate lines were used in order to increase (through a bacterial artificial chromosome [BAC] insertion containing a DNA fragment including the *STAT92E* gene under the endogenous STAT promotor) or decrease (through RNAi knockdown) *STAT92E* levels in a *GMR*>dsRNA background. Neither ectopic expression nor reduction of *STAT92E* caused an eye phenotype alone **(Figure 4.4 B & C)**. In conjunction with expression of the repeat dsRNA, ectopic expression of *STAT92E* did not lead to any noticeable changes to the eye compared to the control **(Figure 4.4 A' & B')**, while knockdown led to a modest return of red pigmentation within the eye **(Figure 4.4 C')**, though this could be due to the high level of red pigment observed when the construct is expressed alone **(Figure 4.4 C).** Indeed, minor structural

disruptions observed through expression of the dsRNA alone were still present **(Figure 4.4 C')**.

These results suggest that STAT92E makes a minor (if any) contribution to the repeat dsRNA pathology. Given that 'non-self' viral recognition through Dicer-2 appears vital for the dsRNA-mediated pathogenesis **(see Chapter 3)** (45), it is plausible that this interaction is through an antiviral cell death response, though other roles performed by the JAK/STAT pathway cannot be discounted. If Dicer-2-mediated upregulation of *STAT92E* in response to viral challenge is indeed a conserved mechanism in invertebrates (53, 392), then the minor suppression of the dsRNA pathology following *STAT92E* reduction **(Figure 4.4 C')** may represent the inhibition of a single immune pathway controlled by Dicer-2 upon sensing the dsRNA. Quantitating *STAT92E* expression in flies expressing the dsRNA in Dicer-2 wildtype and mutant backgrounds would aid in determining if this represents a valid hypothesis.

The JAK/STAT pathway is rate-limiting for the induction of some viral genes and the overall control of viral load following infection with DCV and CrPV (391). However, constitutive activation of the pathway via *Hopscotch* does not result in a similar upregulation and is therefore not sufficient to drive the response alone (451). This indicates that antiviral genes may integrate signals from a number of separate pathways following viral infection, and thus blocking a single pathway may only have minimal effect on overall antiviral function.

Taken together, the reduction of either *Upd3* or *STAT92E* appear to have opposing (albeit minor in the case of *STAT92E*) effects on the dsRNA pathology. This dichotomy may reflect the observation that *STAT92E* activity can be initiated from effector molecules outside of the canonical JAK/STAT pathway (295, 392, 456). This would in part mirror observations that Dicer-2 is required for dsRNA pathology but functions in this manner independently of its canonical role in the RNAi pathway, which suppresses the pathology (45, 343) **(Appendix A)**. Further examination of the JAK/STAT pathway would aid in confirming these interactions and provide additional insight into whether *STAT92E* operates independently of the JAK/STAT pathway in an antiviral role.

4.2 Mitochondrial quality control mediates repeat dsRNA pathology

A wealth of evidence now places mitochondria at the centre of innate immunity in addition to its role as the powerhouse of the cell (140). Mitochondrial participation in immunity is multi-faceted; they act as a platform for RIG-I-like receptor signalling through MAVS (102, 457), generate mitochondrial ROS (mROS) to kill invading pathogens (458, 459) and trigger inflammation upon injury/stress via the release of mROS (460, 461) and/or mitochondrial DNA (94, 462). Indeed, the release of damage-associated molecular patterns (DAMPs) and other pro-inflammatory molecules derived from damaged mitochondria can lead to systemic inflammatory disease (96, 460, 462) and drive neuroinflammation (139, 463).

As such, a number of mitochondrial quality control pathways are in place to remove damaged/dysfunctional mitochondria and prevent persistent inflammation **(Figure 4.5)** (464-466). Mitochondria are dynamic organelles and constantly undergo the processes of fusion, in order to share metabolites and healthy mtDNA (467), and fission (mediated by Dynamin-related protein 1 – Drp1), to sequester away harmful material into mitochondria that are subsequently shuttled towards degradation (466, 468). Mitophagy provides the means for damaged mitochondria to be degraded by autophagic machinery to prevent their accumulation (469). The importance of mitophagy is demonstrated by the observation that inherited loss-of-function mutations in mitophagy regulators *PINK1* and *Parkin* cause autosomal recessive early-onset PD (99, 100).

The conservation of mitochondrial quality control pathways between vertebrates and *Drosophila* has proven useful in dissecting the pathways underlying mitochondrial function/dysfunction **(Figure 4.5)** (470). This includes modelling key PD features through the use of *PINK1* and *parkin* mutants (332, 334) and dissecting their regulatory roles in mitophagy (471). Of particular relevance to expanded repeat disease, over-expression of *PINK1* is neuroprotective in a *Drosophila* mutant Huntington (mHtt) model of HD through the upregulation of mitophagy (472), suggesting that mitochondrial quality control aids in removing HD-specific polyQ expansions. Thus, to determine whether mitochondrial quality control is rate-limiting in an RNA model of HD (a different gene product), the repeat dsRNA

Figure 4.5: Overview of mitochondrial quality control pathways. Mitochondrial fission, mediated by Drp1, allows the separation of healthy (green) and dysfunctional (red) mitochondria. The damaged mitochondria are depolarized, leading to the accumulation of PINK1 on the outer mitochondrial membrane (OMM). The E3 ubiquitin ligase Parkin is then recruited to the damaged mitochondria, adding ubiquitin chains (Ub, yellow circles) to protein substrates, including ECSIT. Ubiquitinated mitochondria attract autophagy receptors including p62 and Optineurin, followed by the recruitment of LC3-positive phagosomes (in orange), thus facilitating degradation of the damaged mitochondria. Mammalian protein names are denoted in black, *Drosophila* orthologues (if any) are denoted in blue. Proteins underlined are tested in this chapter.

eye model (*GMR*>dsRNA) was used to screen against several genes known for roles in mitochondrial homeostasis.

Given the reported neuroprotective role of *PINK1* in HD (472), it was examined for its role in dsRNA pathology. Under homeostatic mitochondrial conditions, PINK1 is imported into the inner mitochondrial membrane (IMM) where it undergoes constitutive degradation (466). However, loss of membrane potential in dysfunctional mitochondria leads to the accumulation of PINK1 on the outer mitochondrial membrane (OMM), where its kinase activity recruits Parkin and progresses mitophagy (473-475). Familial PD mutations in *PINK1* affect its ability to recruit Parkin, which further highlights mitophagy as a key pathway in neurodegeneration (473, 475).

Reducing *PINK1* levels via RNAi knockdown alone did not cause any noticeable phenotype compared to the control **(Figure 4.6 A, B & C)**. In support of neuroprotective *PINK1*-
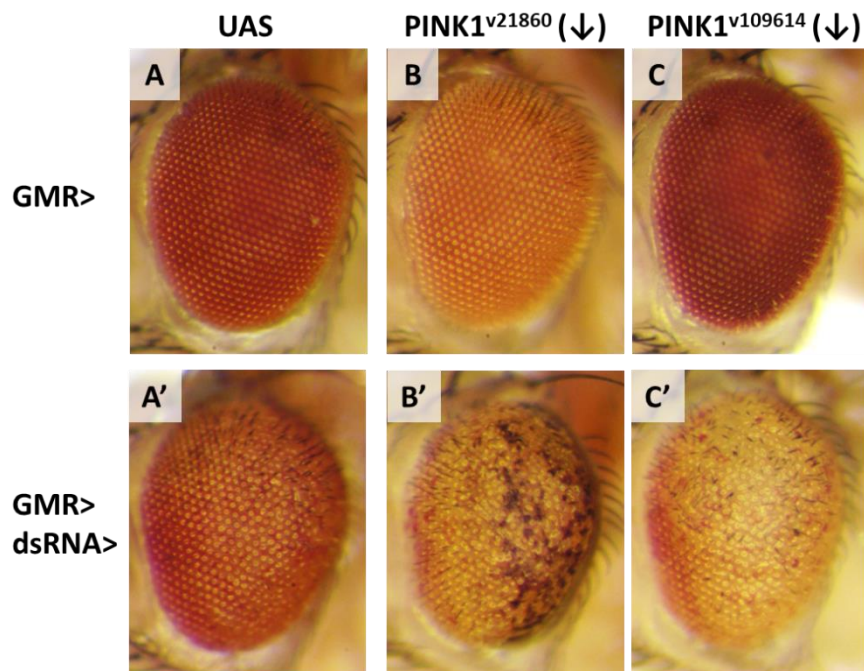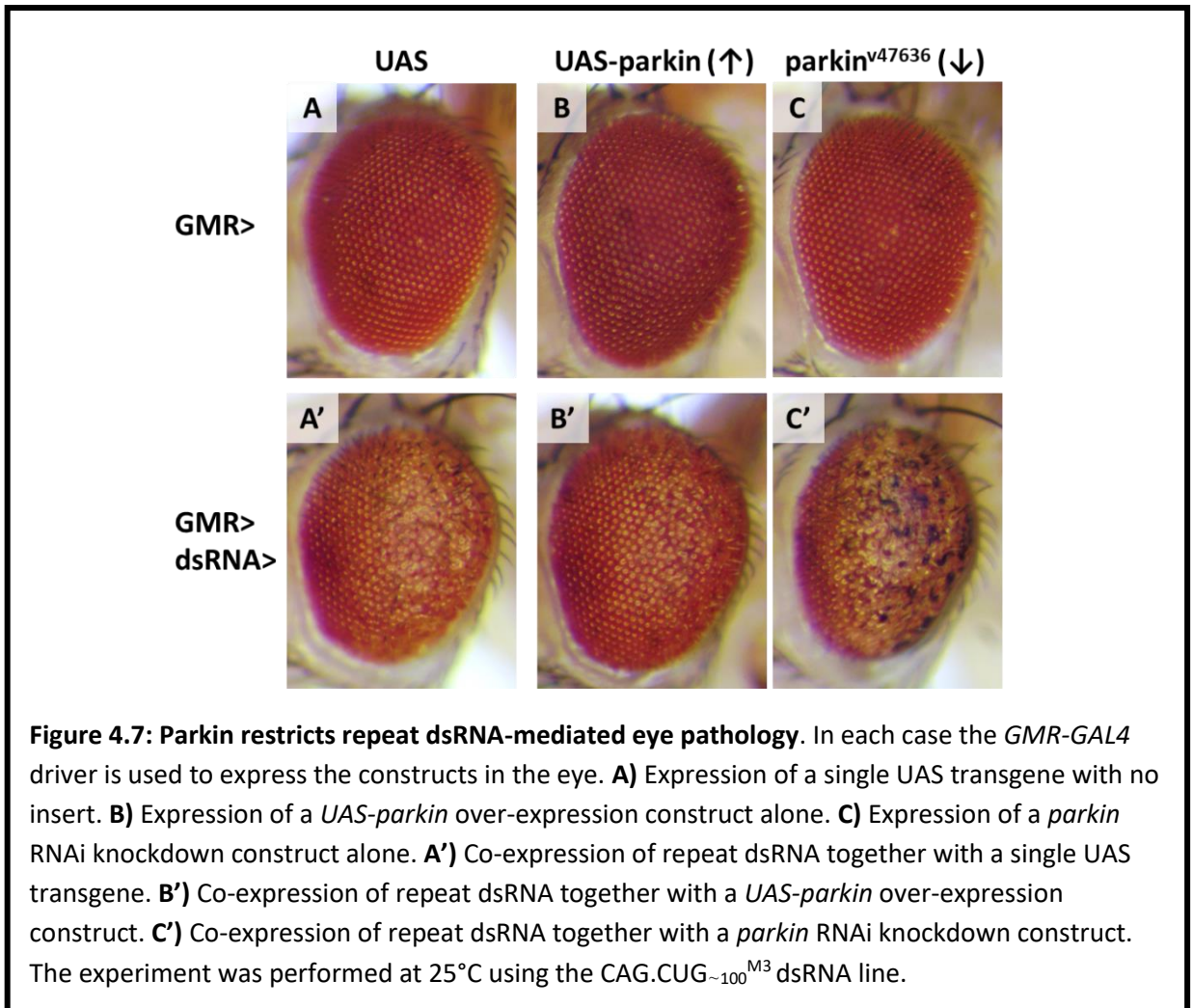
Figure 4.6: Reduction of PINK1 enhances repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B-C)** Expression of two independent *PINK1* RNAi knockdown constructs alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B'-C')** Co-expression of repeat dsRNA together with two independent *PINK1* RNAi knockdown constructs. The experiment was performed at 25°C using the CAG.CUG$_{\sim100}$^{M3} dsRNA line.

mediated mitophagy in HD, reducing *PINK1* expression led to enhanced dsRNA pathology in both knockdown lines **(Figure 4.6 B' & C')**. Co-expression of dsRNA with the *PINK1^{v21860}* RNAi construct led to a reduction in eye pigment and the development of black necrotic areas on the posterior side of the eye **(Figure 4.6 B')**, while co-expression with the *PINK1^{v109614}* RNAi construct displayed a more potent loss of pigmentation but did not develop necrotic areas **(Figure 4.6 C')**. Thus, PINK1 appears to restrict dsRNA pathology.

Given the observed role of PINK1 in mediating expanded repeat disease pathology both here **(Figure 4.6)** and elsewhere (472), the investigation was extended to Parkin, an E3 ubiquitin ligase that acts downstream of PINK1 in the mitophagy pathway. Upon recruitment to dysfunctional mitochondria by PINK1, Parkin is activated and begins to ubiquitinate protein substrates on the OMM, ultimately facilitating the recruitment of autophagic machinery to degrade the material through autophagosomal-lysosomal activity (476). Aside from

Figure 4.7: Parkin restricts repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** Expression of a *UAS-parkin* over-expression construct alone. **C)** Expression of a *parkin* RNAi knockdown construct alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a *UAS-parkin* over-expression construct. **C')** Co-expression of repeat dsRNA together with a *parkin* RNAi knockdown construct. The experiment was performed at 25°C using the $CAG.CUG_{\sim100}^{M3}$ dsRNA line.

mitophagy, Parkin also plays an evolutionarily conserved role in pathogen degradation through ubiquitin-mediated autophagy (477), indicating that it can also regulate the removal of 'non-self' material through a common degradative mechanism. Either over-expression of *parkin* via a UAS construct **(Figure 4.7 B)** or reduction via RNAi knockdown **(Figure 4.7 C)** did not cause any observable eye phenotype compared to the control **(Figure 4.7 A)**. However, over-expression of *parkin* in conjunction with *GMR*>dsRNA led to a suppression of dsRNA pathology, characterised by the return of red pigmentation and slight improvement to ommatidial patterning compared to *GMR*>dsRNA alone **(Figure 4.7 A' & B')**. In contrast, reducing *parkin* expression in a *GMR*>dsRNA background enhanced the dsRNA pathology as observed by the presence of necrotic black spots distributed on the posterior side of the eye **(Figure 4.7 C')**.

The observation that increased *parkin* restricts the dsRNA pathology supports previous work demonstrating that the upregulation of mitochondrial quality control is protective in HD (472), while also indicating that multiple products (RNA and protein) from the *huntington* (*htt*) disease gene affect common pathways. Interestingly, whereas Parkin is necessary for the neuroprotective effect of *PINK1* over-expression, *parkin* over-expression alone is not protective in the HD model, indicating that the ability of *PINK1* to discern between healthy and dysfunctional mitochondria is critical (472).

Downstream of Parkin-mediated ubiquitination activity, a number of autophagic adaptors are recruited to mitochondria including p62/SQSTM1 (Sequestosome 1) and Optineurin, which act to shuttle the damaged mitochondria to autophagosomes for degradation (478, 479). Interestingly, mutations in either *p62* or *optineurin* are both linked to familial and sporadic forms of ALS (480, 481), highlighting defects in mitochondrial quality control as a contributor to motor neuron death. *Drosophila* contains a conserved *p62* orthologue known as *Refractory to Sigma P* (*Ref(2)P*) but lacks a characterised *optineurin* counterpart, suggesting that mammalian mitophagy may have developed additional compensatory or redundant mechanisms to prevent total loss of degradation (465).

Like *p62*, *Drosophila Ref(2)P* has been demonstrated as a participant in mitophagy, acting downstream of *PINK1* and *Parkin* to promote the clustering of damaged mitochondria and their subsequent degradation (482). In addition, *Ref(2)P* also localizes to proteinaceous aggregates in the *Drosophila* brain that form under conditions of ageing, autophagy/proteasome loss of function and in fly models of neurodegenerative disease (483). Finally, *Ref(2)P* acts as a viral restriction factor to control the replication of Sigma virus (484, 485) and Zika virus (322). Thus, *Ref(2)P* contributes to a number of the pathways in the multi-armed *Drosophila* inflammatory system, so it was investigated whether *Ref(2)P* restricts repeat dsRNA pathology within the eye.

Over-expression of *Ref(2)P* through the use of a *UAS-Ref(2)P* construct did not cause any noticeable phenotype compared to a control **(Figure 4.8 A & B)**, and a similar lack of phenotype was observed when *Ref(2)P* was reduced via RNAi **(Figure 4.8 C)**. Over-expression of *Ref(2)P* in a *GMR*>dsRNA background led to no obvious modification of the dsRNA
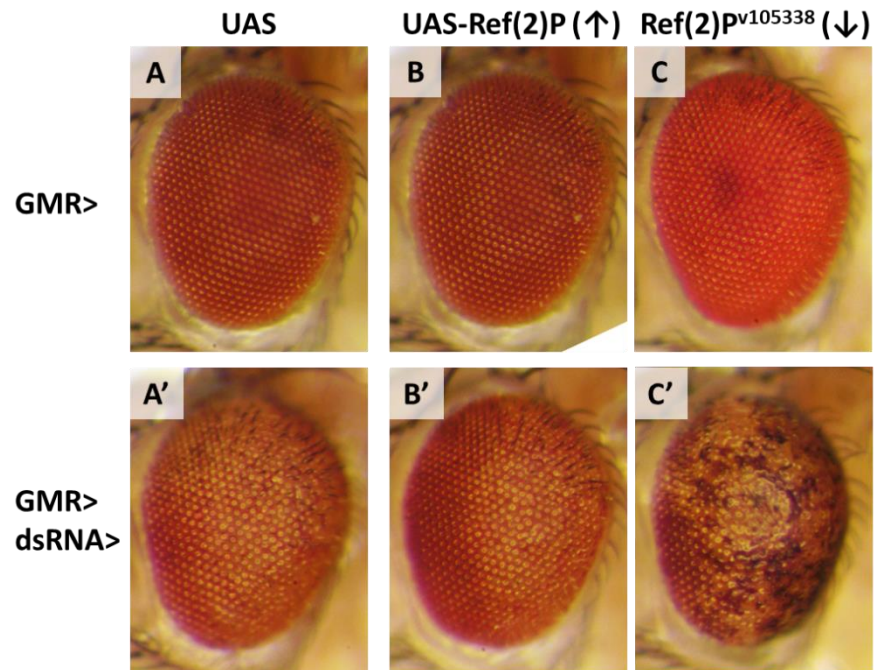
Figure 4.8: Reduction of Ref(2)P enhances repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** Expression of a *UAS-Ref(2)P* transgenic over-expression construct alone. **C)** Expression of a *Ref(2)P* RNAi knockdown construct alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a *UAS-Ref(2)P* transgenic over-expression construct. **C')** Co-expression of repeat dsRNA together with a *Ref(2)P* RNAi knockdown construct. The experiment was performed at 25°C using the CAG.CUG$_{\sim100}^{M3}$ dsRNA line.
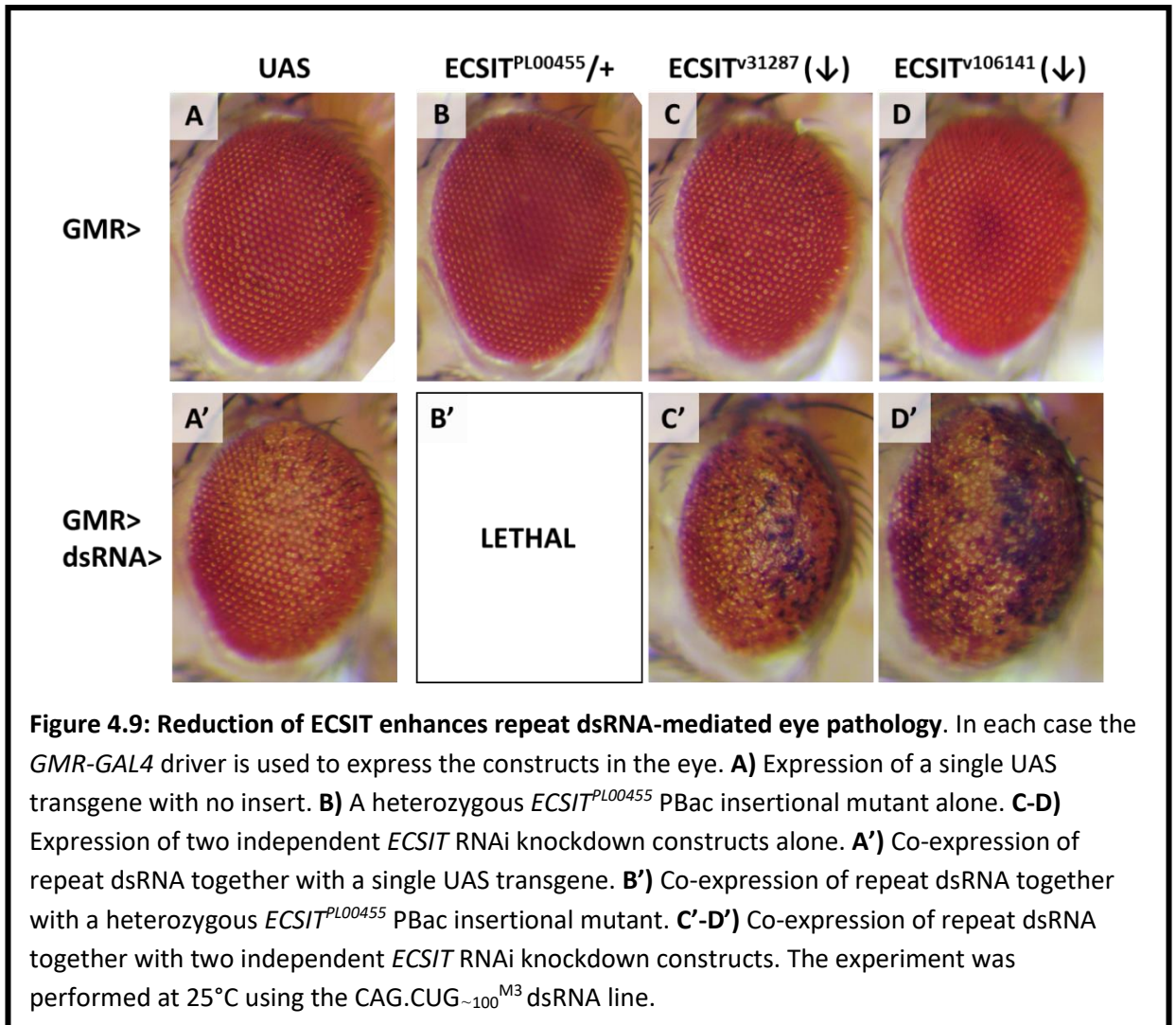
pathology compared to *GMR*>dsRNA alone **(Figure 4.8 A' & B')**. It must be noted that the *UAS-Ref(2)P* carries a GFP fluorescent tag and it is not known if the addition of a tag affects the normal function of the Ref(2)P protein. However, *Ref(2)P* knockdown enhanced the dsRNA pathology, characterised by the presence of black necrotic areas distributed throughout the eye **(Figure 4.8 C')**.

Therefore, *Ref(2)P* appears to play a role in restricting the repeat dsRNA pathology, likely to be through its role as on autophagy receptor. Whether this is through the *Drosophila* mitophagy pathway (482) or as part of an antiviral/autophagy response similar to its role in combating Zika virus (322) is not known. However, given that the dsRNA appears to be detected as 'non-self' or viral material **(Chapter 3)** and dsRNA pathology is restricted by

PINK1 and Parkin **(Figures 4.6 & 4.7)**, it is plausible that both mitophagy and antiviral signalling act together to facilitate the clearance of repeat dsRNA. Another possible explanation is that *Ref(2)P* represents a link between antiviral signalling and mitophagy, where autophagosome formation and cargo degradation downstream of the respective initial signalling cascades represents a common endpoint of resolution. Recent evidence indicates that *Ref(2)P* is induced through a Relish (NF-κB) and STING dependent pathway in response to Zika virus (322). Thus, it would be of interest to determine whether both *Relish* and *STING* also contribute to the dsRNA pathology.

Another protein that links mitochondrial quality control and antiviral signalling is evolutionarily-conserved signalling intermediate in Toll pathways (ECSIT). Initially discovered as a conserved adaptor protein within the Toll/IL-1 signalling pathway (486), recent evidence has demonstrated a key role for ECSIT in bridging RIG-I-like receptors RIG-I and MDA5 to their adaptor molecule MAVS on the mitochondrial membrane following viral challenge (487). In addition, ECSIT participates in mitochondrial complex I (MCI) assembly in both vertebrates and *Drosophila* (488, 489) and is ubiquitinated by Parkin in order to recruit autophagic machinery to damaged mitochondria (490). Finally, ECSIT has been hypothesised to act as a sensor of AD pathogenesis through its role as a mitochondrial signalling hub and its location within an AD susceptibility locus (491). Surprisingly, aside from its conserved roles in Toll signalling and mitochondrial complex I formation (486, 489), knowledge on the functions (conserved or otherwise) of *ECSIT* in *Drosophila* is scarce. However, the known roles of vertebrate *ECSIT* in antiviral signalling and mitochondrial quality control highlight it as a potential contributor to mitochondrial-mediated inflammation in *Drosophila*. Thus, *ECSIT* was tested for its participation in dsRNA pathology.

A heterozygous *ECSIT* insertional mutant displayed no signs of disruption **(Figure 4.9 B)**, while reduction of *ECSIT* through two independent RNAi constructs also did not cause phenotypes alone **(Figure 4.9 C & D)**. Strikingly, the heterozygous *ECSIT* mutant was lethal when combined with *GMR*>dsRNA **(Figure 4.9 B')**, indicating a high level of cellular toxicity. In addition, RNAi knockdown of *ECSIT* led to enhancement of the dsRNA pathology in both independent constructs, as seen by the appearance of black necrotic areas concentrated on

Figure 4.9: Reduction of ECSIT enhances repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** A heterozygous *ECSIT^PL00455* PBac insertional mutant alone. **C-D)** Expression of two independent *ECSIT* RNAi knockdown constructs alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a heterozygous *ECSIT^PL00455* PBac insertional mutant. **C'-D')** Co-expression of repeat dsRNA together with two independent *ECSIT* RNAi knockdown constructs. The experiment was performed at 25°C using the CAG.CUG$_{\sim100}^{M3}$ dsRNA line.

the posterior side of the eye and ommatidial disruption **(Figure 4.9 C' & D')**. Indeed, it is possible that the lethality observed when using the *ECSIT* insertional mutant **(Figure 4.9 B')** could be due to GAL4-driven misexpression of adjacent genes (including *ECSIT* itself), though the direction of pathological alteration appears consistent with the reduction of *ECSIT* enhancing toxicity **(as seen in Figure 4.9 C' & D')**.

The limited knowledge surrounding *ECSIT* function in *Drosophila* means that the mechanisms by which *ECSIT* restricts the dsRNA pathology are largely speculative. However, based on the reported role of ECSIT in mitochondrial quality control (490), and that loss of *ECSIT* leads to enhanced dsRNA pathology reminiscent of known mitophagy regulators *PINK1* and *parkin* **(Figures 4.6 & 4.7)**, mitophagy constitutes a plausible common

mechanism. Similar to *Ref(2)P*, *ECSIT*-mediated restriction of the dsRNA pathology may represent crosstalk between mitophagy and innate inflammatory signalling. In addition to its involvement in antiviral responses, ECSIT-mediated Toll signalling in macrophages leads to antibacterial activity through the production of mitochondrial ROS (mROS) (459), indicating that ECSIT can integrate signals from multiple inflammatory pathways. Notably, the Toll pathway is not only required for the dsRNA pathology but is also induced through the expression of dsRNA (52). Therefore, it would be of interest to determine the effect of *ECSIT* over-expression on the dsRNA eye phenotype, given that loss of *ECSIT* enhances pathology and thus appears to act in a competing pathway to Toll.

Taken together, these results implicate mitochondria as a key component of CAG expanded repeat neurodegenerative disease pathogenesis, potentially through the separate but intrinsically-linked inflammatory and mitochondrial quality control pathways.

4.3 Chapter Discussion

The activation of innate inflammatory pathways is a tightly controlled balancing act; its dampening confers susceptibility to pathogen challenge and often their persistence, whereas chronic immune activation leads to tissue damage and dysfunction and is, not surprisingly, a common feature in neurodegenerative disease (428, 492). A range of diverse genetic lesions that constitute causal/risk factors in neurodegenerative disease affect components of the broader inflammatory response; either by inhibiting neuroprotective functions such as autophagy and mitophagy, or the amplification of pro-inflammatory signalling (68). The relative conservation of inflammatory mechanisms coupled with lower functional redundancy among pathways means that *Drosophila* provides a robust model organism to investigate inflammation in the context of neurodegenerative disease development and progression (355, 440).

Previous studies using the *Drosophila* model of expanded repeat disease described here have implicated both Dicer-2- and Toll-dependent inflammatory signalling as drivers of disease pathology in the eye tissue model (45, 52). This chapter identifies additional components of the broader inflammatory system that contribute to the repeat dsRNA
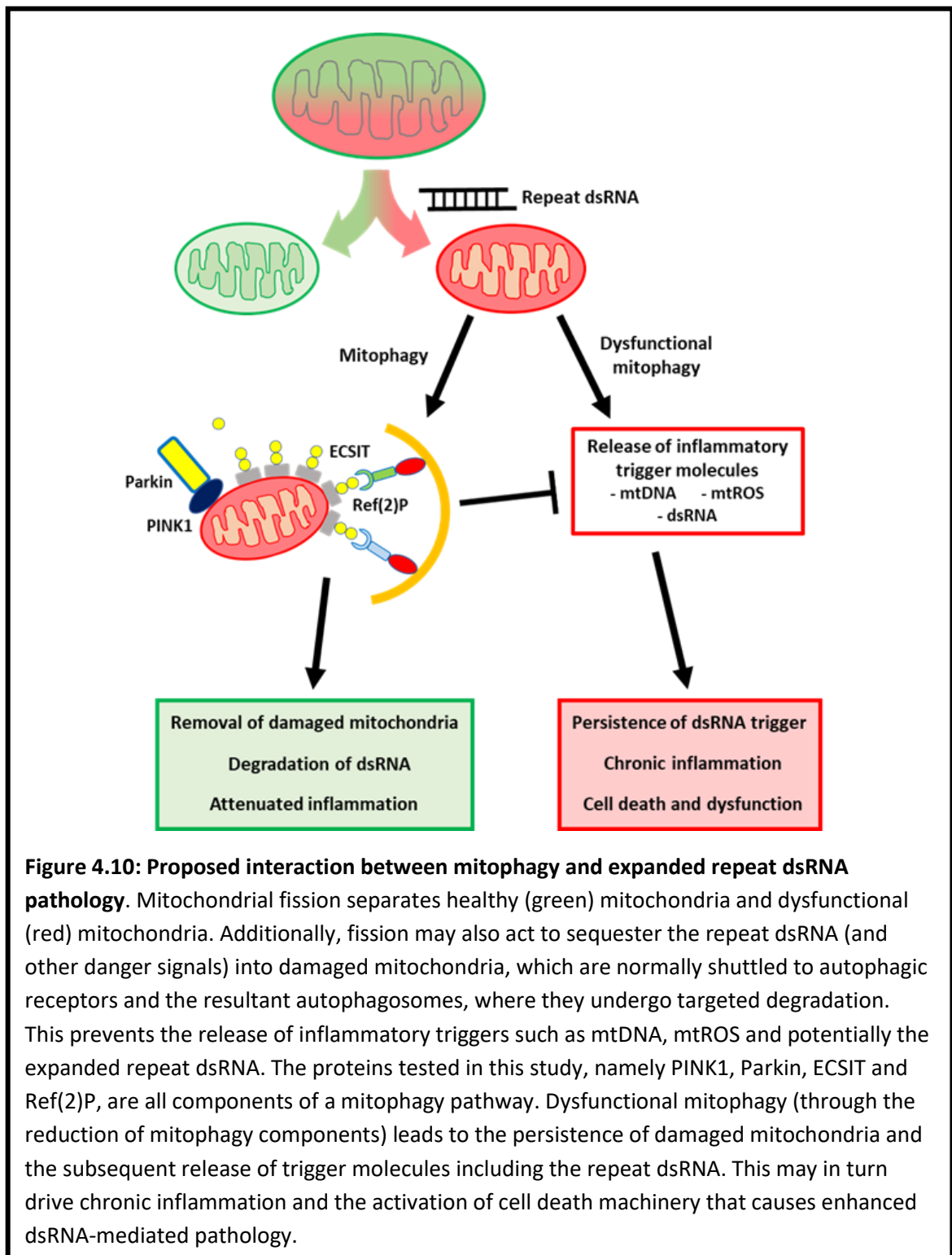
Figure 4.10: Proposed interaction between mitophagy and expanded repeat dsRNA pathology. Mitochondrial fission separates healthy (green) mitochondria and dysfunctional (red) mitochondria. Additionally, fission may also act to sequester the repeat dsRNA (and other danger signals) into damaged mitochondria, which are normally shuttled to autophagic receptors and the resultant autophagosomes, where they undergo targeted degradation. This prevents the release of inflammatory triggers such as mtDNA, mtROS and potentially the expanded repeat dsRNA. The proteins tested in this study, namely PINK1, Parkin, ECSIT and Ref(2)P, are all components of a mitophagy pathway. Dysfunctional mitophagy (through the reduction of mitophagy components) leads to the persistence of damaged mitochondria and the subsequent release of trigger molecules including the repeat dsRNA. This may in turn drive chronic inflammation and the activation of cell death machinery that causes enhanced dsRNA-mediated pathology.

mediated pathology, in particular a number of proteins that participate in mitochondrial quality control, thus implicating mitochondrial turnover as a key process in CAG repeat expansion disease **(Figure 4.10)**. Mitochondria represent a vital signalling hub for innate immunity and inflammation, both as a signalling platform (102) and as a mediator through the release of inflammatory factors such as mROS and mtDNA in response to stress and other insults (493). As such, pathways that ensure mitochondrial integrity and function are of vital importance in preventing uncontrolled inflammation and cell death (469, 494).

Loss of canonical mitophagy regulators *PINK1* or *parkin* both enhance the dsRNA pathology considerably, providing evidence that mitophagy is a key restrictive process in the presence of repeat dsRNA. Additionally, over-expression of *parkin* partially rescues the dsRNA pathology, which suggests that the ability of mitophagy to degrade dsRNA may be limited by the boundaries of endogenous upregulation. This could be further tested by over-expressing *parkin* in conjunction with a stronger dsRNA line. Over-expression of *PINK1* is neuroprotective in a *Drosophila* model of HD generated through expression of *mHtt* (472), and in future should be tested with the dsRNA model used in this study. When taken together, the results described here and in Khalil *et al.,* indicate that upregulation of mitophagy may represent a promising therapeutic target in HD, and that multiple products (RNA and protein) of the same disease-gene (in this case the expanded CAG repeat in *Htt*) are restricted by a common pathway. Intriguingly, recent evidence has described the occurrence of mitophagy operating either in parallel with Parkin (495) or completely independent of Parkin (496), which likely highlights a level of functional redundancy/compensation present in order to prevent compete ablation of mitophagy upon homeostatic insult. Indeed, both PINK1 and Parkin appear largely dispensable for mitophagy under normal physiological conditions (497, 498), and as such the stress-induced PINK1/Parkin axis may represent an entirely distinct mechanism to basal mitophagy.

While the functions of PINK1 and Parkin mainly encompass their role in mitophagy regulation, there are a number of proteins that participate in mitophagy in addition to roles mediating other aspects of the broader mitochondrial inflammatory response. Ref(2)P (functionally conserved with mammalian p62) is a viral restriction factor (484, 485) that functions in mitochondrial quality control as an autophagic receptor, acting as a conduit

between ubiquitinated mitochondria and autophagosomes (482). Indeed, these functions are often linked; Ref(2)P-mediated antiviral autophagy of Zika virus is induced through Relish/STING dependent inflammatory signalling (322). Thus, it is not surprising that reduction of *Ref(2)P* also enhances the dsRNA pathology, though whether this restrictive role derives from its participation in antiviral immunity or mitophagy, or both, is a question that requires further dissection.

Unlike *parkin*, over-expression of *Ref(2)P* does not ameliorate pathology, which indicates that it is not a rate-limiting molecule in the upregulation of *Drosophila* mitophagy. In support of this, several lines of study have reported that mammalian p62/Ref(2)P is important for Parkin-mediated mitochondrial clustering (499, 500) but is dispensable for mitophagy itself (499, 501). However, p62-dependent removal of damaged mitochondria is critical in restricting NLRP3 inflammasome signalling (502), which suggests that the participation of p62 in mitophagy may be context dependent. In *Drosophila*, *Ref(2)P* is able to rescue mitochondrial dysfunction in *PINK1* mutants in a *parkin*-dependent manner which demonstrates that, in invertebrates at least, *Ref(2)P* does indeed function downstream of *PINK1* and *parkin* as part of a mitochondrial quality pathway (482). This in part may reflect functional overlap between mammalian autophagy adaptor proteins that bind both ubiquitin and autophagosome components (469) that is likely to be lower in *Drosophila*. For instance, Optineurin also functions as a Parkin-mediated selective autophagy receptor that displays partial overlap with p62 in mammalian cells (479) but lacks a functional orthologue in *Drosophila*. Thus, *Ref(2)P* may perform a similar but less-redundant role in *Drosophila* mitochondrial quality control.

Alternatively, does Ref(2)P interact with repeat dsRNA via its participation in Relish/STING-mediated antiviral autophagy? In mammals, STING functions as a sensor of cytosolic DNA (399), while the *Drosophila* STING orthologue appears be capable of sensing both DNA and RNA viral material (323, 393) in addition to acting downstream of Relish in Zika virus infection (322). Notably, the *Drosophila* STING counterpart has only very recently been characterised (322, 323), and so *GAL4-UAS* expression constructs that facilitate genetic manipulations of *STING* could not be obtained for this project. The *Drosophila* Ref(2)P protein contains an N-terminal Phox and Bem 1 (PB1) domain, a ZZ zinc finger domain and a

ubiquitin associated (UBA) domain at the C-terminal end (484). The PB1 domain is required for viral interactions (484, 485), while the UBA and PB1 (to a lesser extent) domains are essential for protein aggregation and clearing dysfunctional mitochondria (482). Thus, analysis of *Ref(2)P* mutants lacking either the PB1 or UBA domains in the context of repeat dsRNA pathology would be informative in determining whether loss of mitochondrial quality control or antiviral signalling (or both) contribute to enhancement of the pathology.

The finding that *ECSIT* also appears to restrict dsRNA pathology, in conjunction with a lack of knowledge regarding its functionality in *Drosophila*, raises several intriguing questions. Is the reported participation of ECSIT in mammalian mitochondrial quality control (490) conserved in Drosophila? Is the role of ECSIT as a mitochondrial-localised antiviral signalling adaptor (487) also conserved, given that a *Drosophila* orthologue of MAVS is yet to be characterised? If not, does the conserved role of *ECSIT* in Toll signalling (486) represent an alternative innate immune pathway that responds to 'non-self' dsRNA? *ECSIT* also facilitates the TLR-dependent release of mROS from mammalian macrophages in order to kill bacterial pathogens (459) and is integral both in vertebrates and *Drosophila* for the formation of mitochondrial complex I (MCI) (488, 489). Thus, similar to *Ref(2)P*, *ECSIT* could plausibly restrict dsRNA pathology through its participation in a range of mitochondrial-associated pathways. The mammalian ECSIT protein comprises of an N-terminal mitochondrial targeting sequence (MTS) and two binding domains (486, 503). The binding domains are designated as TNF-associated receptor factor 6 (TRAF6) binding domain and TAK1 binding domain respectively and are required for NF-κB signalling through Toll receptors (503). Additionally, the C-terminal TAK1 binding domain is sufficient for interactions with the RNA sensors RIG-I and MDA5 and is thus important for mediating signal transduction between the receptors and the mitochondrial MAVS adaptor protein (487). *Drosophila* ECSIT has been demonstrated to bind to both fly and mammalian TRAF6 (486), and indeed *Drosophila* contains a conserved TAK1 that is important for NF-κB signalling (504, 505). Thus, it is reasonable to suggest that this binding domain may also be present in *Drosophila*. Whether the MTS is also conserved is not known. Therefore, much like Ref(2)P, analysis of mutated ECSIT proteins would aid in identifying the role of *ECSIT* in mediating dsRNA pathology.

90

Taken together, mitochondrial quality control, in particular mitophagy, appears vital for restricting expanded repeat dsRNA eye pathology in the model used in this study. A plausible hypothesis is that this constitutes a method by which the repeat dsRNA can be sequestered into mitochondria destined for degradation via mitophagy, thus preventing the accumulation of dsRNA as an inflammatory trigger **(Figure 4.10)**. Reducing the expression of mitophagy components (*PINK1*, *parkin*, potentially also *Ref(2)P* and *ECSIT* in this context) below a functional threshold leads to dysfunctional mitophagy, allowing the persistence and release of molecules (dsRNA, mtDNA and mtROS) that can propagate a damaging inflammatory response. The expansion of the dsRNA eye screen to additional components of mitochondrial quality control pathways, including mitochondrial fission and fusion which precede mitophagy, will challenge this hypothesis and should be considered as part of future studies.

Based on preliminary work presented in this chapter, the participation of the *Drosophila* JAK/STAT pathway in mediating the dsRNA pathology appears minimal, which may reflect the ability of alternate inflammatory pathways to compensate for loss of JAK/STAT signalling. Reduction of the *Upd1* ligand did not affect the dsRNA eye phenotype, while *Upd3* appeared to play a minor role in restricting the pathology. All three *Unpaired* ligands signal through a single receptor known as *Domeless* (441) and display a level of redundancy in their function (455), suggesting that any contribution(s) to dsRNA pathology observed via reducing *upd* expression may be compensated for. Reduction of the downstream transcriptional activator *STAT92E* led to a mild suppression of the dsRNA eye phenotype, though whether this is due to the increased red pigment level in the RNAi line itself is not known and could be tested with an independent *STAT92E* knockdown or mutant. *STAT92E* can be activated independently of the canonical JAK/STAT pathway in *Drosophila* following injury or viral challenge (53, 295) and so whether any suppression represents the inhibition of the JAK/STAT-mediated transcriptional program or that of an alternate STAT-activating pathway could not be discerned in this study. Therefore, extending the dsRNA eye phenotype screen to the JAK/STAT receptor *Domeless* or its adaptor molecule *Hopscotch* would provide further insight into the participation of JAK/STAT signalling in the presence of repeat dsRNA.

The most compelling results in this chapter implicate mitochondrial quality control in repeat dsRNA-mediated pathology. The efficient turnover of mitochondrial material is essential to maintain a pool of functional mitochondria and to prevent the accumulation of mitochondrial associated trigger molecules including mtROS and mtDNA that can propagate inflammatory signalling and neurodegeneration (139, 506). Evidence provided in this chapter and elsewhere (472) has implicated dysfunctional mitochondrial turnover as a driver of expanded repeat disease. The repeat dsRNA used in this study has been demonstrated to induce an inflammatory response (52), and so a plausible scenario exists where dsRNA is sequestered into damaged mitochondria destined for degradation through mitophagy, thus maintaining a healthy pool of mitochondria and preventing inflammation **(Figure 4.10)**. Supporting this, mitochondrial fission events often produce asymmetrical daughter mitochondria, where one displays decreased membrane potential and is targeted for mitophagy (468). Thus, going forward, it would be of interest to test regulatory components of both mitochondrial fission and fusion in the context of dsRNA pathology – does the inhibition of fission prevent the shuttling and subsequent degradation of dsRNA? Additionally, the investigation of mitochondrial quality control in other expanded repeat models could provide a common contributing mechanism to expanded repeat disease.

CHAPTER 5: The contribution of glial cell subtypes in repeat dsRNA-mediated neurodegeneration

Long thought of as simply support for neuronal cells, a wealth of experimental evidence has now implicated glial cells as key drivers in the development of neurodegenerative disease, providing a non-cell autonomous mechanism for diseases previously viewed of as purely neuron-centric in pathogenesis (225).

Much of this renewed focus has targeted the resident CNS immune cells, microglia, for their ability to transition to a "reactive" state in response to specific stimuli such as pathogenic insult and brain injury that can promote CNS repair or drive neuronal toxicity (177, 507). However, recent evidence has also uncovered a similar change of state in astrocytes via microglial signalling that led to neuronal cell death, further highlighting the importance of non-cell autonomous communication in driving neurodegenerative disease (247). It is noteworthy that the changes of state observed in both microglia and astrocytes are not simply the gain of neurotoxic signalling, but also the loss of their normal homeostatic functions performed within the CNS, including the promotion of cellular repair, phagocytic clearance of neuronal debris and trophic support (177, 179). Finally, the disruption of non-neuronal cells responsible for forming the blood-brain barrier (BBB) can also promote neurodegeneration through processes including the accumulation of toxic systemic and pathogenic molecules and diminished BBB transport efficiency (508). Thus, it is clear that glial cells can mediate neurodegeneration through both gain-of-function and loss-of-function mechanisms, underlying the importance of a tightly regulated CNS environment.

Despite the lack of distinct one-for-one orthologues between humans and *Drosophila*, many of the functional and morphological features between vertebrate and invertebrate glia are conserved (310). Like their vertebrate counterparts, there exists distinct glial subtypes within the *Drosophila* CNS, differentiated through both their described functions and their relationships with neuronal cells (160). Their utility as a relatively simple *in vivo* model, as well as the range of widely available genetic and molecular tools to explore the CNS, highlight *Drosophila* as an excellent model to gain further insight into glial cell biology and the non-cell autonomous axis of neurodegenerative disease. Previous work using *Drosophila*

has highlighted glial cells as a crucial non-cell autonomous driver of expanded repeat dsRNA-mediated pathology, where pan-glial expression of the dsRNA leads to striking reductions in both neuronal function and survival (52). In order to tease apart the contributions made to this high level of pathology by glial cell subtypes, GAL4 driver lines specific to the individual subtypes were obtained **(Table 5.1)**.

There are several considerations to take into account when using *Drosophila* to investigate the role of glial cells in neurodegeneration. Firstly, the GAL4 expression pattern is derived from an endogenous regulatory enhancer element, which relies on the putative expression pattern of the chosen enhancer to display robust tissue specificity (314). Consequently, although GAL4 drivers are identified for specificity as best possible, there exists the possibility of expression "leakage" into non-targeted tissue types. Indeed, while the glial GAL4 drivers described here **(Table 5.1)** were chosen on the basis on glial subtype-specificity according to previous large-scale analysis of glial-specific drivers (292), some display trace levels of non-cell type exclusive tissue expression. One such example is the cortex glial-specific *R54H02-GAL4* driver used in this study **(Table 5.1)**; while the expression pattern is

Table 5.1: Summary of the glial subtype-specific drivers. Other frequently used tissue-specific drivers are listed for reference.

GAL4-Driver	Denoted as	Generic glial subtype	*Drosophila* Gene	Human Orthologue(s)
R54C07-GAL4	SPG-GAL4	Subperineural	Mdr65	ABCB1/4/5/11
nrv2-GAL4	nrv2-GAL4	Wrapping	nervana2	ATP1B1/4
R75H03-GAL4	TEG-GAL4	Tract-ensheathing	Drgx	PHOX2A/B
R56F03-GAL4	NEG-GAL4	Neuropil-ensheathing	CG9657	SLC5A1/2/4/9/10/11
R86E01-GAL4	ALG-GAL4	Astrocyte-like	btn	MEOX1/2
R54H02-GAL4	CG-GAL4	Cortex	wrapper	None
GAL4-Driver	**Denoted as**	**Generic cell subtype**	***Drosophila* Gene**	**Human Orthologue(s)**
Repo-GAL4	Repo-GAL4	All glial cells except midline glia	repo	None
Elav-GAL4	Elav-GAL4	All neuronal cells	elav	ELAVL1/2/3/4
GMR-GAL4	GMR-GAL4	Differentiated photoreceptor cells	Synthetic enhancer element	N/A

largely specific to cortex glia, evidence of GFP-reporter expression was also observed in surface glia and the non-CNS Malpighian tubules (309). Thus, the potential of non-cell type exclusive expression must be factored into the interpretation of the results, particularly in cases where evidence has already been presented. Secondly, the relative strength of expression of each driver must be considered. As with the tissue-specificity, a comparison of the chosen drivers regarding expression was performed and major quantitative differences in expression were not noted (292).

Indeed, a number of the most widely used GAL4 drivers also display evidence of non-tissue specific expression. The developing eye-specific driver *GMR-GAL4* has been reported to express in some neuronal cells, in addition to residual expression in the trachea and larval wing discs (509, 510). *Elav-GAL4*, a commonly used pan-neuronal driver, has also shown transient expression in embryonic glial cells and wing discs (511, 512). Thus, GAL4-UAS non-cell type exclusive expression is not a phenomenon exclusive to the glial subtype drivers utilized in this chapter, but must be considered in the interpretation nonetheless.
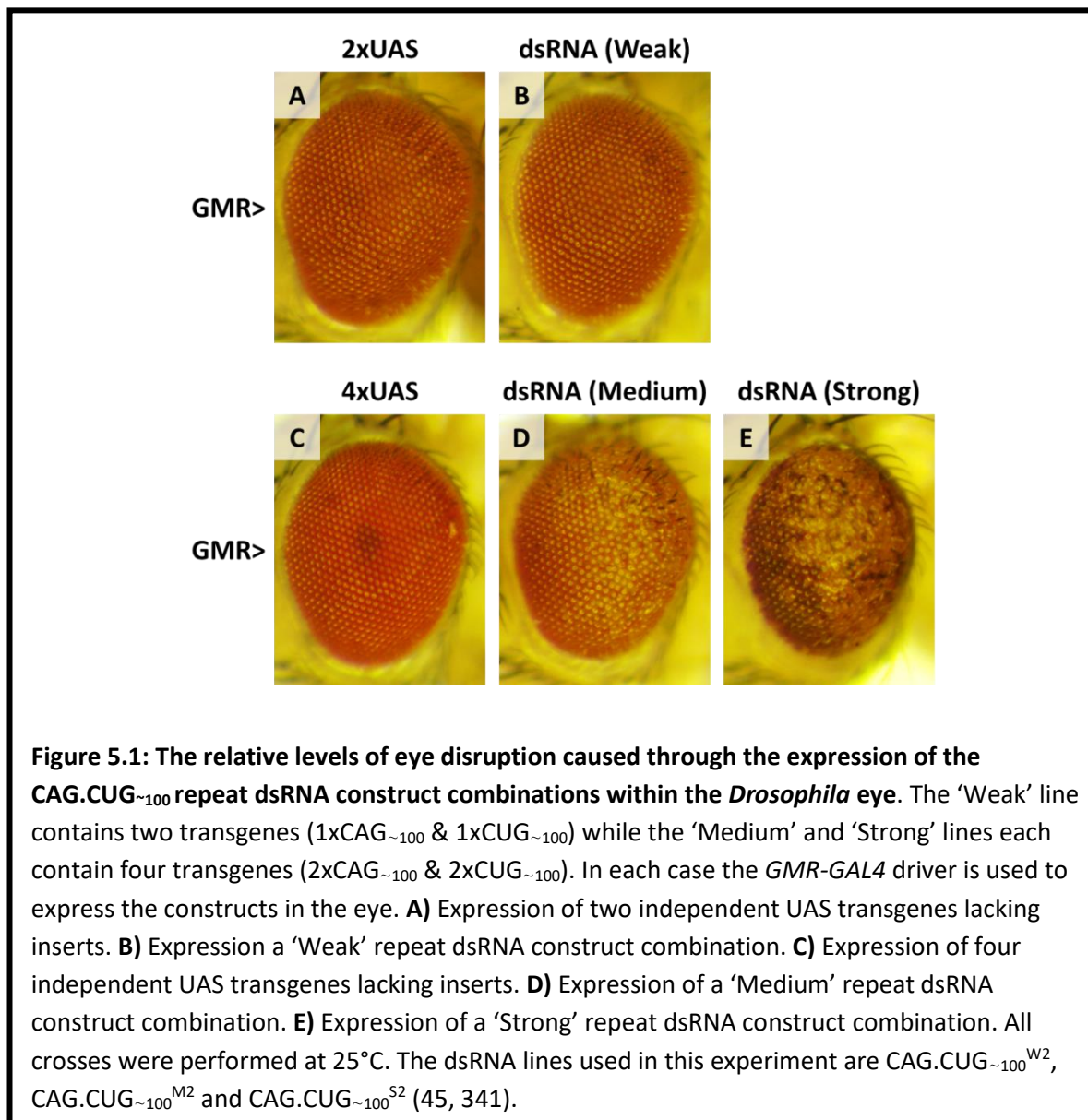
With these considerations in mind, the glial subtype-specific driver lines utilized in this chapter **(Table 5.1)** were selected for their high degree of tissue specificity (292). The data presented here can be thought of as a preliminary investigation using the best available genetic tools, designed to gain an insight into the glial subtypes contributing to the expanded repeat dsRNA-mediated pathology, therefore paving the way for future studies of a more targeted nature.

5.1 Phenotypical confirmation of the expanded repeat dsRNA construct combinations

One advantage of the established expanded repeat dsRNA model (45) is the spectrum of severity of disruptive eye phenotypes caused through the expression of different combinations of repeat transgenic insertions (341). Thus, one line each of the 'Weak', 'Medium' and 'Strong' dsRNA expression phenotypes were selected to be used in this chapter. The independent use of these different phenotypes provides a range of pathological severities with which to interrogate the glial subtypes, potentially highlighting

any toxicity thresholds present. Indeed, pan-glial expression of either a 'Medium' or 'Strong' dsRNA construct combination has been previously shown to cause lethality, while the 'Weak' construct combination is viable but displays a neurodegenerative phenotype and reduced survival (52). Thus, in addition to neurodegeneration, viability and survival can also provide insights into glial cell contributions to the dsRNA pathology.

The independent dsRNA lines were first expressed in an eye-specific manner to confirm their relative pathology **(Figure 5.1)**. Since the 'Weak' line comprises of two repeat transgenes ($1xCAG_{\sim100}$/$1xCUG_{\sim100}$) compared to four transgenes ($2xCAG_{\sim100}$/$2xCUG_{\sim100}$) for both the

Figure 5.1: The relative levels of eye disruption caused through the expression of the CAG.CUG$_{\sim100}$ repeat dsRNA construct combinations within the *Drosophila* eye. The 'Weak' line contains two transgenes ($1xCAG_{\sim100}$ & $1xCUG_{\sim100}$) while the 'Medium' and 'Strong' lines each contain four transgenes ($2xCAG_{\sim100}$ & $2xCUG_{\sim100}$). In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of two independent UAS transgenes lacking inserts. **B)** Expression a 'Weak' repeat dsRNA construct combination. **C)** Expression of four independent UAS transgenes lacking inserts. **D)** Expression of a 'Medium' repeat dsRNA construct combination. **E)** Expression of a 'Strong' repeat dsRNA construct combination. All crosses were performed at 25°C. The dsRNA lines used in this experiment are CAG.CUG$_{\sim100}$[W2], CAG.CUG$_{\sim100}$[M2] and CAG.CUG$_{\sim100}$[S2] (45, 341).

'Medium' and 'Strong' lines, separate UAS construct-containing lines representing the appropriate number of transgenes were used as controls.

Eye-specific expression of the chosen 'Weak' dsRNA construct combination leads to no apparent structural disruptions or loss of pigmentation compared to the 2xUAS control **(Figure 5.1 A-B)**. This result in is agreeance with previously observed results, where no phenotypical effects are produced through the expression of 'Weak' dsRNA construct combinations (341). In contrast, expression of the 'Medium' dsRNA construct combination results in both structural defects and loss of pigmentation compared to the 4xUAS control **(Figure 5.1 C-D)**. In line with this, the 'Strong' dsRNA line enhances this disruption, along with the appearance of black necrotic spots scattered throughout the eye **(Figure 5.1 E)**. Taken together, these results confirm the pathology gradient of the dsRNA construct combinations when expressed within the eye.

5.2 Pan-glial expression of repeat dsRNA is highly pathogenic

To confirm that this pathology gradient is also present in glial cells, the pan-glial driver *Repo-GAL4* was utilized to express the dsRNA construct combinations in order to both recapitulate the previously observed neuronal dysfunction (52) and to serve as a standard to which the contribution of the specific glial subtypes could be assessed. Of note, *Repo-Gal4* expresses in all glial cells except midline glia (347). Midline glia are distinct in that they derive from the meso-ectoderm as opposed to the neuroectoderm and undergo programmed cell death before adulthood (513, 514) and thus will not be discussed here.

In concordance with previous results, pan-glial expression of either the 'Medium' or 'Strong' dsRNA construct combinations both resulted in complete lethality, highlighting the susceptibility of glial cells when challenged with the repeat dsRNA. In contrast, the 'Weak' dsRNA line was viable, allowing the use of the dsRNA-expressing flies in the RING assay to assess locomotor function. An impact on locomotor function was observed in 1 day old 'Weak' dsRNA flies **(Figure 5.2 A)** shown by a significantly decreased climbing ability (p<0.0001) which persisted to at least 8 days (p<0.0001) **(Figure 5.2 B)**. The high level of locomotor dysfunction mirrors previous observations, where pan-glial expression of a

A 1 Day Old

B 8 Days Old

C

D

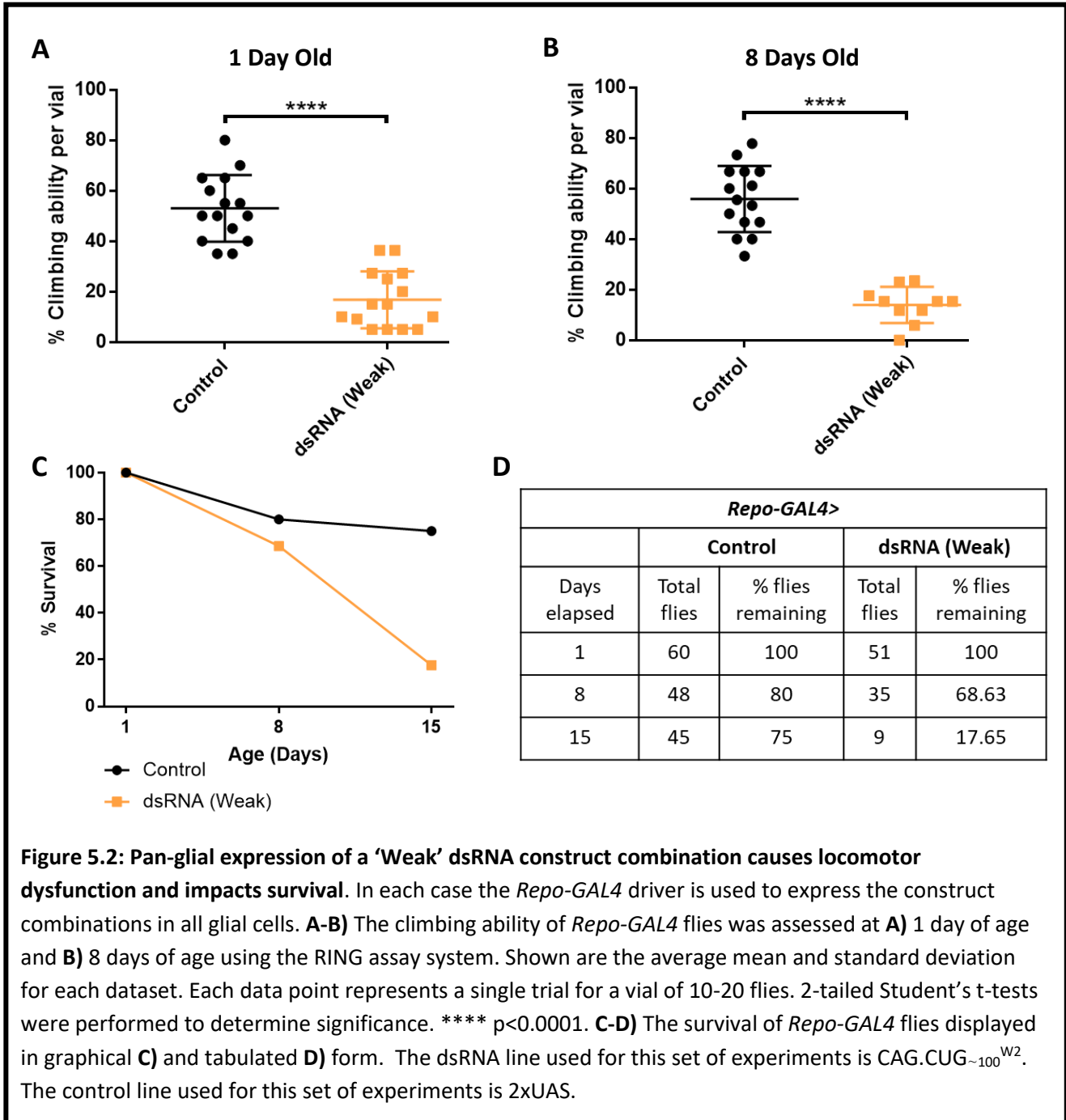| Repo-GAL4>				
	Control		dsRNA (Weak)	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining
1	60	100	51	100
8	48	80	35	68.63
15	45	75	9	17.65

Figure 5.2: Pan-glial expression of a 'Weak' dsRNA construct combination causes locomotor dysfunction and impacts survival. In each case the *Repo-GAL4* driver is used to express the construct combinations in all glial cells. **A-B)** The climbing ability of *Repo-GAL4* flies was assessed at **A)** 1 day of age and **B)** 8 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. ******** $p<0.0001$. **C-D)** The survival of *Repo-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim100}^{W2}$. The control line used for this set of experiments is 2xUAS.

'Weak' dsRNA construct combination did not cause lethality but severely impacted the climbing ability of the affected flies (52). In addition, the 'Weak' dsRNA flies displayed impaired survival compared to control flies **(Figure 5.2 C)**, to the point where only 9 flies (17.65% of the total collected at day 1) remained after 15 days **(Figure 5.2 D)**. As a result, RING assays were unable to be performed on the *Repo-GAL4* flies at 15 days due to low numbers in the dsRNA group.

The high level of neurotoxicity caused through pan-glial expression of repeat dsRNA demonstrates the sensitivity of glial cells to repeat dsRNA. Though pan-neuronal expression of repeat dsRNA also leads to a neurodegenerative phenotype (45), the impact on both locomotor function and viability are reduced in magnitude when compared to that of pan-glial expression. Given that locomotor function is a phenotypical readout of neurons, these observations also highlight glial cells as important non-cell autonomous determinants of dsRNA-mediated neuronal dysfunction.

Following on from successful replication of the severe neurodegenerative phenotype observed through pan-glial expression of the repeat dsRNA, the next stage involved repeating the locomotor assessment of dsRNA-expressing flies using glial subtype-specific GAL4 drivers. The purpose of these experiments was to tease apart the specific *Drosophila* glial subsets and attempt to pair their known biological functions within the CNS and their contribution in driving the dsRNA-mediated neurodegeneration, subsequently providing insight into the potential biological pathways that are rate-limiting in the expanded repeat disease model.

5.3 Subperineural glial expression of repeat dsRNA is pathogenic

Sub-perineural glia (SPG), along with ***perineural glia*** (PG) represent the glial subtypes (also known as surface glia) responsible for the formation of the *Drosophila* blood-brain barrier (BBB), a protective covering designed to separate the central and peripheral nervous systems (PNS) from the circulating hemolymph. In particular, subperineural glia are critical for establishing both diffusive and chemical barriers between the two areas (280, 515). Connecting the large sheet-like subperineural cells are septate junctions, which are

analogous to the tight junctions located between epithelial cells in the vertebrate BBB (280). Subperineural glial cells share common progenitors with perineural and cortex glia within both the larval brain and ventral nerve cord (281, 285). Additionally, within the PNS they arise from several sensory organ progenitors (SOPs), one of which also generates wrapping glial cells (283). During development subperineural glial cells do not proliferate, instead expanding greatly in size in order to connect to one another via septate junctions (280).

Maintaining BBB integrity is recognized as critical to normal neuronal function in both vertebrates and invertebrates; disruption of the barrier allows the migration of circulating systemic molecules and exogenous microbes into the tightly regulated CNS, often leading to detrimental neuroinflammation and subsequent CNS dysfunction (271, 516). As such, breakdown of the BBB has been linked to a number of neurodegenerative diseases, including AD and HD (508). Therefore, the effect of BBB-specific expression of repeat dsRNA was investigated through the use of a GAL4 driver specific to subperineural glia (denoted as *SPG-GAL4*). This driver has been previously shown to be uniformly expressed in all subperineural glia cells with no known off-target expression (292).

Targeted expression of the 'Weak' dsRNA construct combination in subperineural glial cells led to a significant climbing deficiency after 1 day (p=0.0035) **(Figure 5.3 A)** that further progressed at day 15 (p<0.0001) **(Figure 5.3 B)**. However, the observed locomotor dysfunction was not accompanied by a detrimental impact on survival **(Figure 5.3 C & D)**, suggesting that neurodegeneration and survival are not always intrinsically linked to one another as observed with pan-glial expression of the repeat dsRNA **(Figure 5.2)**. To confirm the contribution of subperineural glia to the dsRNA-mediated neuronal pathology, the 'Medium' and 'Strong' dsRNA construct combinations were examined under control of the *SPG>GAL4* driver. Strikingly, subperineural-specific expression of the 'Strong' construct combination resulted in complete lethality, and therefore could not be assessed for neuronal function. However, this result provides an immediate indication of the importance of subperineural glia in mediating the dsRNA pathology. This is supported by the results obtained in flies expressing the 'Medium' dsRNA construct combination in subperineural cells **(Figure 5.4)**. The 'Medium' flies develop a climbing defect that is present after 1 day (p=0.0412) **(Figure 5.4 A)** and is further enhanced at 15 days (p=0.0083) **(Figure 5.4 B)**. In
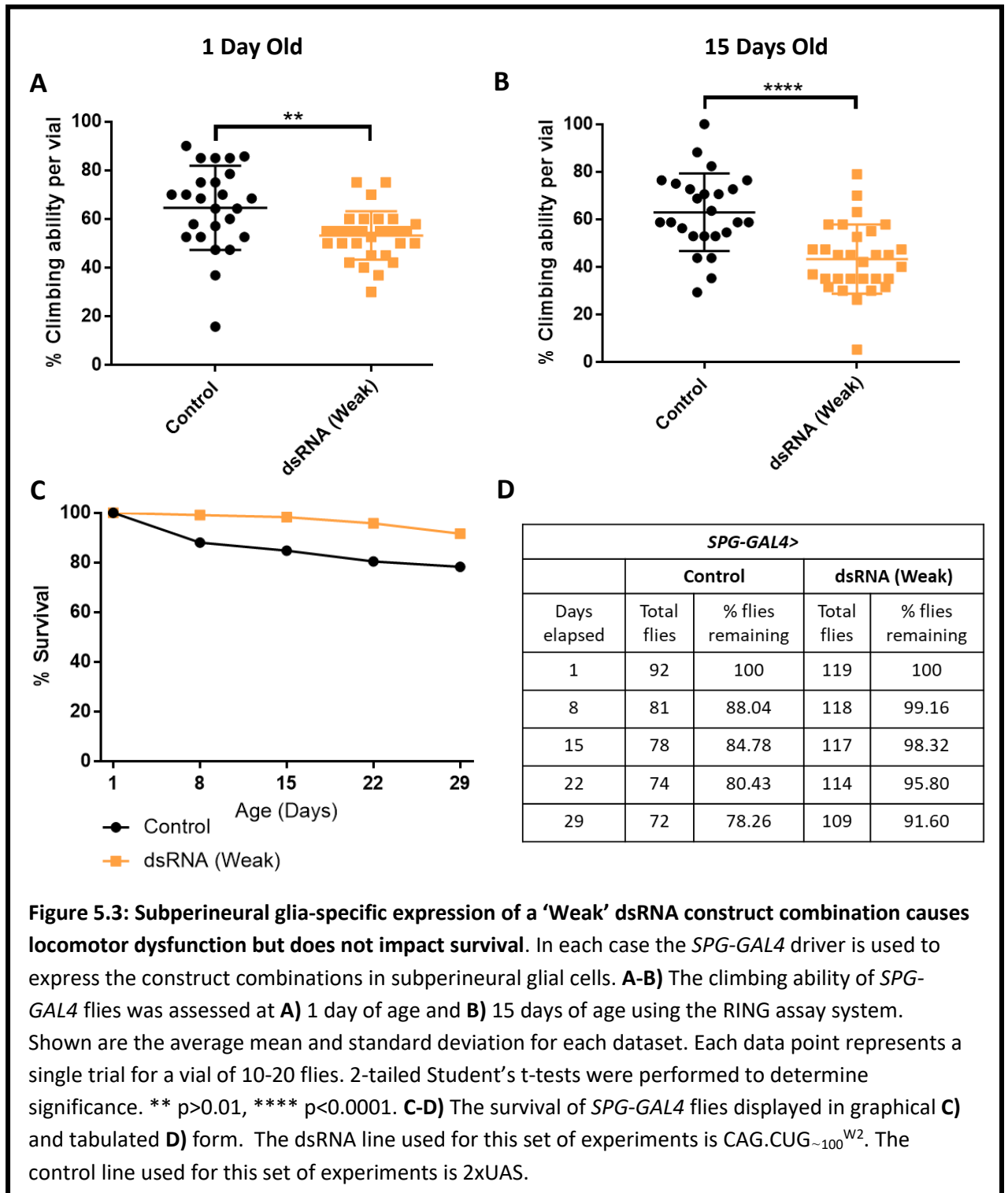
100

1 Day Old

15 Days Old

Figure 5.3: Subperineural glia-specific expression of a 'Weak' dsRNA construct combination causes locomotor dysfunction but does not impact survival. In each case the *SPG-GAL4* driver is used to express the construct combinations in subperineural glial cells. **A-B)** The climbing ability of *SPG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. ** $p > 0.01$, **** $p < 0.0001$. **C-D)** The survival of *SPG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim 100}$^{W2}. The control line used for this set of experiments is 2xUAS.
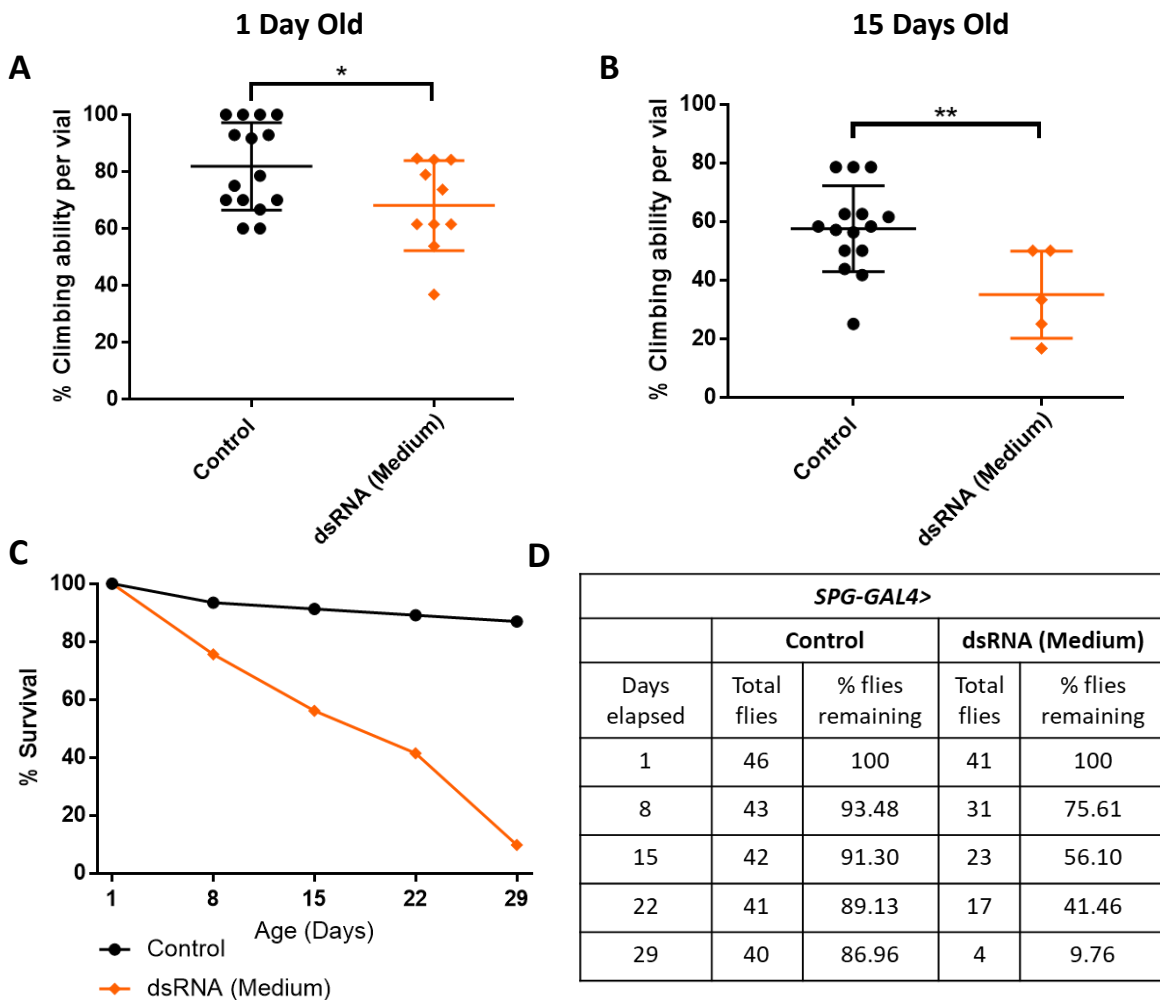
1 Day Old

A

15 Days Old

B

C

D

SPG-GAL4>				
	Control		**dsRNA (Medium)**	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining
1	46	100	41	100
8	43	93.48	31	75.61
15	42	91.30	23	56.10
22	41	89.13	17	41.46
29	40	86.96	4	9.76

Figure 5.4: Subperineural glia-specific expression of a 'Medium' dsRNA construct combination impacts locomotor function and survival. In each case the *SPG-GAL4* driver is used to express the construct combinations in subperineural glial cells. **A-B)** The climbing ability of *SPG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. * $p<0.05$, ** $p<0.01$. **C-D)** The survival of *SPG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim100}$^{M2}. The control line used for this set of experiments is 4xUAS.

addition, the survival of the flies is severely impacted, with only four SPG>dsRNA ('Medium') flies (9.76%) remaining after 29 days **(Figure 5.4 C & D)**. Given the high mortality rate for the 'Medium' flies, it is again possible that the surviving flies represent the healthiest subjects, thus performing to a better standard in the locomotor assay. Regardless, the 'Medium' flies tested still developed a significant decline in climbing ability, further highlighting the rate-limiting role of subperineural glia in the neurodegenerative phenotype. Formation and maintenance of the BBB is essential for protecting the CNS from circulating systemic factors and pathogenic agents, whilst providing a tightly regulated environment to facilitate proper neuronal signalling. As such, it is not surprising that the presence of pathogenic dsRNA specifically within the SPG leads to a high level of neurodegeneration and mortality. It can be speculated that expression of the dsRNA leads to permeabilization of the BBB, allowing in the migration of systemic microbes and immune cells that can both exacerbate inflammation and kill cells within the CNS. This raises the question of whether a non-cell autonomous inflammatory response directed against subperineural cell-localized dsRNA precedes and plays a causal role in BBB breakdown, in addition to the post-BBB breakdown response to circulating factors.

In concordance with these results is recent work investigating the pathology of expanded polyQ tracts within *Drosophila* glial cells. Yeh and colleagues demonstrated that BBB-specific expression of the expanded polyQ disease proteins Ataxin3 (Atxn3) and Huntington (Htt) each cause BBB leakage and restrict lifespan (277). In addition, the polyQ proteins affect BBB development at the early pupal stage, while only a modest impact on permeability was observed after the BBB had already been formed (277). This suggests that disruption to the development of the BBB, as opposed to any disturbance of the fully formed barrier itself, leads to the permeabilization and the associated CNS dysfunction. The work of Yeh *et al* taken together with the results described here indicate that both the RNA and protein gene products derived from repeat expansion lesions initiate CNS dysfunction when expressed within subperineural glial cells. Whether or not the dsRNA affects the BBB in a similar fashion/timepoint to the polyQ disease proteins is unknown, though it can be hypothesised that the dsRNA causes dysfunction during development (given that the neuronal dysfunction was observed from day 1) and with ageing (as the dysfunction worsened with age).

Aside from forming a second BBB layer on top of the subperineural cells, little is known regarding additional functions carried out by perineural glia (517). Thus, whether or not the perineural glia contribute to the dsRNA-mediated pathology should also be examined in future as a method of determining if the strong phenotype observed in subperineural cells forms part of a wider disruption to the BBB or is specific to other functions performed by subperineural glia.

5.4 Wrapping glial expression of repeat dsRNA is pathogenic

Wrapping glia are unique compared to other glial subtypes discussed here in that they are specific to the PNS rather than the CNS. The wrapping glial cells themselves are ensheathed within surface glia that form a peripheral barrier referred to as part of the *Drosophila* BBB, though is more reminiscent of the vertebrate blood-nerve barrier (BNB) (518). The primary function of wrapping glia is to enclose individual axons projecting both into and out of the CNS (280). In this manner, they are roughly homologous to the CNS-localised ensheathing glia (EG), in particular tract-ensheathing glial (TEG) cells. This is supported by the observation that a considerable number of glial-specific GAL4 drivers that express in TEG also display expression in wrapping glia (292). When comparing the *Drosophila* PNS to that of vertebrates, wrapping glia closely resemble the differentiated Schwann cells that form non-myelinating Remak fibres (287, 288). Wrapping glia stem from progenitors in shared with subperineural cells in the ventral nerve cord; the neuro-glioblast NB1-3 and a ventral SOP (vSOP) (283). Also similar to subperineural cells, wrapping glia do not proliferate but lengthen considerably during development to ensheath peripheral nerves (283).

While considered separate entities, the PNS is frequently implicated in CNS-related disorders including PD and ALS (519). Consistent with this, a number of genes studied for their role in *Drosophila* PNS development have been identified as major contributors in models of human neurodegenerative disease (520). Of particular relevance is a study linking the *Drosophila* sensory organ development regulator *senseless* to the pathogenesis of spinocerebellar-ataxia 1 (SCA1), a disease grouped with HD as a CAG-mediated repeat expansion disease (521). Therefore, wrapping glia provide an excellent tissue to investigate potential contributions that the PNS makes in the development of repeat dsRNA pathology.

The driver *nervana2-GAL4* (*nrv2-GAL4*) was selected to drive repeat dsRNA expression within wrapping glia. While initially reported to express in nerve cells (348, 522), further studies revealed that the expression was largely restricted to glial cells (280, 523, 524). Recent studies using the *nrv2-GAL4* driver report strong expression specifically restricted to wrapping glia in addition to residual expression within some subperineural glial cells (288, 289). Thus, while potential non-wrapping glial cell expression of the dsRNA must be factored into the observed results, the *nrv2-GAL4* provides the most specific tool to explore if peripheral dsRNA pathology can non-cell autonomously lead to nerve cell dysfunction/death.

Wrapping glial-driven expression of the 'Weak' dsRNA construct combination did not cause any observable neuronal dysfunction in RING assays performed at 1 day of age (p=0.8176) **(Figure 5.5 A)** and at 8 days of age (p=0.7370) **(Figure 5.5 B)**. In addition, the 'Weak' dsRNA flies displayed a lower rate of mortality over 22 days (85.91% survival) compared with control flies (64.13% survival) **(Figure 5.5 C-D)**. This suggests that either expression of dsRNA within PNS glia is not pathogenic, or that the 'Weak' dsRNA construct combination is not potent enough to pass the threshold required for cellular dysfunction. To further explore this, both the 'Medium' and 'Strong' dsRNA construct combinations were also expressed in a wrapping glia-specific pattern. After 1 day both sets of dsRNA flies were not significantly affected in nerve cell function ('Medium' p=0.2663 & 'Strong' p=0.0626), though the 'Strong' flies did exhibit a trend of reduced climbing ability **(Figure 5.6 A)**. However, after 8 days both the 'Medium' and 'Strong' flies displayed highly significant climbing defects (p<0.0001 & p<0.0001 respectively) **(Figure 5.6 B)**. In addition, a striking decline in survival was observed for both sets of dsRNA-expressing flies between 8-15 days; almost complete mortality was observed **(Figure 5.6 C-D)**. Therefore, the stronger dsRNA construct combinations cause a high level of pathology when expressed in peripheral glia, clearly displaying a non-CNS contribution to neuronal dysfunction.

These results also provide strong evidence of a pathogenic threshold in wrapping glia that, when exceeded, leads to cellular dysfunction. It is plausible that these observations reflect the ability of wrapping glia to effectively respond to and remove minor threats, an ability potentially superseded by a damaging inflammatory response when challenged by a more
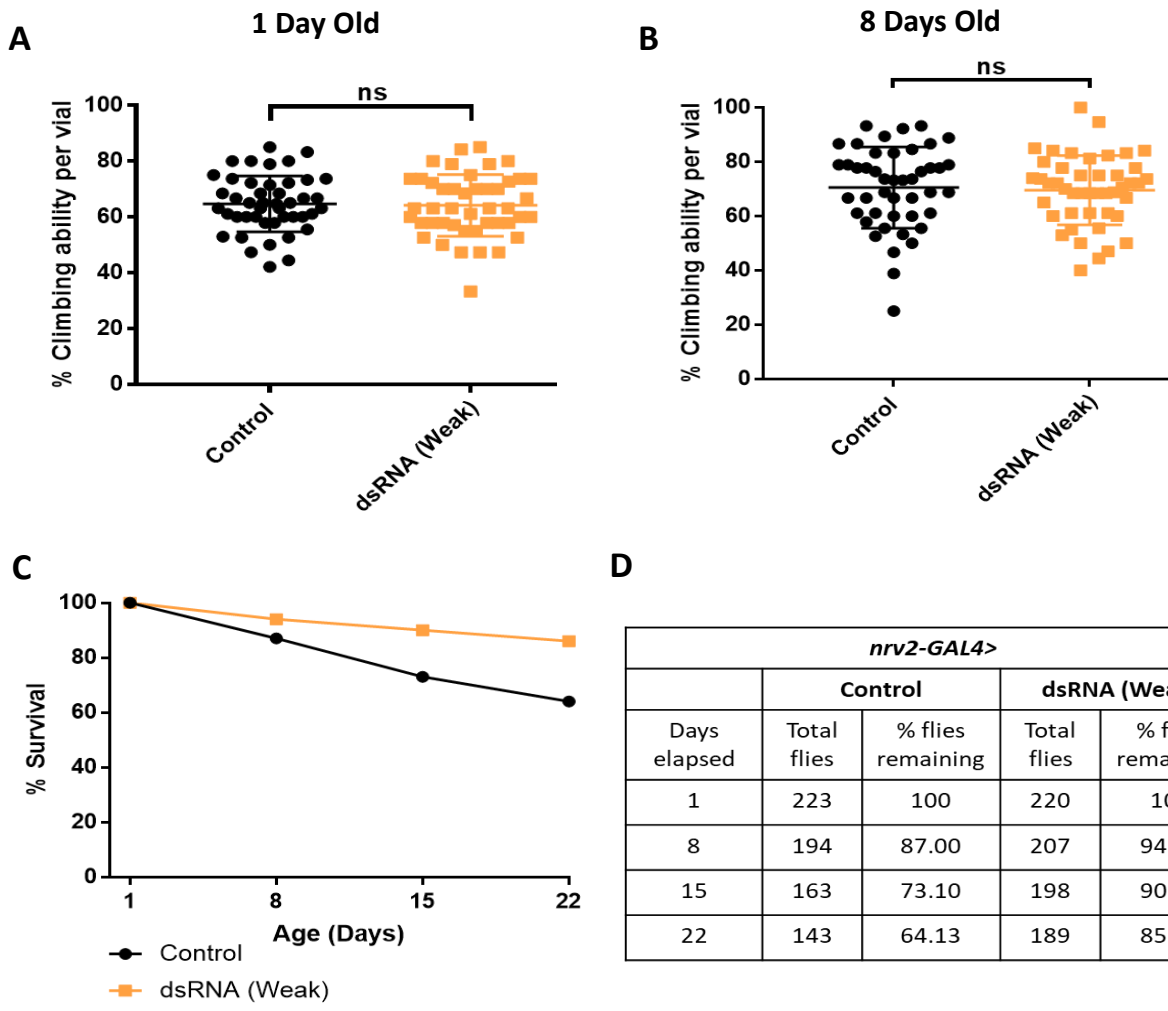
A 1 Day Old

B 8 Days Old

C

D

nrv2-GAL4>				
	Control		**dsRNA (Weak)**	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining
1	223	100	220	100
8	194	87.00	207	94.10
15	163	73.10	198	90.00
22	143	64.13	189	85.91

Figure 5.5: Wrapping glia-specific expression of a 'Weak' dsRNA construct combination does not lead to defects in locomotor function or survival. In each case the *nrv2-GAL4* driver is used to express the construct combinations in wrapping glial cells. **A-B)** The climbing ability of *nrv2-GAL4* flies was assessed at **A)** 1 day of age and **B)** 8 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. ns p>0.05. **C-D)** The survival of *nrv-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA lines used for this set of experiments are CAG.CUG$_{\sim 100}$^{W2}. The control line used for this set of experiments is 2xUAS. The collection of experimental data and analysis was performed under supervision by Karen Raymond.
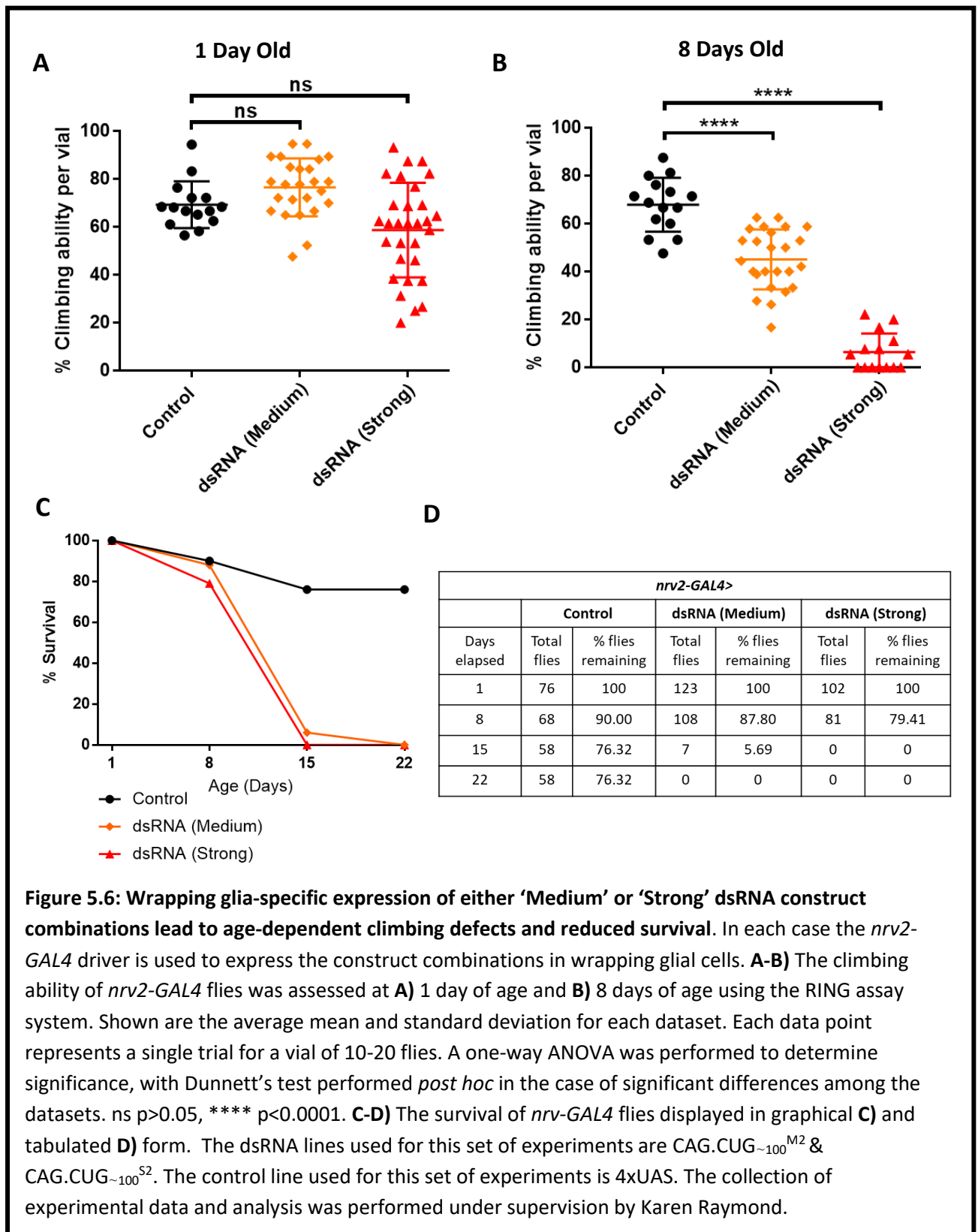
106

Figure 5.6: Wrapping glia-specific expression of either 'Medium' or 'Strong' dsRNA construct combinations lead to age-dependent climbing defects and reduced survival. In each case the *nrv2-GAL4* driver is used to express the construct combinations in wrapping glial cells. **A-B)** The climbing ability of *nrv2-GAL4* flies was assessed at **A)** 1 day of age and **B)** 8 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. A one-way ANOVA was performed to determine significance, with Dunnett's test performed *post hoc* in the case of significant differences among the datasets. ns $p>0.05$, **** $p<0.0001$. **C-D)** The survival of *nrv-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA lines used for this set of experiments are CAG.CUG$_{\sim100}^{M2}$ & CAG.CUG$_{\sim100}^{S2}$. The control line used for this set of experiments is 4xUAS. The collection of experimental data and analysis was performed under supervision by Karen Raymond.

substantial 'non-self' molecule load. However, whether wrapping glia can function in a phagocytic capacity is not well known, though given their close morphological resemblance to CNS ensheathing glia it appears likely that this could be a shared function (292). In addition, wrapping glia-derived signalling is critical for both the proliferation of perineural glia and the expansion of septate junction contacts between subperineural glial cells, highlighting their important role in BBB formation (289). Therefore, the expression of dsRNA within wrapping glia may lead to disruptions to BBB integrity, allowing systemically-derived molecules and cells to enter the nervous system where they can contribute to the inflammatory response.

Notably, these results are similar to the outcomes observed with subperineural glial-specific expression of the dsRNA **(Section 5.3)**, further supporting the idea that dsRNA-mediated BBB dysfunction plays a key role in the glial-derived phenotypes in this model. Based on previous observations using *nrv2-GAL4* (288), it is possible that the dsRNA construct combinations may be expressed weakly in subperineural glia when using this driver and that this expression could contribute to the pathology in the 'Medium' and 'Strong' lines **(Figure 5.6)**. Observations that the 'Weak' dsRNA construct combination is pathogenic in subperineural glia **(Figure 5.3)** but not in wrapping glia **(Figure 5.5)** suggest it is unlikely that this low level of residual expression would be sufficient to cause any form of cellular dysfunction, though this would require further confirmation. If indeed dsRNA-mediated breakdown of the BBB is a common pathogenic mechanism in both subperineural and wrapping glial cells, the stronger phenotypes observed in the *SPG*>dsRNA flies may represent the fact that the BBB surrounding both the brain and the peripheral nerves are affected as opposed to only peripheral nerves in *nrv2*>dsRNA flies.

Whether the high mortality observed in 'Medium' and 'Strong' dsRNA lines is a consequence of significant neuronal dysfunction or a separate systemic mechanism is unknown. However, ubiquitous expression of the 'Weak', 'Medium' and 'Strong' dsRNA construct combinations all cause complete lethality (data not shown). This suggests that the mortality observed here stems from neuronal dysfunction, given that *nrv2*>dsRNA flies are able to successfully emerge from pupation.

Taken together, these results demonstrate that peripheral wrapping glia are able to negatively influence CNS function when challenged with repeat dsRNA, potentially through the inhibition of correct BBB formation and the subsequent infiltration of systemically-derived cells and molecules/danger signals into the CNS.

5.5 Ensheathing glial expression of repeat dsRNA is pathogenic or mildly neuroprotective based on dsRNA construct combination

Ensheathing glia (EG) represent the most morphologically diverse glial subtype; they are the first of the glial subtypes able to infiltrate the neuropil, their flattened cell bodies extending to form boundaries to isolate distinct neuropil compartments (279, 285). In addition, an anatomically distinct form of ensheathing glia appear to wrap axon tracts as they project from the neuropil, similar in morphology to peripheral wrapping glia that ensheath nerve fibres outside of the CNS (292). Both ensheathing and astrocyte-like glia (collectively termed as neuropil glia) share common progenitors during embryogenesis in both the brain and ventral nerve cord (285). Embryonically-derived neuropil glia (primary glia) are unique in that they undergo cell death during pupal development, followed by the generation of adult (secondary) neuropil glia through a second wave of gliogenesis (291). Whether all secondary neuropil glia derive from common progenitors is not well understood.

The role of ensheathing glia in a phagocytic capacity within the *Drosophila* CNS has been well documented, including the engulfment of axonal debris following injury (293) and removal of apoptotic neuronal cells during development (525). Ensheathing glia express components of the Draper pathway that are vital for recognition and glial engulfment of neuronal debris (293). This process is tightly regulated by differentially spliced Draper isoforms that can either promote axonal engulfment or terminate the phagocytic activity to restore the glial cells to a resting state (351). The age-related decline of Draper translation observed in the ensheathing glia of aged flies leads to a striking reduction in phagocytic responses (296). Importantly, neurodegeneration has been demonstrated to be an outcome of defective Draper-mediated phagocytic activity (297, 308), highlighting the importance of ensheathing glia function in the context of neurodegenerative disease. Thus, expression of

the repeat dsRNA was investigated in ensheathing cells to determine if their dysfunction formed part of the pathology in *Repo*>dsRNA flies.

Of note, the glial cell specific GAL4 driver screen performed by Kremer *et al.* did not identify an expression pattern that was specific to all (neuropil-associating and tract-associating) ensheathing glial cells, potentially because such a driver may not exist (292). As a result, two separate EG-specific lines were used for this set of experiments; *R75H03-GAL4* (*TEG*-GAL4) drives expression in **tract ensheathing glia** (TEG) specifically, while *R56F03-GAL4* (*NEG-GAL4*) drives expression specifically within **neuropil ensheathing glia** (NEG) and a small population of TEG cells (292). Therefore, the results have been interpreted in the knowledge that not all ensheathing glia are expressing the repeat dsRNA in each case.

Using the tract ensheathing glia-specific driver to express the 'Weak' dsRNA construct combination in flies led to a significant reduction in climbing ability after 1 day (p=0.0001) **(Figure 5.7 A)** that persisted at day 15 (p=0.0001) **(Figure 5.7 B)**. In addition, *TEG*>dsRNA ('Weak') flies experienced a decreased rate of survival (45.30% survival at day 29) compared to the control flies (83.48% survival at day 29) **(Figure 5.7 C-D)**. Thus, the results show that tract ensheathing glia-specific expression of a 'Weak' dsRNA construct combination caused detrimental impacts on both neuronal function and survival, suggesting that tract-associating ensheathing glia contribute to the dsRNA-mediated pathology.

To confirm the participation of tract ensheathing glia in the neurodegenerative phenotype, either 'Medium' or 'Strong' dsRNA construct combinations were also expressed using the *TEG-GAL4* driver. Interestingly, while the 'Strong' dsRNA flies displayed a climbing defect at day 1 (p=0.0080), the 'Medium' flies displayed improved climbing ability compared to the control (p=0.0001) **(Figure 5.8 A)**. The 'Medium' line continued to display improved climbing ability at day 15 (p=0.0011), while remarkably the 'Strong' flies also significantly improved compared to the control (p=0.0332) **(Figure 5.8 B)**. Survival of both of the dsRNA lines were not noticeably impacted compared to the control, though any potential impact on survival could be masked by the poor survival of the control line **(Figure 5.8 C-D)**. Thus, in tract ensheathing glia, the 'Weak' dsRNA construct combination causes a higher level of pathology than either of the stronger dsRNA construct combinations. This contrasts with the
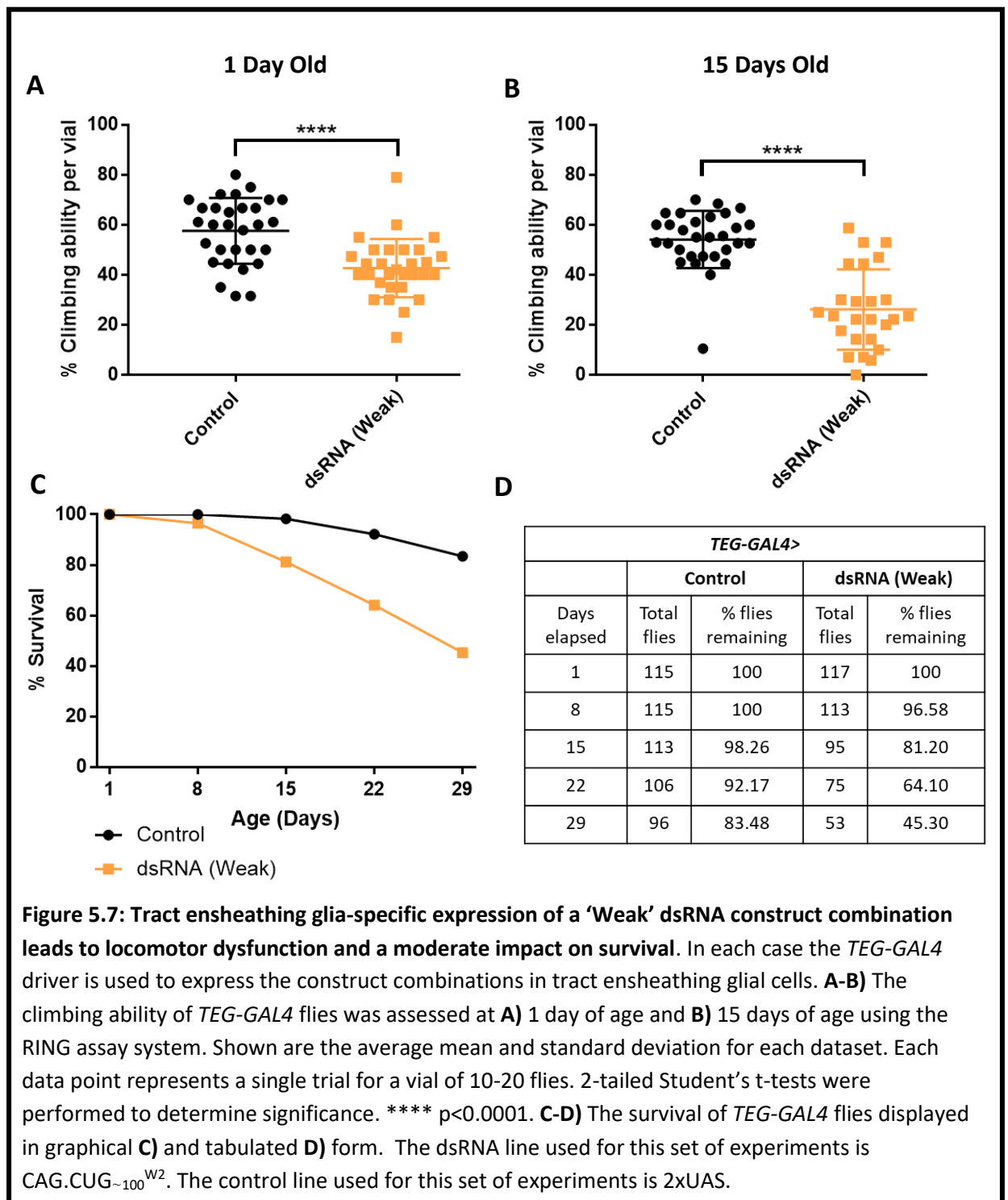
A 1 Day Old

B 15 Days Old

C

D

TEG-GAL4>				
	Control		dsRNA (Weak)	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining
1	115	100	117	100
8	115	100	113	96.58
15	113	98.26	95	81.20
22	106	92.17	75	64.10
29	96	83.48	53	45.30

Figure 5.7: Tract ensheathing glia-specific expression of a 'Weak' dsRNA construct combination leads to locomotor dysfunction and a moderate impact on survival. In each case the *TEG-GAL4* driver is used to express the construct combinations in tract ensheathing glial cells. **A-B)** The climbing ability of *TEG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. **** $p<0.0001$. **C-D)** The survival of *TEG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim100}$^{W2}. The control line used for this set of experiments is 2xUAS.
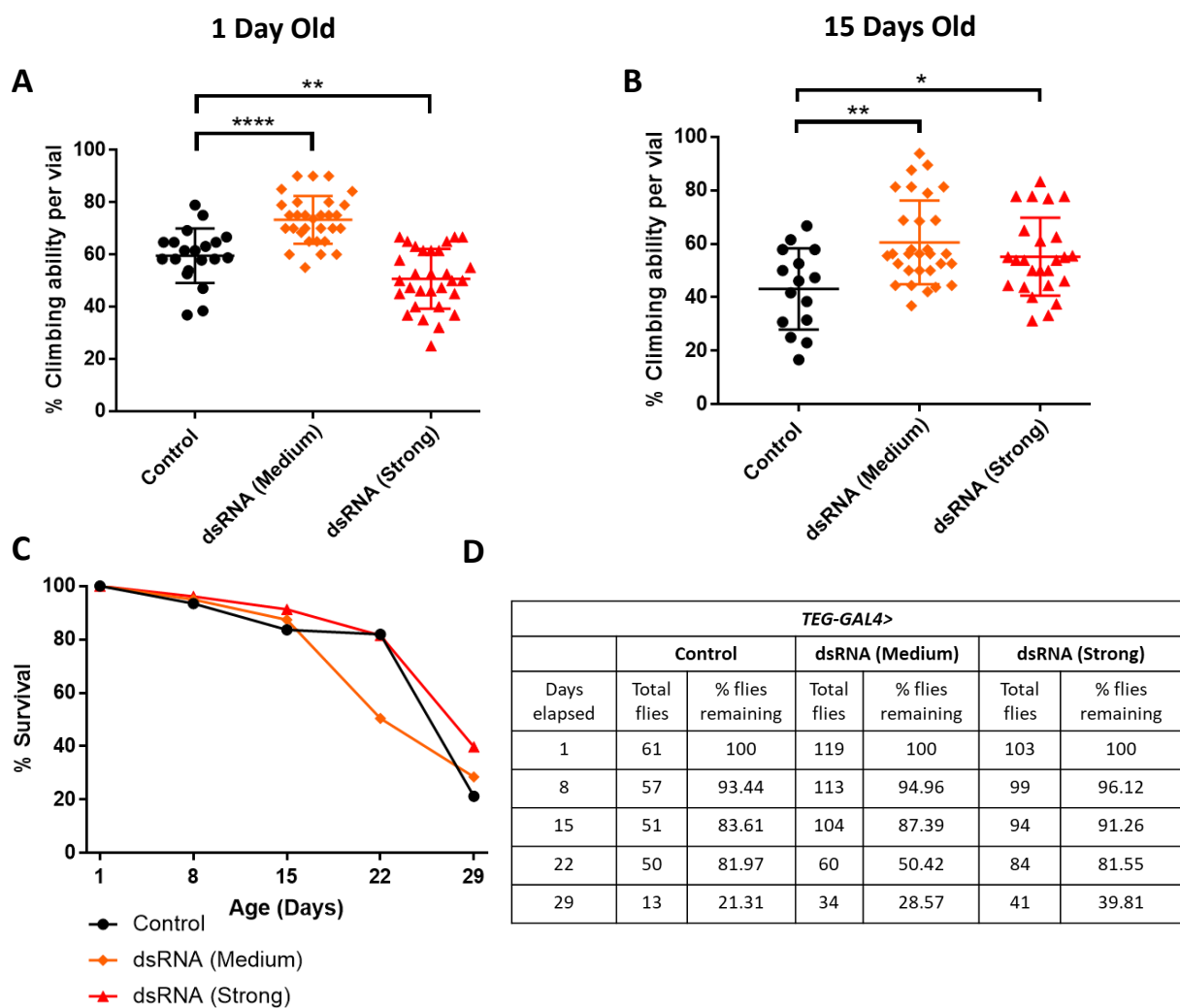
1 Day Old

15 Days Old

Figure 5.8: Tract ensheathing glia-specific expression of either 'Medium' or 'Strong' dsRNA construct combinations cause varied effects on locomotor function whilst not impacting survival. In each case the *TEG-GAL4* driver is used to express the construct combinationss in tract ensheathing glial cells. **A-B)** The climbing ability of *TEG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. A one-way ANOVA was performed to determine significance, with Dunnett's test performed *post hoc* in the case of significant differences among the datasets. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. **C-D)** The survival of *TEG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA lines used for this set of experiments are $CAG.CUG_{\sim100}^{M2}$ & $CAG.CUG_{\sim100}^{S2}$. The control line used for this set of experiments is 4xUAS.

pathology gradient observed in *SPG*>dsRNA flies **(Figures 5.3 & 5.4)**, demonstrating how different glial cell subtypes are affected by the presence of 'non-self' molecules.

To determine if this phenomenon is specific to tract ensheathing glia or encompasses the broader ensheathing glial cell morphologies, the dsRNA construct combinations were expressed specifically in the neuropil-associating EG (NEG) using the *NEG-GAL4* driver. In concordance with the *TEG*>dsRNA ('Weak') findings, NEG-specific expression of the 'Weak' construct combination led to a significant reduction in climbing ability at day 1 (p<0.0001) **(Figure 5.9 A)** that persisted to day 15 (p<0.0001) **(Figure 5.9 B)**. Survival was not impacted compared to the control **(Figure 5.9 C-D)**, though again the control line displayed reduced survival. Given the consistent climbing defects observed in flies expressing the 'Weak' dsRNA construct combination in either tract or neuropil ensheathing glia, it appears that repeat dsRNA causes dysfunction in these cells using weaker construct combinations.

However, a consistent gradient of dsRNA-mediated pathology (Weak<Medium<Strong) is not observed; NEG-specific expression of the 'Medium' construct combination displays improved climbing ability compared to the control (p=0.0001), while climbing improvement is also observed in the 'Strong' dsRNA flies (p=0.0376) **(Figure 5.10 A)**. However, an age-related decline in climbing ability is observed in both sets of dsRNA flies, whereby the 'Medium' and 'Strong' flies no longer display any significant climbing improvement compared to the control (p=0.1356 & p=0.7064 respectively) **(Figure 5.10 B)**. In addition, the 'Strong' dsRNA flies display a reduction in survival, though this appears to predominantly occur after the final RING assay timepoint of day 15, while the 'Medium' flies are not impacted **(Figure 5.10 C-D)**. The lack of pathology in the stronger *NEG*>dsRNA lines is consistent with the results observed using the *TEG-GAL4* driver **(Figure 5.8)**, suggesting that this phenomenon is common to all ensheathing glia as opposed to specific morphologies. Supporting this is the finding in Kremer *et al* that the majority of ensheathing glia-specific GAL4 lines expressed to some degree in both tract and neuropil ensheathing glia, suggesting that many commonalities exist between the distinct ensheathing glial morphologies (292).
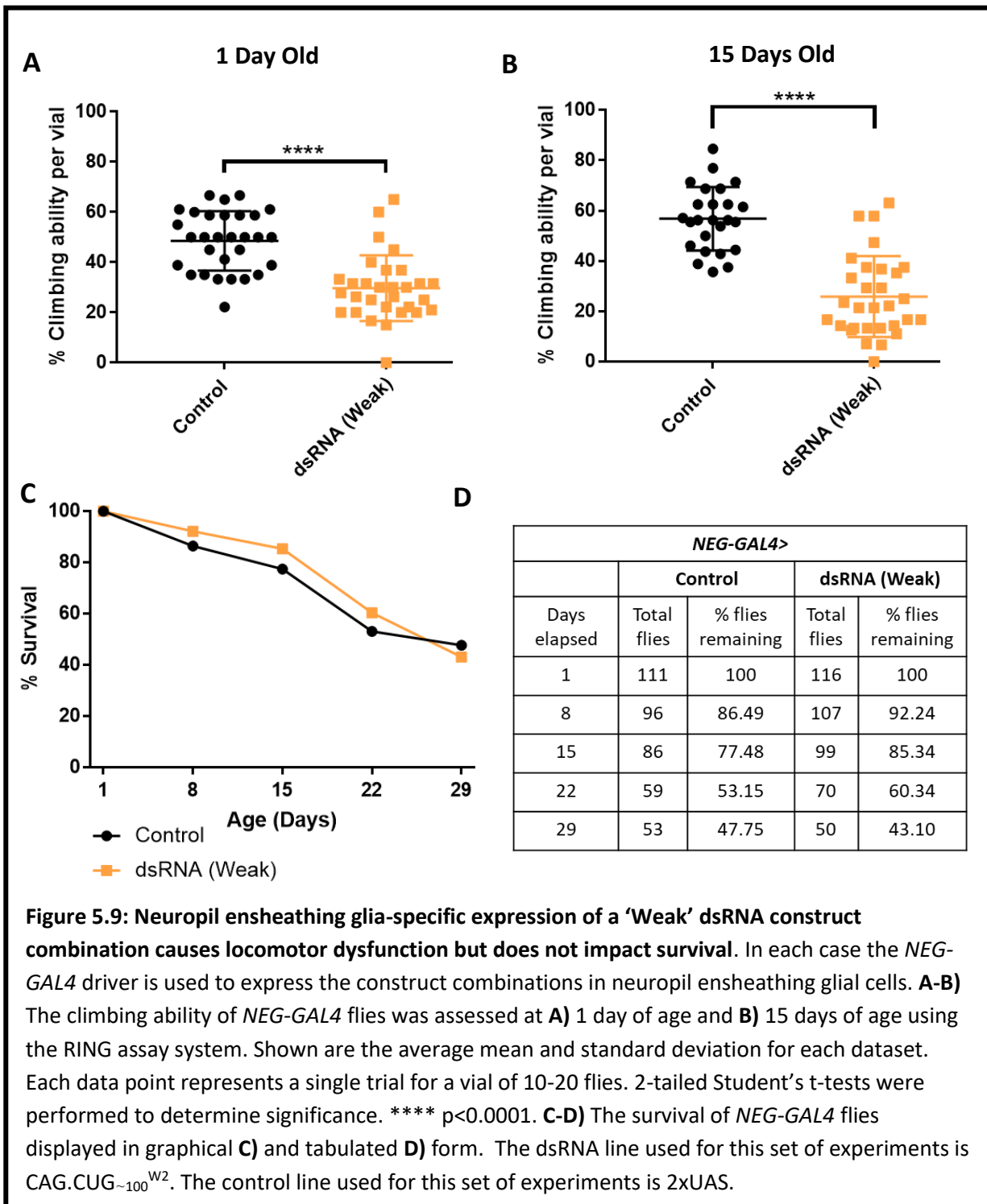
A 1 Day Old

B 15 Days Old

C

D

	NEG-GAL4>				
		Control		dsRNA (Weak)	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining
1	111	100	116	100
8	96	86.49	107	92.24
15	86	77.48	99	85.34
22	59	53.15	70	60.34
29	53	47.75	50	43.10

Figure 5.9: Neuropil ensheathing glia-specific expression of a 'Weak' dsRNA construct combination causes locomotor dysfunction but does not impact survival. In each case the *NEG-GAL4* driver is used to express the construct combinations in neuropil ensheathing glial cells. **A-B)** The climbing ability of *NEG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. **** $p < 0.0001$. **C-D)** The survival of *NEG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim 100}^{W2}$. The control line used for this set of experiments is 2xUAS.
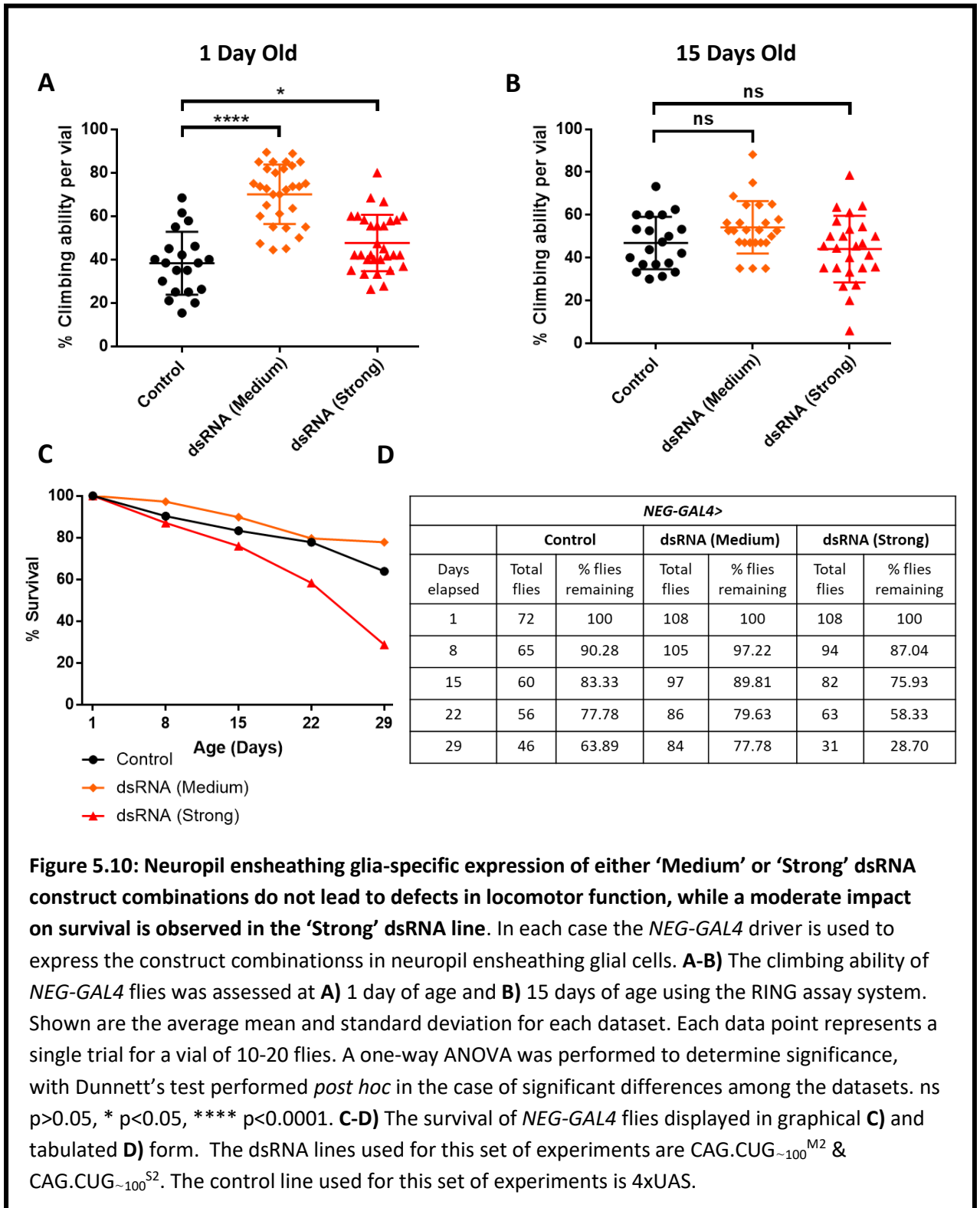
Figure 5.10: Neuropil ensheathing glia-specific expression of either 'Medium' or 'Strong' dsRNA construct combinations do not lead to defects in locomotor function, while a moderate impact on survival is observed in the 'Strong' dsRNA line. In each case the *NEG-GAL4* driver is used to express the construct combinationss in neuropil ensheathing glial cells. **A-B)** The climbing ability of *NEG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. A one-way ANOVA was performed to determine significance, with Dunnett's test performed *post hoc* in the case of significant differences among the datasets. ns $p > 0.05$, * $p < 0.05$, **** $p < 0.0001$. **C-D)** The survival of *NEG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA lines used for this set of experiments are CAG.CUG$_{\sim 100}^{M2}$ & CAG.CUG$_{\sim 100}^{S2}$. The control line used for this set of experiments is 4xUAS.

The observation with both ensheathing glia drivers in that the 'Weak' dsRNA construct combination displays a higher degree of pathology than both the 'Medium' and 'Strong' construct combinations raises the question as to whether the neuroprotective functions of ensheathing glia are upregulated to scale with the magnitude of the perceived threat or injury. This idea is supported by recent work showing that STAT92E-mediated feed-forward signalling of the Draper phagocytic pathway is amplified and becomes more widespread across glial cells within the *Drosophila* CNS in response to the severity of axonal injury (295). Therefore, it is plausible that the stronger dsRNA lines induce an enhanced phagocytic response that is capable of removing/degrading the 'non-self' dsRNA and subsequently avoiding glial dysfunction. In contrast the 'Weak' dsRNA construct combination may only induce a modest phagocytic response that is unsuccessful in removing the threat, leading to a persistent inflammatory response that is damaging within the CNS. It must be noted that the reported scaled phagocytic response is through the detection of axonal injury rather than glial dysfunction (295), though the possibility exists that ensheathing glia are able to recognize the 'non-self' dsRNA on neighbouring glia of the same subtype and act accordingly. While *Drosophila* glia show limited physical interactions between both cells of the same subtype and neighbouring subtypes (292), a non-cell autonomous signalling mechanism could allow neighbouring glial cells to assist in the clearance of dysfunctional cells. Whether this process could involve other glial subtypes is not certain; neuropil ensheathing glia form defined boundaries with cortex glia and surround astrocyte glial cell bodies, while astrocyte processes have also been observed projecting into the layer of ensheathing glia within the neuropil (292).

Recent work has also demonstrated the important role of Draper-mediated phagocytosis in neuropil glia; facilitating the removal of apoptotic neuronal bodies during pupal metamorphosis (525). Thus, an alternative plausible explanation is that the stronger construct combinations induce apoptosis in the affected cells which leads to their subsequent removal, minimizing tissue damage. Conversely, the 'Weak' dsRNA construct combination-derived dsRNA may not warrant the induction of phagocytic and apoptotic machinery, and as such is able to persist and cause problems that manifest in adulthood as neuronal dysfunction.

Taken together, the ensheathing glia appear to be able to resist the pathology associated with the presence of the stronger dsRNA construct combinations, whilst expression of a 'Weak' dsRNA construct combination causes ensheathing glial dysfunction and subsequent neurodegeneration. While the mechanism(s) regarding this disparity between the dsRNA construct combinations is currently unknown, the ability of glial cells to detect and scale the magnitude of their phagocytic response to 'non-self' material may play a key role. In addition, this process may involve communication between distinct glial subtypes. Recent experimental work has demonstrated the requirement of an axon-derived degeneration cues to induce Draper-mediated signalling (526); whether damaged ensheathing glial cells (and indeed any subtype of glial cells) are able to produce similar 'eat me' signals to trigger phagocytic activity from neighbouring glia is also not well understood. Temporal control of the ensheathing glia-specific drivers using an inducible inhibitor of GAL4 such as GAL80 could also be considered to determine the critical timepoint at which the 'non-self' repeat dsRNA is either removed or allowed to persist.

5.6 Astrocyte-like glial expression of repeat dsRNA is pathogenic or mildly neuroprotective based on dsRNA construct combination

Astrocyte-like glia (ALG) comprise the second *Drosophila* glial subtype that associates with the neuropil; infiltrating the region with dense branch-like processes (279, 298). As previously discussed, primary both astrocyte-like and ensheathing glia derive from common progenitors during embryogenesis before undergoing cell death, after which secondary neuropil glia are generated via a set of uncharacterised precursors (291). Recent work has demonstrated the requirement of Notch pathway signalling for the differentiation of newly generated neuropil glia into astrocytes in the larval ventral nerve cord (527), while it is unclear what signalling pathways control the differentiation of secondary neuropil glia into the adult astrocyte-like and ensheathing glia (528).

The highly ramified structure of *Drosophila* astrocyte-like cells bears a striking morphological resemblance to mammalian astrocytes, in addition to sharing a number of functional similarities including synaptic remodelling and neurotransmission (160, 298). Furthermore, astrocyte-like glia have also been demonstrated to display phagocytic activity during

Drosophila development; steroid signalling prompts larval astrocytes to transform into *bona fide* phagocytes capable of both pruning neurons and eliminating neuronal debris (302). Interestingly, astrocyte-like glia do not appear to act in this manner in the adult *Drosophila* brain; they lack expression of key engulfment effectors *Drpr/dCed6* and do not respond to neuronal injury (293). However, work in mouse has shown that mammalian astrocytes continue to display phagocytic activity in the adult CNS, in part through MEGF10, the orthologue of the *Drosophila* Draper (188).

In order to investigate if the presence of 'non-self' repeat dsRNA can affect astrocyte function and thus contribute to the observed pan-glial pathology observed **(Figure 5.2)**, the astrocyte-like glia-specific *R86E01-GAL4* (*ALG-GAL4*) driver line was obtained. ALG-specific expression of the 'Weak' dsRNA construct combination led to a significant reduction in climbing ability at day 1 compared to the control (p<0.0001) **(Figure 5.11 A)** that persisted through to day 15 (p<0.0001) **(Figure 5.11 B)**. However, the survival of the *ALG*>dsRNA ('Weak') flies was not affected, displaying a similar survival trajectory to the control **(Figure 5.11 C-D)**. The loss of climbing ability without a decline in survival suggests that the presence of the 'Weak' dsRNA construct combination in astrocyte-like glia may be adequate to cause neuronal dysfunction but not of a pathogenic level to cause mortality. Similar results were observed when the weak line was expressed specifically within subperineural **(Figure 5.3)** and neuropil ensheathing glia **(Figure 5.9)**, while the 'Weak' line expressed in tract ensheathing glia caused increased mortality in addition to a climbing defect **(Figure 5.7)**.

Conversely, expression of either the 'Medium' or 'Strong' dsRNA construct combinations did not lead to climbing dysfunction; rather the 'Medium' displayed significantly improved climbing ability compared to the control (p=0.0062) whereas the 'Strong' line was not affected (p=0.0687) **(Figure 5.12 A)**. At 15 days the 'Medium' line maintained a climbing improvement (p=0.0016), while the 'Strong' line again displayed no difference compared to the control (p=0.2998) **(Figure 5.12 B)**. Interestingly, both dsRNA construct combinations were severely impacted in terms of survival **(Figure 5.12 C-D)**. However, because the increased mortality occurred after the day 15 RING assay timepoint, the potential death of
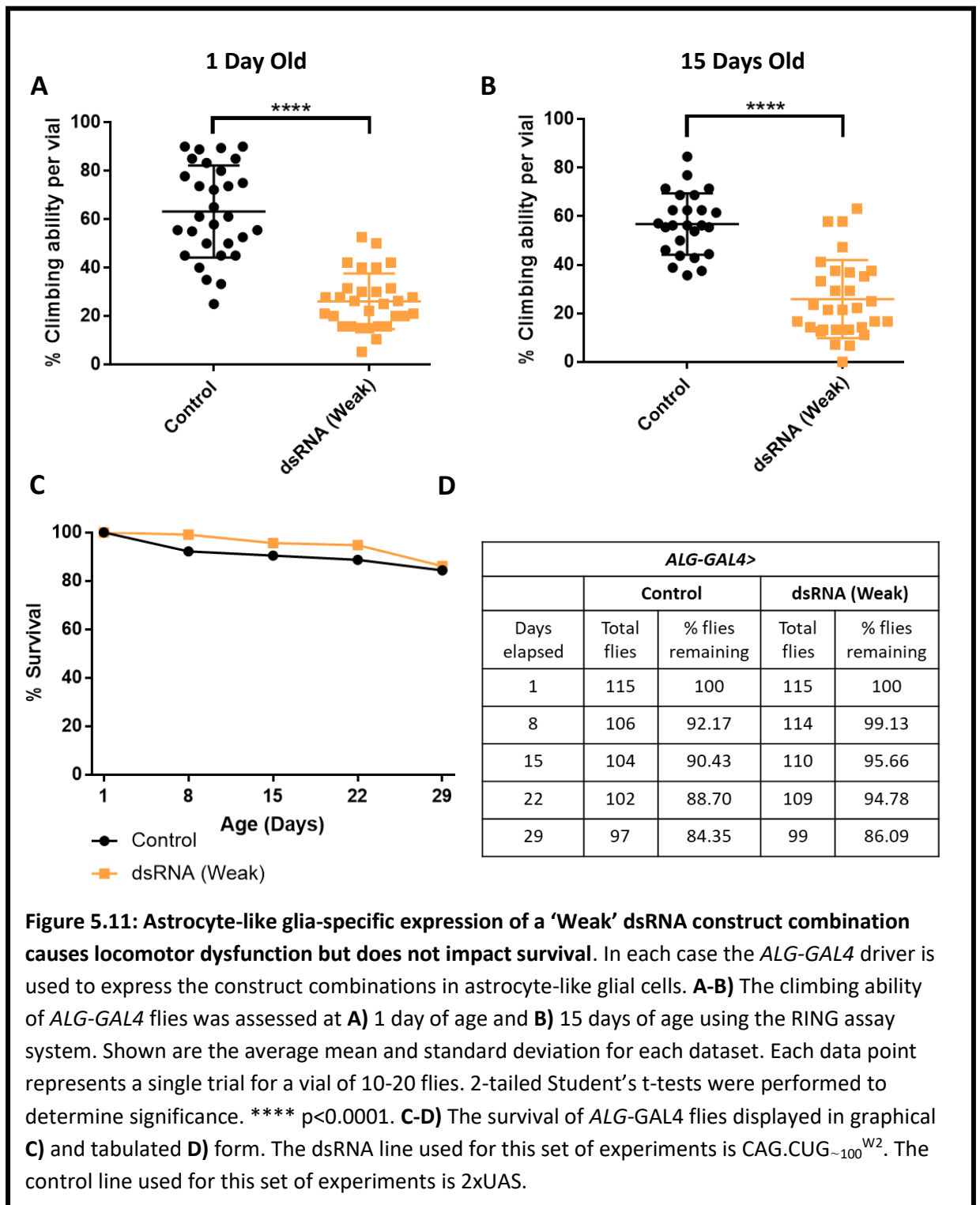
Figure 5.11: Astrocyte-like glia-specific expression of a 'Weak' dsRNA construct combination causes locomotor dysfunction but does not impact survival. In each case the *ALG-GAL4* driver is used to express the construct combinations in astrocyte-like glial cells. **A-B)** The climbing ability of *ALG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. **** $p<0.0001$. **C-D)** The survival of *ALG*-GAL4 flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim 100}^{W2}$. The control line used for this set of experiments is 2xUAS.
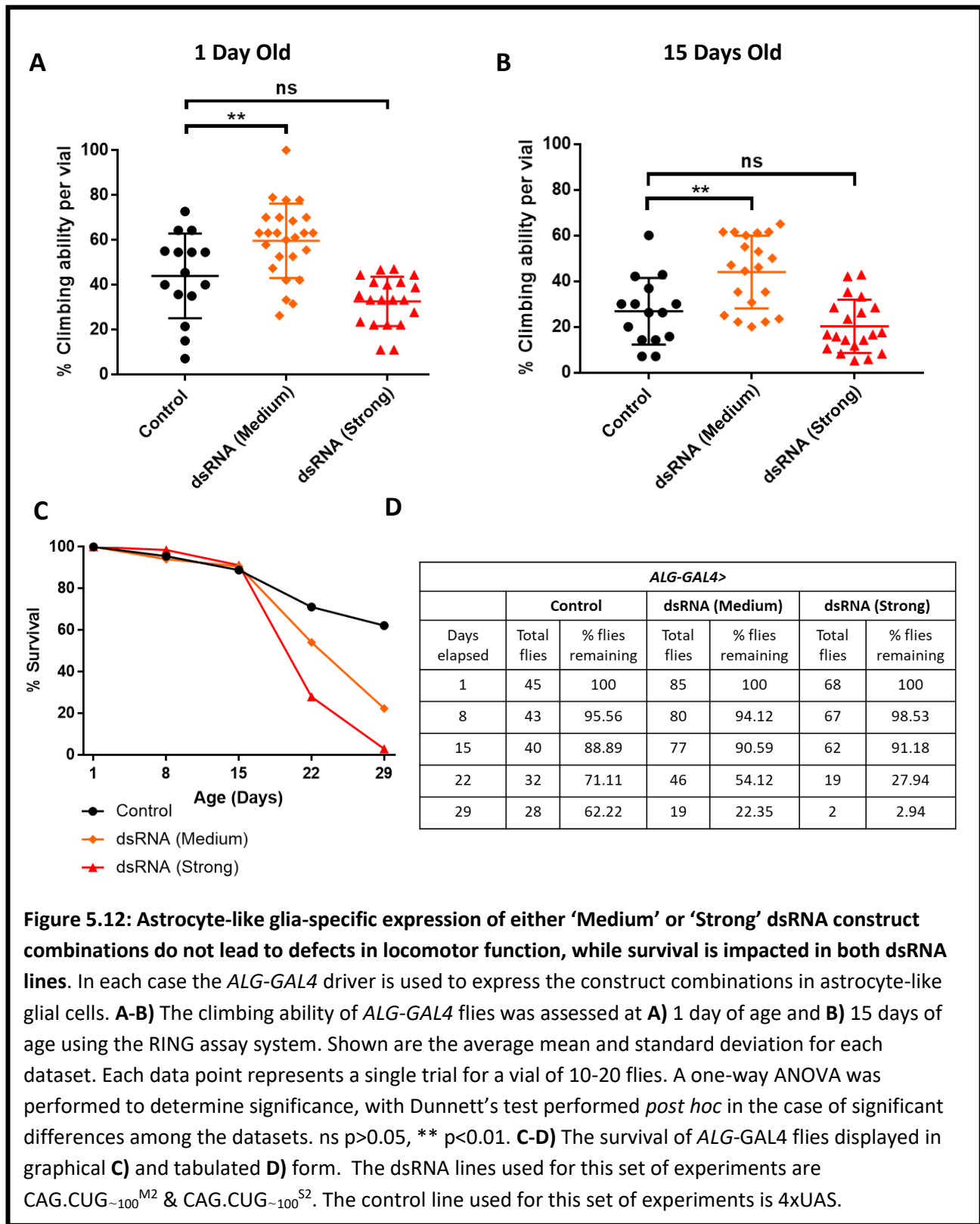
A 1 Day Old

B 15 Days Old

C

D

ALG-GAL4>						
	Control		dsRNA (Medium)		dsRNA (Strong)	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining	Total flies	% flies remaining
1	45	100	85	100	68	100
8	43	95.56	80	94.12	67	98.53
15	40	88.89	77	90.59	62	91.18
22	32	71.11	46	54.12	19	27.94
29	28	62.22	19	22.35	2	2.94

Figure 5.12: Astrocyte-like glia-specific expression of either 'Medium' or 'Strong' dsRNA construct combinations do not lead to defects in locomotor function, while survival is impacted in both dsRNA lines. In each case the *ALG-GAL4* driver is used to express the construct combinations in astrocyte-like glial cells. **A-B)** The climbing ability of *ALG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. A one-way ANOVA was performed to determine significance, with Dunnett's test performed *post hoc* in the case of significant differences among the datasets. ns $p>0.05$, ** $p<0.01$. **C-D)** The survival of *ALG*-GAL4 flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA lines used for this set of experiments are CAG.CUG$_{\sim100}$^{M2} & CAG.CUG$_{\sim100}$^{S2}. The control line used for this set of experiments is 4xUAS.

the most severely affected flies is not a factor when assessing the climbing performance of the 'Medium' and 'Strong' flies.

The *ALG*>dsRNA RING assay results presented bear a striking similarity to those obtained when using the ensheathing glia drivers **(Section 5.5)**, namely the loss of climbing ability in flies expressing the 'Weak' dsRNA construct combination and the somewhat paradoxical improvement/lack of decline in flies expressing the more potent 'Medium' or 'Strong' construct combinations. This raises the question of whether scaled phagocytic signalling is a shared mechanism amongst all neuropil glia. Given that astrocyte-driven CNS debris clearance appears to be restricted to larval development (293, 302), this scenario would implicate the developmental phase as the critical stage at which the dsRNA can either be degraded effectively or persist into adulthood and instigate cellular dysfunction. Restricting EG/ALG-specific expression of the dsRNA to different developmental stages in the flies using a temperature-inducible GAL80 construct or drug-inducible GeneSwitch construct would be highly informative to this end and would likely highlight the critical temporal window for intervention.

Cell-to-cell communication between mammalian astrocytes has been well-established (529) and potential evidence of glial cell communication after severe axonal injury in adult flies has been recently reported (295). Therefore, if hypothesising on the underlying mechanisms in an adult-centric manner, it is possible that the more pathogenic dsRNA construct combination-mediated toxicity in ALG induces the release of signals to nearby EG cells, instructing the clearance of the affected cells and resolving the insult. Conversely, neuronal injury signals in vertebrates can also induce an astrocytic change of state into so-called 'reactive' astrocytes (179). After acute injury astrocytes respond to initiate debris clearance and form a "scar" to prevent the spreading of inflammation (250, 302). However, reactive astrocytes can also be induced into a neurotoxic state through microglial signalling, leading to the release of pro-inflammatory molecules and neuronal cell death (247).

Whether a similar transformation in *Drosophila* astrocyte-like glia can occur is unknown; indeed they are capable of phagocytic activity and repair after CNS injury (302), though a clear microglial orthologue capable of inducing a pro-inflammatory astrocytic state is yet to be described. However, a recent report of a novel microglial-like cell type (termed MANF Immunoreactive Cells or MiCs) appearing under certain conditions in the *Drosophila* pupal

CNS (303) may provide the closest microglial orthologue currently described. The MiCs expressed both the glial engulfment receptor Draper and immune transcription factor Relish, though the ability of these cells to communicate with both neurons and glial cells has not been investigated. Interestingly, MiCs were found to localise to the neuropil region of the brain, an area occupied by astrocytic processes (303), raising the possibility of a localised interaction between the two cell types. Indeed, MiCs were only detected using the pan-glial driver *Repo-GAL4* and did not appear when using glial subtype-specific driver lines, suggesting that the appearance of MiCs either represent a global glial cell response or a failure of the glial subtype drivers to match the expression strength of *Repo-GAL4* (303). However, the specific glial drivers used in this study have all been reported to express at a stronger level than two independent *Repo-GAL4* drivers (292), thus not excluding the potential of signalling between astrocyte-like glia and MiCs. Further study into the conditions required for MiC induction and their signalling program will be important for shedding light on the exciting possibility of microglial-like immune activity within *Drosophila* and in response to the expression of repeat dsRNA.

5.7 Cortex glial expression of repeat dsRNA causes limited pathogenicity

Cortex glia (CG) are situated within the cortical region of the *Drosophila* CNS, forming a sponge-like structure of membranes that surround and ensheath individual neuronal cell bodies (279). Cortex glia share common progenitors with surface glia in both the larval brain and ventral nerve cord during development (285). Embryonically-derived cortex glial cells are maintained through to adulthood, though proliferation does occur during the larval stages to form secondary cortex glia (282). Given their close contact with neuronal bodies, cortex glial cells have been suggested to provide trophic and metabolic support to neurons (530), though little more is known about their normal biological roles within the CNS. Despite this, recent work has highlighted the importance of cortex glia in proper neuronal function. Compromised cortex glial development through the loss of *ceramide phosphoethanolamine synthase (cpes)* prevented adult cortex glia from successfully ensheathing neuronal cell bodies, leading to light-induced epileptic seizures in a *Drosophila* model of photosensitive epilepsy (PSE) (306).

122

Therefore, experiments were undertaken to test if the presence of repeat dsRNA within cortex glial cells could cause a decline in function, contributing to the high level of neurotoxicity observed using *Repo-GAL4*. Using the *CG-GAL4* driver, the dsRNA construct combinations were expressed specifically within cortex glia and the resulting *CG*>dsRNA flies were assayed for locomotor function and survival. Expression of the 'Weak' dsRNA construct combination in cortex glial cells did not lead to locomotor dysfunction in flies 1 day old (p=0.2799) **(Figure 5.13 A)**, however after 15 days **(Figure 5.13 B)** the dsRNA flies displayed better climbing ability compared to the control flies (p=0.0436). In addition, the survival of 'Weak' flies was not impacted **(Figures 5.13 C & 5.13 D)**. This suggests that the 'Weak' dsRNA construct combination is not sufficiently abundant nor pathogenic to drive neurodegenerative processes when expressed specifically within cortex glia.

Expression of the either 'Medium' or 'Strong' dsRNA construct combinations led to significantly reduced climbing ability in flies after 1 day (p=0.0421 & p=0.0065 respectively) **(Figure 5.14 A)**. However, the reduced climbing ability of both dsRNA groups did not display age-related progression compared to the control flies (p=0.9993 & p=0.9999 respectively) after 15 days **(Figure 5.14 B)**. Though the climbing ability of both the 'Medium' and 'Strong' dsRNA flies were unaffected with age, the survival of the 'Medium' flies was severely impacted, with only 12.5% of flies surviving after 29 days, compared to 71.7% of the control flies and 64.17% of the 'Strong' flies **(Figure 5.14 C & D)**. Given that the dying flies likely represent those most sensitive to any form of potential dsRNA-mediated pathology, there exists the possibility that any locomotor dysfunction in the 'Medium' flies after 15 days is masked by way of the death of the affected flies, thus leaving only the "fittest" flies remaining. However, the survival of the 'Strong' flies is not impacted in a similar fashion, indicating that other stochastic factors such as bacterial growth may be responsible for the increased mortality of the 'Medium' flies.

Cortex glia are unique in that they alone populate the cortical regions of the *Drosophila* CNS and are thus the only glial cells in direct contact with neuronal cell bodies (292). As such, it stands to reason that tightly regulating cortex glia homeostasis is of vital importance in maintaining neuronal function. The observation that cortex glial cells express *draper (drpr)* and *ced6*, vital components responsible for the clearance of apoptotic cells within the

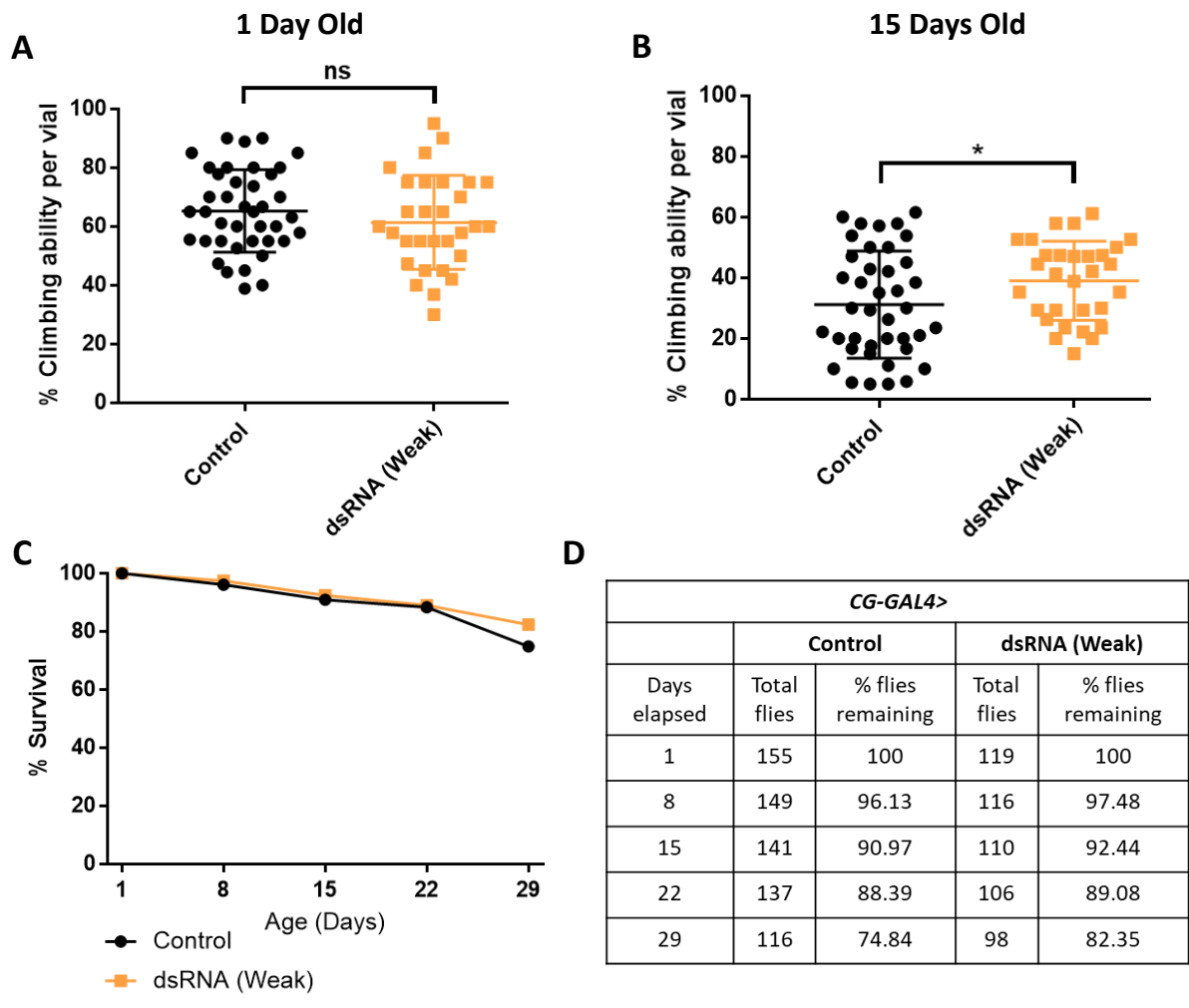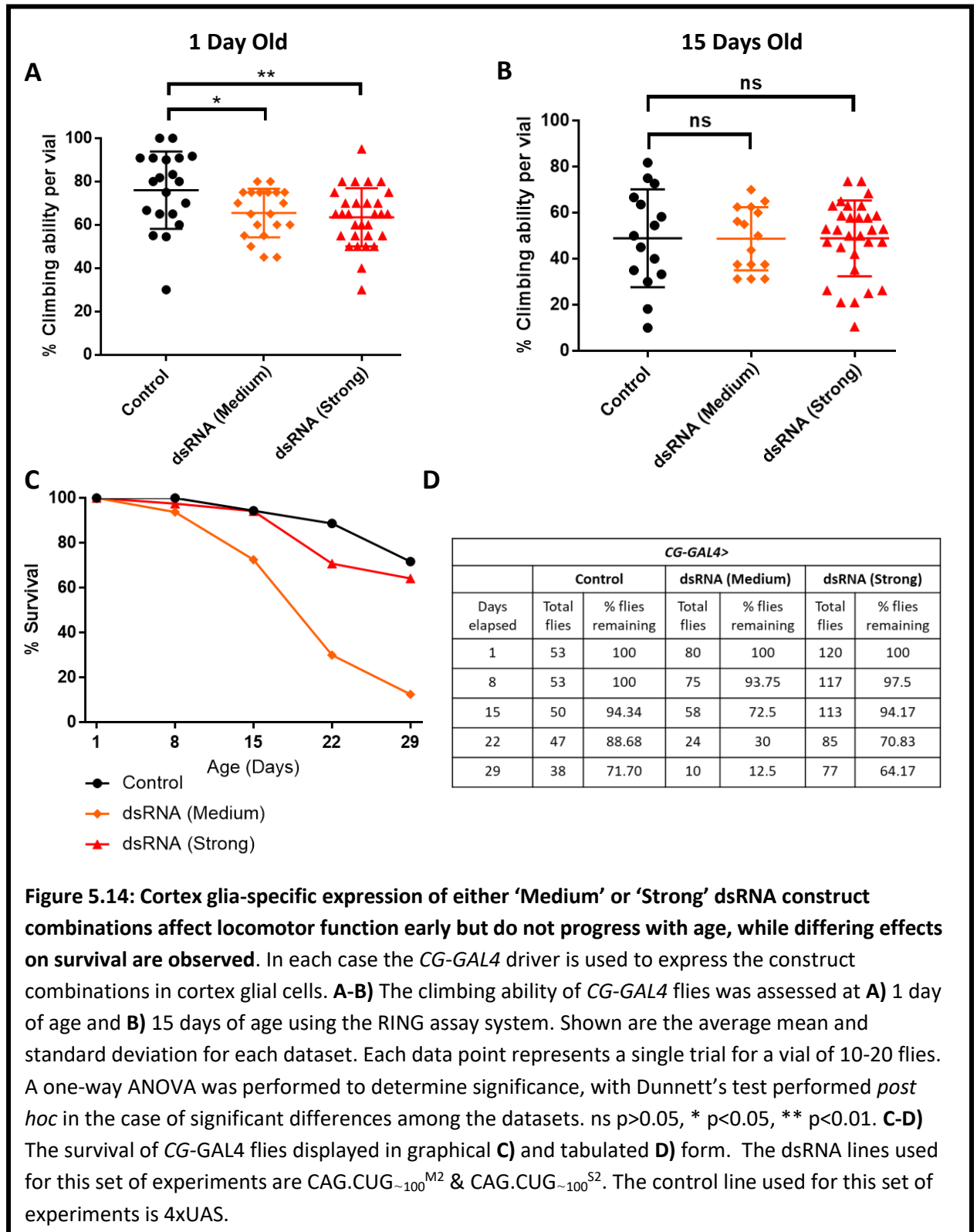**A** 1 Day Old

B 15 Days Old

C

D

CG-GAL4>				
	Control		dsRNA (Weak)	
Days elapsed	Total flies	% flies remaining	Total flies	% flies remaining
1	155	100	119	100
8	149	96.13	116	97.48
15	141	90.97	110	92.44
22	137	88.39	106	89.08
29	116	74.84	98	82.35

Figure 5.13: Cortex glia-specific expression of a 'Weak' dsRNA construct combination does not affect locomotor dysfunction or survival. In each case the *CG-GAL4* driver is used to express the construct combinations in cortex glial cells. **A-B)** The climbing ability of *CG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. 2-tailed Student's t-tests were performed to determine significance. ns $p>0.05$, * $p<0.05$. **C-D)** The survival of *CG-GAL4* flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA line used for this set of experiments is CAG.CUG$_{\sim100}^{W2}$. The control line used for this set of experiments is 2xUAS.
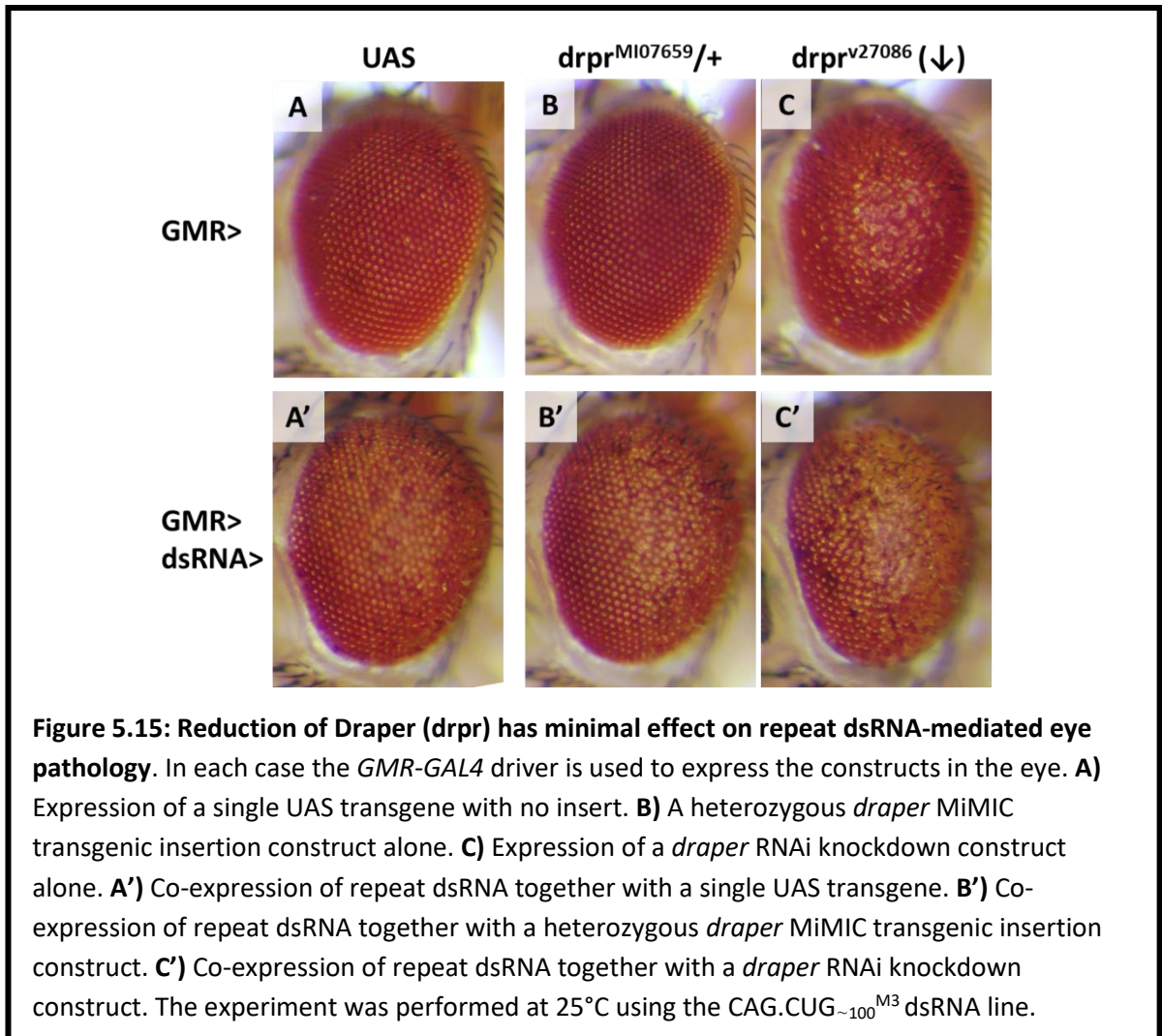
Figure 5.14: Cortex glia-specific expression of either 'Medium' or 'Strong' dsRNA construct combinations affect locomotor function early but do not progress with age, while differing effects on survival are observed. In each case the *CG-GAL4* driver is used to express the construct combinations in cortex glial cells. **A-B)** The climbing ability of *CG-GAL4* flies was assessed at **A)** 1 day of age and **B)** 15 days of age using the RING assay system. Shown are the average mean and standard deviation for each dataset. Each data point represents a single trial for a vial of 10-20 flies. A one-way ANOVA was performed to determine significance, with Dunnett's test performed *post hoc* in the case of significant differences among the datasets. ns $p>0.05$, * $p<0.05$, ** $p<0.01$. **C-D)** The survival of *CG*-GAL4 flies displayed in graphical **C)** and tabulated **D)** form. The dsRNA lines used for this set of experiments are CAG.CUG$_{\sim100}$^{M2} & CAG.CUG$_{\sim100}$^{S2}. The control line used for this set of experiments is 4xUAS.

Drosophila CNS (531), has led to the proposal that cortex glial cells exhibit phagocytic function within the adult *Drosophila* cortex where known phagocytic ensheathing glia are unable to act (293). In support of this, recent evidence has shown that cortex glial-specific knockdown of *draper* leads to the accumulation of apoptotic neuronal corpses during development, leading to age-dependent neurodegeneration (308).

Given the lack of neurodegeneration as observed in **Figure 5.13** & **Figure 5.14**, it could be speculated that cortex glia are capable of clearing 'non-self' dsRNA in development in a cell-autonomous fashion. This could potentially occur through the incorporation of dsRNA into neuronal corpses targeted for degradation if *draper* is required, given its role as an extracellular sensor. Interestingly, cortex glial expression of the 'Medium' dsRNA construct combination causes a high level of mortality, though it is unclear as to whether this mortality represents an age-dependent failure of phagocytic machinery or stochastic factors, particularly given that the 'Strong' flies do not show a similar level of mortality. As it stands, cortex glia remain vastly understudied (160) and so further research will be critical to understanding the normal biological functions of cortex glia and how they can respond to neuronal insults such as the repeat dsRNA utilized here.

5.8 Phagocytic effectors contribute modestly to the dsRNA eye pathology

The lack of neuronal dysfunction observed when the stronger dsRNA construct combinations are expressed in neuropil glia (and cortex glia) **(see sections 5.5, 5.6 & 5.7)** raises the possibility that non-cell autonomous phagocytosis is a neuroprotective mechanism in response to dsRNA challenge. The importance of the glial engulfment receptor Draper in mediating CNS phagocytosis is well documented (310). In addition, Draper has been demonstrated to clear Aβ42 and ameliorate behavioural phenotypes in a *Drosophila* model of AD (532), highlighting its importance as a neuroprotective agent in neurodegenerative disease models. Indeed, glial cells migrate to the eye imaginal disc during development to aid in the formation of visual circuitry (292, 533) and remain important within the adult eye supporting photoreceptor cells (534). Therefore, the established dsRNA eye screening model **(described in Chapter 4)** was utilized to determine if Draper can suppress expanded repeat dsRNA-mediated eye pathology.
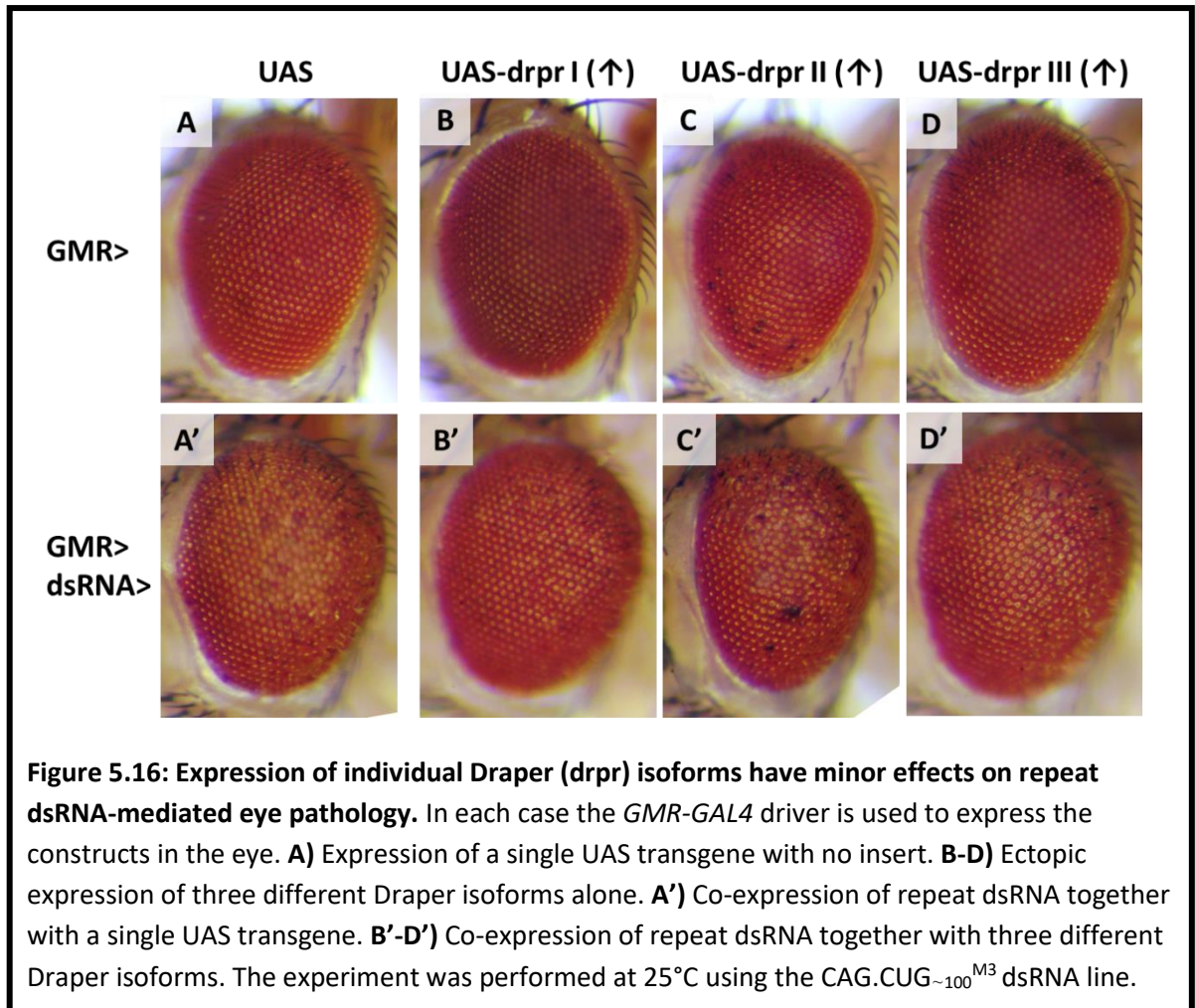
Figure 5.15: Reduction of Draper (drpr) has minimal effect on repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** A heterozygous *draper* MiMIC transgenic insertion construct alone. **C)** Expression of a *draper* RNAi knockdown construct alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a heterozygous *draper* MiMIC transgenic insertion construct. **C')** Co-expression of repeat dsRNA together with a *draper* RNAi knockdown construct. The experiment was performed at 25°C using the CAG.CUG$_{\sim 100}$^{M3} dsRNA line.

Draper expression decreases with age in *Drosophila*, impairing glial cells in their response to CNS injury (296). In addition, RNAi silencing of *draper* leads to degeneration in muscle cells as well as the CNS (297). As such, the potential contribution of Draper to the dsRNA pathology was first investigated in flies with reduced *draper* expression **(Figure 5.15)**. The introduction of a heterozygous insertion mutation did not show any structural disruptions alone **(Figure 5.15 B)**, while RNAi knockdown of *draper* alone caused disrupted ommatidial patterning compared to the control **(compare Figure 5.15 A & C)**. This supports previous observations that *draper* silencing leads to tissue degeneration (297). The heterozygous *draper* insertional mutant had no obvious effect on the dsRNA pathology **(Figure 5.15 B')**, while combination of the RNAi *draper* knockdown with *GMR*>dsRNA led to a loss of pigmentation and ommatidial disruption **(Figure 5.15 C')**, though this appears to be an additive effect when compared to expression of the individual components alone **(Figure**

5.15 C & A'). Taken together, these results suggest that Draper is important under normal homeostatic conditions but may not play a neuroprotective role in response to 'non-self' dsRNA challenge.

Of note, the eye was used as the tissue model for this experimental work, as opposed to glial cells within the CNS where Draper has been heavily characterised (293, 294). Indeed, the observation that loss of Draper specifically within the eye causes disruptions to eye patterning suggests that Draper may perform a similar homeostatic role within the eye. Additionally, apoptotic cell death is a normal and required process during eye development (535), and so the known role of Draper in processing apoptotic cells during CNS development (308) may also be key to ensure proper structural patterning within the eye.

In order to further assess if Draper can contribute to dsRNA pathology, three alternate Draper isoforms (Draper-I, Draper-II & Draper-III) under the control of UAS sites were individually expressed **(Figure 5.16)**. Previous work characterising these isoforms revealed that Draper-I is the predominant isoform present within the CNS and strongly promotes glial engulfment functionality following axonal injury (351). In contrast, Draper-II acts to negatively regulate Draper-I signalling and glial engulfment, thus representing a novel mechanism for the fine-tuning of glial phagocytosis under injury conditions (351). The role of Draper-III in regulating glial engulfment is unknown; expression of Draper-III is not sufficient to drive nor inhibit phagocytosis (351).

Expression of Draper-I or Draper-III alone **(Figure 5.16 B & 5.16 D respectively)** did not lead to any obvious phenotypic changes compared to the control **(Figure 5.16 A)**. However, expression of Draper-II alone **(Figure 5.16 C)** led to the appearance of black necrotic spots and slight abnormalities in eye shape towards the posterior side which were fully penetrant among scored eyes. This suggests that constitutive inhibition of Draper-mediated engulfment is detrimental to normal eye structure. Intriguingly, expression of Draper-II was not observed during development as opposed to both Draper-I and Draper-III (351). Furthermore, Draper is critical for the clearance of apoptotic neuronal bodies during development (308, 525). Therefore, the necrotic spots may represent neuronal corpses, allowed to persist into adulthood due to a curtailed phagocytic response. Co-expression of

Figure 5.16: Expression of individual Draper (drpr) isoforms have minor effects on repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B-D)** Ectopic expression of three different Draper isoforms alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B'-D')** Co-expression of repeat dsRNA together with three different Draper isoforms. The experiment was performed at 25°C using the CAG.CUG$_{\sim100}^{M3}$ dsRNA line.

Draper-I with *GMR*>dsRNA led to a restoration of red pigmentation in the eye and patterning **(Figure 5.16 B')** when compared to expression of the dsRNA alone **(Figure 5.16 A')**. The restoration of pigmentation and patterning was also observed when Draper-III was co-expressed, though these changes were almost negligible **(Figure 5.16 D')**. Expression of Draper-II in conjunction with dsRNA shows necrotic spots along with minor disruptions to eye shape and structure **(Figure 5.16 C')**, consistent with the phenotype being the additive effect of repeat dsRNA or Draper-II expression alone **(Figure 5.16 A' & C respectively)**.

Thus, promotion of glial engulfment through Draper-I activity may play a minor neuroprotective role in response to the presence of repeat dsRNA, while blockage of this phagocytic activity causes dysfunction to normal eye development regardless of whether dsRNA is present. However, it is important to note that endogenous expression of Draper was not ablated in this experiment, and thus may contribute to the phenotypic contributions

(or lack thereof) observed. Indeed, glial expression of Draper-II in wild type flies completely ablates glial engulfment while overexpression of either Draper-I or Draper-III failed to enhance phagocytic activity beyond that of control flies (351). Since overexpression of Draper-I appears to suppress both the roughness and pigment loss caused via dsRNA expression **(compare Figure 5.16 A' & B')**, it is plausible that enhanced Draper engulfment activity through increased Draper-I may prove beneficial when challenged with disease gene products. Supporting this, overexpression of Draper-I in a wild type Draper background reverses Aβ42 accumulation and the associated neurological phenotypes in a *Drosophila* model of AD (532).

Taken together, these results indicate that Draper may play a minor restrictive role in repeat dsRNA pathology. Loss of Draper or negative regulation of engulfment through Draper-II expression causes a disruptive eye phenotype alone, highlighting the important homeostatic function of Draper-mediated signalling. However, Draper loss does not further enhance the dsRNA pathology, suggesting that other degradative pathways may be upregulated in response to the presence of 'non-self' dsRNA. In contrast, overexpression of Draper mildly suppresses the dsRNA pathology and could reflect increased phagocytic capacity within the eye.

Activation of Draper-mediated phagocytic activity often requires signalling and/or recognition of so-called "eat-me" signals that present on apoptotic cells or neuronal debris (308). Phosphatidylserine (PS) is a conserved and well characterised example; the phospholipid becomes exposed on the outer membrane of apoptotic cells to be recognized by phagocytic machinery, including Draper in *Drosophila* (536, 537). Indeed, proteins can also act as ligands via their binding to cells destined for phagocytosis/apoptosis. This includes macroglobulin complement-related (Mcr), a thioester-containing protein (TEP) closely related to mammalian complement proteins (538, 539). Mcr was first characterised as a required binding component for the phagocytosis of the pathogenic yeast species *Candida albicans* in *Drosophila* (540), thus demonstrating its importance in inflammation. Two independent studies have also demonstrated that Mcr is critical for the formation of septate junctions (SJs), connections between epithelial cells that form paracellular barriers

to separate specialized organ compartments, including the *Drosophila* blood-brain barrier (BBB) (541, 542).

More recent evidence has uncovered a dual role for Mcr in activating Draper-mediated phagocytosis; inducing autophagy in neighbouring cells during development and recruiting macrophages to epithelial wounding sites (543). Notably, the mammalian Draper orthologue Megf10 is bound and activated by the complement protein C1q in order to phagocytose apoptotic cells (544), indicating that this interaction is highly conserved. C1q is important in initiating debris elimination through the complement cascade by marking pathogens and excess synaptic material (189, 545). However, C1q can also promote microglia-mediated neurodegeneration via synaptic pruning in AD mouse models (230) and through its secretion from microglia to induce neurotoxic astrocyte reactivity, a process observed in a number of neurodegenerative diseases (247). Therefore, the process by which ligands activate phagocytic machinery needs to be tightly controlled to avoid elimination of essential cells.

Thus, *Mcr* was altered in a *GMR*>dsRNA background to determine if it participated in dsRNA pathology. Introduction of an *Mcr* P-element insertional mutation did not cause any noticeable eye phenotype alone **(Figure 5.17 B)**, while RNAi knockdown of *Mcr* alone led to a very minor disruption to eye patterning compared to the control **(Figure 5.17 A & C)**. The RNAi knockdown-induced disruption may indicate that, like Draper, the importance of Mcr in clearing apoptotic cells during development is extended to the eye.

While the heterozygous *Mcr* mutation appeared to mildly suppress the dsRNA pathology **(Figure 5.17 A' & B')**, RNAi knockdown of *Mcr* was lethal in a *GMR*>dsRNA background **(Figure 5.17 C')**, suggesting that Mcr could be important for restricting dsRNA pathology. The *Mcr^EY07421* P-element insertion contains UAS sites at the 3' end which may lead to the misexpression of neighbouring genes (including *Mcr*) when combined with *GMR-GAL4*. Thus, the possibility of misexpression leading to the suppression of the dsRNA pathology must be considered. Indeed, both *Mcr* alterations modify the dsRNA eye pathology, highlighting Mcr as a candidate for future targeted studies concerning its role in dsRNA-mediated pathogenesis. Additionally, transcript analysis of *GMR*>*Mcr^EY07421* could be undertaken to determine the nature of *Mcr* alteration in this line. Finally, the use of an endogenous *Mcr*
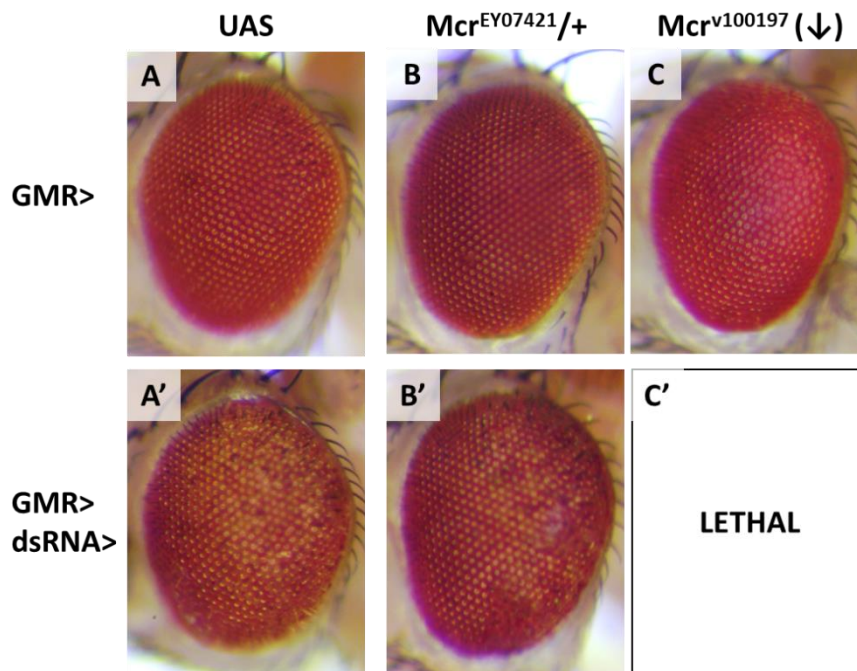
Figure 5.17: Effect of Mcr alteration on repeat dsRNA-mediated eye pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert. **B)** A heterozygous P-element transgenic insertion alone. **C)** Expression of an *Mcr* RNAi knockdown construct alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B')** Co-expression of repeat dsRNA together with a heterozygous P-element transgenic insertion. **C')** Co-expression of repeat dsRNA together with an *Mcr* RNAi knockdown construct. The experiment was performed at 25°C using the CAG.CUG$_{\sim100}$^{M3} dsRNA line.

mutant line could be used to test for potentially confounding misexpression, though none were available for testing.

The lethality observed when combining *GMR*>dsRNA with the *Mcr* RNAi knockdown **(Figure 5.17 C')** may be caused through the inhibition of homeostatic *Mcr* developmental functions compounded with dsRNA-mediated cellular dysfunction. Alternatively, it could potentially reflect the loss of the ability of Mcr to aid in clearance of the dsRNA in cells targeted for phagocytosis. Of note, a previous microarray analysis identified Mcr as significantly upregulated in response to the presence of repeat dsRNA (341), suggesting that Mcr may indeed form part of a protective response directed against 'non-self' dsRNA. Given the importance of Mcr in development (541-543), a *GAL4* inhibitor such as the temperature-sensitive *GAL80* could be used in order to block *Mcr* RNAi knockdown during development.

This would allow adult flies to emerge **(lethal in Figure 5.17 C')** and permit further examination of the apparent "helpful or harmful" interactions between Mcr and the repeat dsRNA.

5.9 Chapter Discussion

Once thought of as purely neuron-centric in terms of cause and consequence, a large body of research has brought glial cells to the forefront as non-cell autonomous drivers of neurodegenerative disease pathogenesis (225, 546). Emerging evidence has demonstrated that both loss of normal homeostatic CNS function and/or gain of pathogenic signalling function can promote neurodegeneration, highlighting the requirement for a tightly regulated CNS environment (242, 247, 547). The experiments presented in this chapter describe a preliminary study into the contributions made by individual *Drosophila* glial subtypes in mediating dsRNA pathology and how their normal biological functions can provide insight into the underlying non-cell autonomous disease mechanisms. The extreme level of neuronal dysfunction observed via pan-glial expression of the repeat dsRNA was first recapitulated to serve as a point of comparison when determining the contributions made by glial subtypes. As previously observed, only flies expressing the weaker dsRNA construct combination were viable but suffered severe neurodegeneration and mortality. This strongly indicated a non-cell autonomous mechanism underlying the pathology and warranted the further dissection of glial cell functionality in this disease model. As summarised in **Figure 5.18**, expression of repeat dsRNA specifically within subperineural or wrapping glia appeared to most faithfully recapitulate the graded level of pathology observed through pan-glial dsRNA expression. The expression of dsRNA in subperineural glial cells resulted in significant pathology, including complete lethality in the 'Strong' dsRNA flies and climbing deficiencies in the 'Weak' and 'Medium' flies. In addition, expression of either the 'Medium' or 'Strong' dsRNA construct combinations specifically within peripheral wrapping glia led to severe neurodegeneration and mortality, while the 'Weak' dsRNA construct combination did not cause any pathology.
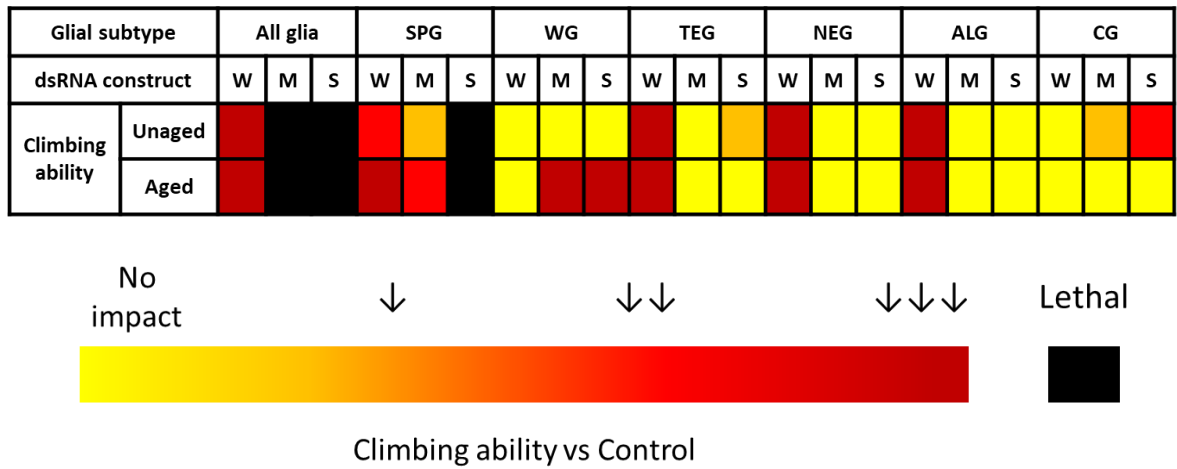
What commonalities exist between subperineural and wrapping glial cells? Firstly, both cell types are derived from a shared set of progenitors in the ventral nerve cord and PNS (283).

Secondly, while subperineural glia constitute the main structural aspect of the invertebrate BBB, signals derived from wrapping glia are required for the expansion of septate junctions connecting subperineural cells, thus both demonstrating importance in BBB development (289). Thirdly, both cell types do not proliferate through development but instead expand greatly in size postembryonically (283). Therefore, it appears unlikely that either cell type can be compensated for through proliferation in the event of death or dysfunction. Finally, the septate junctions that connect subperineural cells appear most sensitive to pan-glial expression of the HD polyQ-expanded protein (277), further supporting the idea that subperineural and wrapping glial-specific expression of dsRNA both share BBB dysfunction as a common cause of the observed pathology. Whether any non-neuronal cells within the CNS augment the observed pathology through neuroinflammatory signalling is unknown, given that any pro-inflammatory roles of *Drosophila* glial cells are not well characterised at this stage.

Importantly, disruptions to BBB integrity have been reported in a mouse model of HD (548), in addition to post-mortem tissue samples and induced pluripotent stem cells (iPSCs) derived from HD patients (548, 549). Further work in HD mice has demonstrated that BBB breakdown is an early pathological event that precedes symptom onset (276). The extent of systemic molecule and cellular infiltration into the CNS in HD models is yet to be explored in detail, though hyperactivation of peripheral immune cells in HD has been linked to CNS dysfunction in several studies (550, 551).

In contrast to a scale of progressive pathology, repeat dsRNA-mediated neurotoxicity in neuropil glia (TEG, NEG & ALG) appears largely restricted to the weakest dsRNA construct combination used, while the stronger dsRNA construct combinations only display limited neuronal dysfunction and but lead to age-dependent mortality in NEG and ALG flies **(Figure 5.18)**. This goes directly against the established pathology gradient observed in the *Drosophila* eye **(Figure 5.1)** and raises the possibility of neuroprotective functions in these glial cells. Both ensheathing and astrocyte-like glia display phagocytic function through the Draper glial engulfment pathway to remove axonal debris (293, 302). Indeed, astrocyte-like glia only appear to have this capability during development (293), which potentially highlights the developmental stages as a critical "make or break" timepoint whereby the
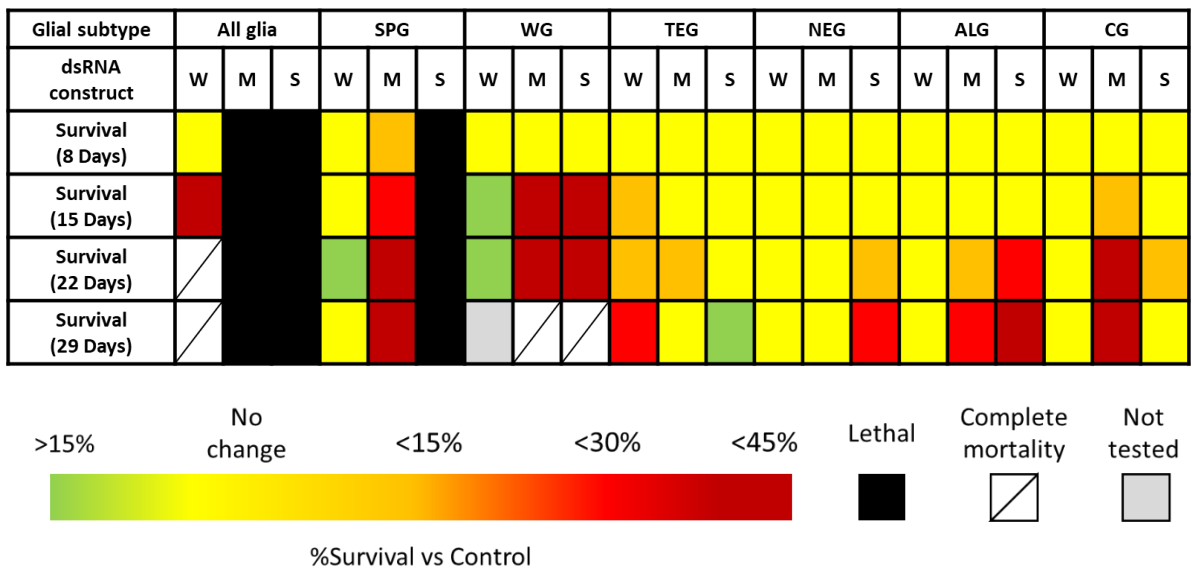
A

Glial subtype	All glia			SPG			WG			TEG			NEG			ALG			CG		
dsRNA construct	W	M	S	W	M	S	W	M	S	W	M	S	W	M	S	W	M	S	W	M	S
Climbing ability — Unaged																					
Climbing ability — Aged																					

No impact → ↓ → ↓↓ → ↓↓↓ → Lethal

Climbing ability vs Control

B

Glial subtype	All glia			SPG			WG			TEG			NEG			ALG			CG		
dsRNA construct	W	M	S	W	M	S	W	M	S	W	M	S	W	M	S	W	M	S	W	M	S
Survival (8 Days)																					
Survival (15 Days)																					
Survival (22 Days)																					
Survival (29 Days)																					

>15% — No change — <15% — <30% — <45% — Lethal — Complete mortality — Not tested

%Survival vs Control

Figure 5.18: Summary of results for individual glial cell subtypes expressing expanded repeat dsRNA. The GAL4 drivers used are listed in Table 5.1. W, M and S refer to the 'Weak', 'Medium' and 'Strong' dsRNA construct combinations respectively. **A)** Summary of RING assay results. In each case the % climbing ability is compared to the relevant control flies. No impact indicates no significant loss of climbing ability, ↓ $p<0.05$, ↓↓ $p<0.01$, ↓↓↓ $p<0.001$. Lethal indicates that no flies successfully emerged from pupation. For all glial subtypes, Unaged represents RING assays taken at Day 1. Aged represents the last timepoint tested for individual subtypes. For All glia and WG, Aged represents Day 8. For SPG, TEG, NEG, ALG and CG, Aged represents Day 15. **B)** Summary of survival results. Colour code indicates the difference in percentage flies remaining between the experimental flies and the appropriate control flies. Lethal indicates that no flies successfully emerged from pupation. Complete mortality indicates the death of all flies before the testing timepoint. Not tested indicates that the flies did not suffer complete mortality but were not tested at the timepoint. SPG, subperineural glia; WG, wrapping glia; TEG, tract ensheathing glia; NEG, neuropil ensheathing glia; ALG, astrocyte-like glia; CG, cortex glia

repeat dsRNA is either degraded or persists to exert toxicity within the CNS. Furthermore, the Draper pathway can be upregulated globally within the *Drosophila* CNS in order to scale with the severity of axonal injury (295). Thus, it is tempting to speculate that the stronger dsRNA construct combinations elicit a more substantial phagocytic or apoptotic (or both) response capable of handling the increased 'non-self' material load, whereas the 'Weak' dsRNA construct combination induces a dampened response that allows the dsRNA to persist and cause the subsequent neuronal dysfunction.

Can glia with phagocytic function recognise and engulf dysfunctional glial cells as well as neuronal debris? Inhibition of Draper prevents the clearance of axonal debris (293, 294) and so it would be of great interest to determine whether proper neuronal function can be maintained in flies expressing ensheathing/astrocyte-specific dsRNA when phagocytic capacity is ablated. Intriguingly, astrocyte-like glia can also initiate neurite clearance and synaptic pruning in development through non-Draper pathways (301, 302), indicating that a level of functional redundancy may be present to prevent complete ablation of phagocytic function upon pathogenic challenge.

When compared to the mammalian CNS, ensheathing glia and astrocyte-like glia appear to be roughly related to microglia and astrocytes respectively as far as function and morphology are concerned. Microglia act as resident phagocytes in the CNS, migrating to sites of neuronal injury and pathogenic insult (177), while astrocytes display phagocytic activity through the Draper orthologue MEGF10 in order to engulf synapses, in addition to a conserved role in neurotransmitter homeostasis (160, 188). However, both microglia and astrocytes can exhibit functional changes of state upon CNS insult/injury to promote pro-inflammatory signalling and neuronal cell death (179, 507). Furthermore, these functional and transcriptional shifts towards pathology become more pronounced with age and coincide with neuronal decline (248, 552, 553). Though ensheathing and astrocyte-like glial cells of the *Drosophila* CNS have not yet been shown to undergo similar helpful-to-harmful shifts in function, aged flies are significantly delayed in their engulfment response to axonal debris after injury which is paired with a concomitant decrease in Draper protein levels (296). Indeed, flies expressing the stronger dsRNA constructs in either neuropil ensheathing or astrocyte-like glia display age-dependent mortality **(Figure 5.18 B)**, which may represent a

136

similar age-related decline in glial engulfment activity. Thus, it will be an exciting future prospect to determine if *Drosophila* neuropil glia also take on a neuroinflammatory change of state with age, particularly in the context of neurodegenerative disease models such as the repeat dsRNA model used in this work.

Finally, recent evidence has uncovered the appearance of microglial-like MANF Immunoreactive Cells (MiCs) that appear within the developing CNS under conditions of upregulated immune signalling, autophagy and silencing of the neurotrophic factor MANF (303). Through their expression of Draper and the immune regulator Relish, MiCs appear to display the capacity for both phagocytic activity and immune signalling (303), raising the intriguing possibility of an interaction with neuropil glia upon dsRNA challenge. However, further characterisation surrounding the conditions under which MiCs are induced and their functions within the CNS will need to occur in order to support this hypothesis.

Cortex glia are distinct in this work in that there does not appear to be any defined pattern of pathology based on dsRNA construct combination **(Figure 5.18)**. The 'Weak' dsRNA construct combination displayed no detectable neuronal dysfunction, whereas the 'Medium' and 'Strong' construct combinations displayed age-dependent mortality, though this was more pronounced in the 'Medium' line. Indeed, cortex glia are also unique in terms of CNS localisation; they are the sole occupant of the cortical region, forming vast honeycomb-like structures that ensheath neuronal cell bodies (279). Being the only glial cell type to be in direct contact with neuronal bodies, it stands to reason that cortex glia themselves require protection in order to insulate neurons against CNS injury and pathogenic insults. Cortex glia have been reported to express Draper and other components of the phagocytic machinery (531) but do not appear to function in this capacity in the context of axonal injury (293). However, cortex glial specific expression of Draper is vital for the programmed clearance of apoptotic neurons during development (308), which could mean that cortex glial-driven phagocytic activity may serve a basal function during development, as opposed to transient upregulation in response to either neuronal injury and/or steroidal signalling in neuropil glia (293, 302). This idea is supported by the fact that expression of the 'Weak' dsRNA construct combination that caused pathology in neuropil glia did not affect flies when expressed in cortex glia **(Figure 5.18)**, suggesting that a basal level of degradation may suffice for turning

over the repeat dsRNA and preventing a heightened neuroinflammatory response. Of course, the possibility exists that neuropil glia can extend membranes into cortex glial territory upon dsRNA insult to aid in removing the threat non-cell autonomously. The normal roles of cortex glia are still not well defined (160), therefore further basic research may uncover novel cortex glia functions that can better describe the results observed here.

Given the key role of Draper in non-cell autonomous phagocytosis and the possibility of this process acting protectively in response to dsRNA, the *Drosophila* eye was used as a qualitative model to determine if Draper could suppress the expanded repeat dsRNA pathology. Upregulation of the engulfment receptor *Draper* also led to a modest suppression in dsRNA pathology, while *Draper* reduction in a *GMR*>dsRNA background did not lead to a reciprocal enhancement. Draper has been well characterised as a glial engulfment receptor that is upregulated in response to axonal injury (293-295, 351) and is capable of clearing Aβ42 in the *Drosophila* brain (532), but whether Draper can also respond to and clear dsRNA material is unknown. Given that *Draper* activation is modulated based on injury severity within the CNS (295), it should be determined if ectopic expression of the positive phagocytic regulator isoform Draper (I) leads to a more pronounced suppression of dsRNA eye pathology caused via a "Strong" dsRNA construct combination (as shown in **Figure 5.1 E**) as opposed to the "Medium" dsRNA construct combination used in **Figure 5.16**.

The reduction of Draper ligand *Mcr* caused lethality upon RNAi reduction in a *GMR*>dsRNA background, suggesting that Mcr may restrict dsRNA pathology. A possible scenario is that *Mcr* reduction in conjunction with dsRNA expression causes lethality due to the loss of function in its roles in septate junction formation and autophagy (541-543). Consistent with this hypothesis, expression of repeat dsRNA in subperineural glial cells, responsible for septate junction formation, was highly pathogenic, while autophagy restricts the dsRNA eye pathology (52). The introduction of a heterozygous *Mcr* insertional mutation supressed the dsRNA pathology, though this may be due to *GMR-GAL4* driven misexpression of *Mcr* and neighbouring genes. Transcript analysis in future could prove helpful in determining how *Mcr* levels are altered in this mutant line. Indeed, that fact that the insertional *Mcr* mutant also modifies the dsRNA pathology further highlights *Mcr* as a candidate gene for future work to further define its role in expanded repeat dsRNA pathogenesis.

The results described in this chapter act as a pilot study into the contributions of individual glial subtypes towards dsRNA-mediated nerve cell dysfunction that has been observed previously (52). Based on pairing the results with the known biological functions of each glial subtype, the findings suggest that BBB breakdown is a key event in driving the pathogenic process, while phagocytosis may be vital for degrading the repeat dsRNA and preventing its persistent activation of inflammation.

While the approach taken in this study focuses on the contribution of single glial subtypes, it is highly likely that non-glial cell autonomous communication and responses from other glial subtypes underlie the results observed. A limitation of the GAL4-UAS system is that only a single specific cell type can generally be manipulated (314), making it difficult to explore the potential for non-cell autonomous mechanisms. However, the establishment of independent binary expression systems including LexA/LexAop (554) and the Q system (555) provide the necessary tools to dissect the non-glial cell autonomous roles hypothesised in this chapter. For example, if a knockdown of *draper* is produced specifically within ensheathing glia, are neighbouring cortex glia and/or astrocyte-like glia more adversely affected by the presence of repeat dsRNA or indeed any disease product or injury? Non-cell autonomous pathology can also arise through gain of toxic pro-inflammatory function, recently demonstrated through communication from microglia to astrocytes in mammals (247). While evidence of glial-derived inflammatory signalling in *Drosophila* is limited, further characterisation of the migratory immunoreactive MiCs (303) may provide evidence supporting the key role glial cells play in CNS disease development and progression.

In summary, the results presented in this chapter highlight the glial cell subtypes involved in BBB development and phagocytosis as promising areas for future research regarding dsRNA-mediated neuronal dysfunction. This opens new avenues for study not only in CAG repeat disorders, but potentially in other forms of repeat expansion neurodegenerative disease.

CHAPTER 6: Final Discussion

6.1 Summary of results

More than 20 dominantly-inherited neurodegenerative disorders are caused by the expansion of a repeat sequence beyond a pathogenic threshold (3). A key question is whether many different pathways or a common pathogenic pathway underlies this group of disorders (4). Additionally, which candidate pathway(s) represent a plausible common mechanism(s) in these diseases? Several expanded repeat loci encode polyglutamine (polyQ) tracts which have been hypothesised to constitute the pathogenic agent in such disorders (termed polyglutamine disorders) (19), though not all disease-causing repeat sequences occur in a coding region of the affected gene. Additionally, several expanded repeat loci are subject to repeat-associated non-AUG (RAN) translation, and the atypical repeat polypeptides translated at such loci have been hypothesised to drive disease pathogenesis (556, 557). However, the relative contribution to disease pathogenesis of the different repeat polypeptides derived from each reading frame of such loci, in particular the 'out of frame' 4 and 5 base repeats, is yet to be determined.

Repeat-containing RNA constitutes a disease gene product common to all dominantly-inherited expanded repeat disorders and is therefore a plausible common pathogenic agent. Bi-directional transcription across these repeat loci, a process observed in all assessed expanded repeat disorders (21), produces complementary RNA sequences that are predicted to form perfectly double-stranded RNA (dsRNA). Our lab has previously established a *Drosophila* model of expanded CAG repeat disease ($rCAG_{\sim 100}.CUG_{\sim 100}$) to investigate expanded repeat dsRNA as a pathogenic agent; discovering that the innate inflammatory system is both required for repeat dsRNA-mediated pathology and upregulated in the presence of the repeat dsRNA (4, 45, 52). The primary aim of this thesis was to further define the innate inflammatory mechanisms underlying expanded repeat dsRNA pathology at both the cellular and molecular pathway level.

One focus of the work (presented in **Chapter 3**) was to investigate how the expanded repeat dsRNA was recognized by pattern recognition machinery, similar to a pathogenic threat.

Using the *Drosophila* eye as a tissue model, expression of expanded repeat dsRNA led to disruptive eye pathology (including ommatidial disruption and areas of necrotic black spots) that has been previously documented in this model (45). However, co-expression of CrPV1A, a viral suppressor protein that antagonizes the antiviral protein Argonaute-2, potently suppressed the eye pathology **(Figure 6.1)**. This suggests that the endogenously-derived *rCAG$_{\sim100}$.CUG$_{\sim100}$* molecules are detected by antiviral sensors as a 'non-self' threat, thus inducing a damaging antiviral inflammatory response that leads to the resultant pathology.

Following on from this, transcript analysis via qRT-PCR was performed on flies expressing either the repeat dsRNA alone or in conjunction with CrPV1A neuronally to uncover potential candidate pathways that participate in the dsRNA-mediated pathology. Supporting previous work, the Toll inflammatory pathway-induced peptide *Drosomycin* was significantly upregulated following dsRNA expression (52). However, this induction was not attenuated by CrPV1A, suggesting that *Drosomycin* likely does not represent the underlying pathogenic pathway leading to dsRNA pathology. Components of the recent-characterised STING/IKKβ/Relish antiviral pathway (393) were not significantly altered, highlighting the complexity and multiplicity of the *Drosophila* antiviral signalling response. Intriguingly, the uncharacterised gene *CG33926*, which is potently induced following viral challenge (393, 394), was significantly downregulated in the presence of repeat dsRNA. Thus, it is likely that the expression of 'non-self' repeat dsRNA induces a distinct but overlapping set of inflammatory genes to that of the *Drosophila* RNA viruses.

The *Drosophila* eye was used as a qualitative tissue model to further define innate inflammatory pathways for their participation in expanded repeat dsRNA-mediated pathology **(Chapter 4)**. The most compelling finding was that mitochondrial quality control is important for restricting the dsRNA-mediated pathology **(Figure 6.1)**; the reduction of key conserved mitophagy genes *PINK1* and *Parkin* led to enhanced eye pathology. In addition, reduction of mitochondrial antiviral genes *Ref(2)P* (*p62* in mammals) and *ECSIT* led to a similar enhancement, though whether this implicates their role(s) in antiviral signalling or mitochondrial quality control (or both) in restricting the dsRNA pathology remains to be seen. Preliminary analysis of the evolutionarily-conserved JAK/STAT antiviral pathway suggests that it participates minimally in mediating dsRNA pathology, which may reflect the
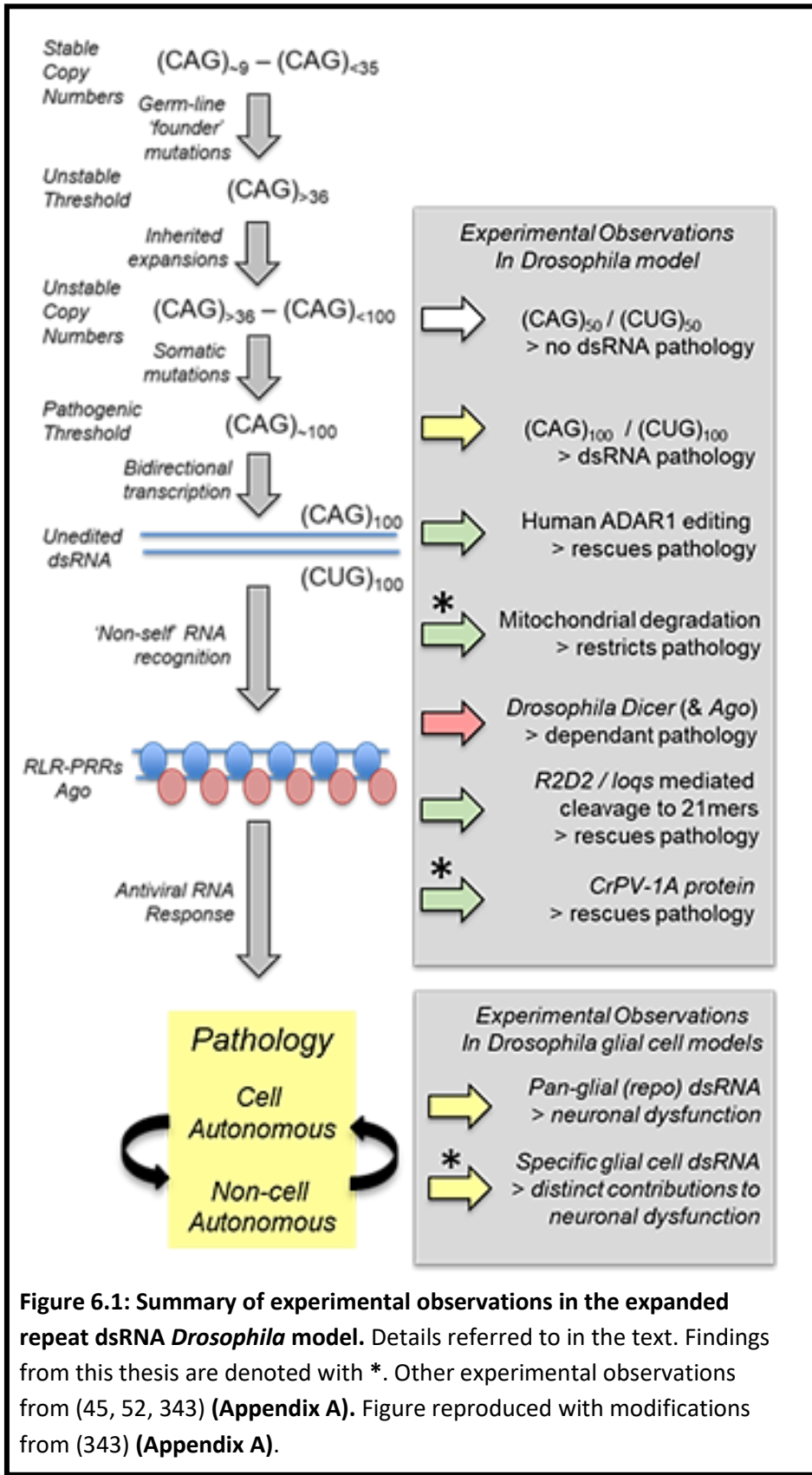
Figure 6.1: Summary of experimental observations in the expanded repeat dsRNA *Drosophila* model. Details referred to in the text. Findings from this thesis are denoted with *. Other experimental observations from (45, 52, 343) **(Appendix A).** Figure reproduced with modifications from (343) **(Appendix A)**.

ability of other pathways to compensate for the loss of JAK/STAT signalling. Taken together, the screening experiments performed in **Chapter 4** highlighted mitochondria as a key target for future investigation regarding dsRNA-mediated pathogenesis.

Based on previous work demonstrating that expression of expanded repeat dsRNA in *Drosophila* glial cells drives severe neuronal dysfunction (52), the experiments in **Chapter 5** aimed to dissect the non-cell autonomous contributions of individual glial cell subtypes to the neurodegeneration observed. The experimental results highlighted that glial cell types involved in the formation and maintenance of the *Drosophila* blood-brain barrier (BBB), namely ***subperineural glia*** and ***wrapping glia***, are sensitive to the expression of expanded repeat dsRNA. Interestingly, glial subtypes capable of phagocytic activity, ***ensheathing glia*** and ***astrocyte-like glia***, appeared resistant to the expression of more pathogenic dsRNA construct combinations, while sensitive to the expression of a weaker construct combination. This indicates that they may respond to repeat dsRNA challenge in a graded potency-dependent manner, reminiscent of glial phagocytic reactivity in *Drosophila* following injury (295). Using the expanded repeat dsRNA eye model, both phagocytic receptor *Draper* and phagocytic ligand *Mcr* modestly restrict the pathology, suggesting that phagocytosis is protective in response to the expression of repeat dsRNA. Thus, this work has implicated the *Drosophila* BBB and phagocytosis as two potential non-cell autonomous determinants of expanded repeat dsRNA-mediated neuronal pathology that warrant further definition **(Figure 6.1)**.

6.2 Implications for expanded repeat neurodegenerative diseases

Though research on expanded repeat neurodegenerative disease has uncovered several commonalities between the individual disease forms, the pathogenic molecular mechanisms that underlie disease development remain unresolved (3). In this regard, the use of a genetically simpler *in vivo* model such as *Drosophila* can be used to model key aspects of expanded repeat disease and dissect candidate pathogenic pathways in a more time effective manner. The conservation of fundamental molecular pathways and cell types between humans and *Drosophila* means that findings obtained in flies are applicable to key mechanistic aspects of the corresponding human condition. This thesis utilizes *Drosophila* to

model one such commonality observed in these diseases – the production of bi-directionally transcribed expanded repeat RNA from affected loci, predicted to form perfectly double-stranded RNA (dsRNA) (21). The work described in this study identifies several mechanisms that participate towards the pathology observed in this expanded repeat dsRNA model.

Firstly, the finding that a viral suppressor protein can effectively ablate the observed dsRNA pathology suggests that the expanded CAG.CUG repeat dsRNA is recognized by the inflammatory system as a 'non-self' molecule, similar to the RNA of a viral pathogen. This supports previous work highlighting inflammatory signalling as a key pathogenic event in HD; both in the CAG.CUG dsRNA *Drosophila* model (45, 52) and in preclinical HD patients (58, 59). Given that bi-directionally transcribed RNA is a common feature across repeat loci (21), these findings may be applicable to the broader range of expanded repeat disorders, including those defined by repeat sequences other than a trinucleotide CAG sequence such as the pentanucleotide sequences that cause SCA10 and SCA31. Additionally, constitutive antiviral inflammation via the 'non-self' recognition of endogenous RNA underlies the pathogenesis of the neurological auto-inflammatory disorder Aicardi-Goutieres syndrome (AGS) and several related disorders (153). The ablation of antiviral RNA sensing machinery has been demonstrated to rescue auto-inflammatory pathology associated with genetic causes of AGS, highlighting antiviral signalling as a promising therapeutic target (152, 156). Indeed, the results described in this study provide evidence that antiviral inflammatory signalling also underlies CAG (and potentially other) expanded repeat disorders, and thus represents a potential therapeutic target.

Secondly, that dysfunctional mitochondrial quality control (namely mitophagy) exacerbates expanded repeat dsRNA pathology further supports the protective nature of mitophagy in HD (472). Taken together with the work describing 'non-self' recognition of expanded repeat dsRNA, mitophagy may represent a method to remove and degrade such molecular triggers before the onset of sustained and damaging inflammatory signalling. Indeed, mitochondrial dysfunction has been highlighted as a key aspect of polyglutamine disorders (558), though the majority of evidence stems from research on the mutant HTT protein and thus is specific to HD. The findings described here provide evidence that mitochondrial dysfunction

underlies repeat RNA pathology, and so may constitute a rate-limiting feature common to other expanded repeat disorders.

Finally, the glial cells that govern development of the *Drosophila* blood-brain barrier (BBB) appear to be highly sensitive to the expression of expanded repeat dsRNA. This finding dovetails with recent evidence demonstrating *Drosophila* BBB sensitivity to expanded polyQ proteins (277), indicating that multiple gene products derived from expanded repeat loci can drive neuronal dysfunction via BBB disruption. In addition, glial cells capable of performing phagocytosis were resistant to the presence of repeat dsRNA, suggesting that these cells (and/or potentially adjacent phagocytic glial cells) can degrade the dsRNA and thus prevent neuronal dysfunction. Thus, BBB integrity and glial-mediated phagocytosis shape as two processes important in restricting expanded repeat dsRNA pathology, further adding to the wealth of experimental evidence now highlighting glial cells as key non-cell autonomous drivers of neurodegeneration (546).

6.3 Considerations for this study

The use of *Drosophila* as an *in vivo* model organism provides a number of advantages, including the range of powerful tools available to genetically dissect candidate pathways and events. However, several caveats were present in this study that need to be taken into consideration when interpreting results.

While the expanded repeat *Drosophila* model used in this thesis highlights co-expressed sense (CAG) and antisense (CUG) repeat sequences (forming dsRNA) as a key pathogenic factor in expanded repeat disease, independent work in a *Drosophila* model of FXTAS has demonstrated that CGG and CCG expanded repeat sequences are toxic when expressed in isolation but lose this toxicity in an RNAi-dependent fashion when co-expressed (49). Although appearing at odds with the dsRNA model used here, there are several key differences between the models that impede their direct comparison. Firstly, while RAN translation does not appear to contribute to the RNA-mediated pathology observed in the CAG.CUG dsRNA model used in this thesis (559), it is plausible that the mechanism contributes to the ssRNA-driven toxicity observed in the FXTAS model (49). Further

characterisation of the *Drosophila* FXTAS model used in Sofola *et al* demonstrated that RAN translation of the sense CGG repeat sequence in a +1 shifted reading frame (GGC) encodes a polyglycine (polyG) tract that drives pathology within the *Drosophila* eye (23). Additionally, both the CGG and CCG repeat sequences are capable on encoding alanine when subject to RAN translation in their respective +2 reading frames (GCG and GCC) (23, 560), the resulting polyalanine (polyA) tract of which has also been demonstrated to cause pathology within the *Drosophila* eye (561). Indeed, the work performed by Sofola *et al* predates the discovery of the RAN translation mechanism (20) and so the respective CGG/CCG repeat lines were not examined for the presence of this phenomenon at the time. Notably however, earlier characterisation of the CGG line found that co-expression of the protein chaperone *Hsp70* suppressed the associated eye pathology (562). Thus, co-expression of the CGG and CCG repeat sequences may inhibit the ability of the sequences to undergo RAN translation into toxic polypeptides, thus ameliorating the eye pathology observed when either sequence is expressed alone (49).

Another key difference relates to the composition of the repeat sequences themselves. The CAG/CUG sequences expressed to generate the expanded repeat dsRNA model used in this thesis are uninterrupted to a length of approximately 100 copies (45). In comparison, the CGG/CCG sequences utilized in Sofola *et al* both contain several interruption triplets interspersed between 90 copies of the respective repeat, shortening the uninterrupted sequence to approximately 70 copies (49, 562). Length of uninterrupted repeat has been demonstrated to be a key determinant in expanded repeat dsRNA-mediated pathology (48, 343) **(Appendix)**, which may mirror the length dependency of mammalian RNA sensors for dsRNA binding and antiviral signalling (563). Thus, the length and composition of the repeat sequences examined by Sofola *et al* may not be of a sufficient threshold to be recognised as 'non-self' by pattern recognition machinery, avoiding a pathogenic antiviral response. Despite differences however, the RNAi antiviral pathway appears to be protective in both *Drosophila* expanded repeat disease models (49, 343) **(Appendix)**. Thus, it would be of interest to determine if other components of the antiviral response (namely Dicer-2 and R2D2) are also rate-limiting in the Sofola *et al* model and, if so, whether these interactions are of a helpful or harmful nature.

When using the eye-based dsRNA pathology as a genetic screening tissue model, *Drosophila* lines containing loss-of-function mutations were not available for some candidate genes. When this was the case, RNAi-based knockdown lines (315, 316) were utilized to reduce expression of candidate genes. However, the important rate-limiting role of RNAi pathway component Dicer-2 (and to a lesser extent Ago2) in the expanded repeat dsRNA pathology means that the use of RNAi-based knockdown to reduce candidate gene expression can potentially interfere with observed changes to the dsRNA pathology. While no consistent phenotypes arose using RNAi knockdowns to implicate RNAi machinery as a confounding factor, the results were interpreted with this possibility in mind. In line with this, insertional mutations can also lead to the misexpression of adjacent genes (including the target itself) when combined with a GAL4 driver. Thus ideally, the findings observed using the dsRNA eye-based screen should be validated with endogenous loss-of-function mutations for each candidate gene if/when they become available. Finally, endogenous heterozygous mutations for a particular gene are not restricted to the specified tissue as dictated by GAL4-UAS expression (for example the eye, neurons or glial cells). Thus, it is possible that impacts on lethality/viability in these flies may be caused via global gene reduction as opposed to tissue-specific effects.

The cellular specificity of GAL4 promoter constructs used in this study, particularly those used to investigate the individual glial subtypes must also be considered. While the GAL4 driver lines used to express the dsRNA in glial cell subtypes were chosen based on their demonstrated specificity (292), one must consider the possibility of off-target tissue expression. Off-target expression and/or mosaicism have been reported in some of the glial cell subtype drivers (292, 309), though these effects are generally at low levels. Indeed, widely-used GAL4 drivers *GMR-GAL4* and *Elav-GAL4* also display trace expression in other tissue types (509, 511). Thus, while it is highly unlikely that any off-target tissue expression is of a sufficient magnitude to influence the work described in this study, the possibility of this must be factored into the interpretation of the results.

Finally, a note regarding the expanded repeat dsRNA *Drosophila* model used in this study. The production of dsRNA in this model is via co-expression of independent *rCAG*$_{\sim100}$ and *rCUG*$_{\sim100}$ constructs *in trans* (45), as compared to natural bi-directional transcription across

expanded repeat loci which occurs via *cis*-acting transcriptional machinery (21). Therefore, only the product(s) of bi-directional transcription across repeat loci, is modelled in this study. Indeed, it is the disease gene product ($rCAG_{\sim100}.rCUG_{\sim100}$), not the mechanism of bi-directional transcription itself, that is implicated in expanded repeat disease pathogenesis. Thus, this model provides an excellent method to investigate how the presence of repeat dsRNA leads to the disease pathology observed.

6.4 Future directions

This study has provided evidence that 'non-self' recognition of expanded repeat dsRNA by the innate inflammatory system contributes to the resultant dsRNA-mediated disease pathology within the *Drosophila* eye, and that co-expression of a viral suppressor protein (CrPV1A) can ablate this pathology. The key next step is to determine the pathway(s) that CrPV1A antagonizes to suppress the dsRNA pathology. The necrotic eye pathology observed suggests that a form of antiviral cell death is activated, however the nature (non-lytic vs lytic) and effectors of the pathway are yet to be determined. Thus, characterisation of cell death in the dsRNA model should be an important future focus. In line with this, the possibility of an interaction(s) between CrPV1A and Dicer-2 mediated antiviral signalling should be investigated, as it is likely to represent a key step of CrPV1A-driven pathology suppression. Of note, this may not necessarily constitute a direct inhibition of Dicer-2 itself by CrPV1A, but of downstream intermediary molecules (possibly including Ago2). Indeed, while the role of Dicer-2 as a pattern recognition receptor is well supported (53-55), little is known regarding the signalling components downstream of Dicer-2 that drive antiviral inflammation – for example, a functional equivalent of MAVS is yet to be identified in *Drosophila*. Thus, the characterisation of Dicer-2 signalling intermediates forms an essential next step in defining the inflammatory mechanism(s) that underlie the expanded repeat dsRNA pathology.

The finding that *Drosomycin* was significantly upregulated while *CG33926* was potently downregulated in response to the expression of repeat dsRNA is intriguing. Additionally, co-expression of *CrPV1A* did not ablate either of these alterations, suggesting that these pathways may represent protective mechanisms. Indeed, the flies tested were at an early

timepoint (0-1 days old), and as such it would be of interest to perform transcript analysis over an extended time course to determine if these changes correlate with the age-dependent neurodegeneration observed in *elavII>*dsRNA flies (45). Furthermore, performing functional assays to confirm that CrPV1A can ablate dsRNA pathology in the neuronal tissue model should be considered. Both lines of further analysis would be valuable in dissecting the antiviral pathways that underlie expanded repeat dsRNA pathogenesis from those that constitute protective responses.

Evidence that mitophagy is vital for restricting dsRNA pathology is consistent with the hypothesis that mitochondrial dysfunction is a key driving factor in neurodegenerative disease (139), and that the processes that govern mitochondrial quality control warrant further investigation with regards to expanded repeat disorders. Mitochondrial fission is responsible for the separation of healthy and dysfunctional mitochondria and precedes mitophagy, so it is plausible that this constitutes a method for the cell to partition the repeat dsRNA (along with other inflammatory triggers) into mitochondria that are targeted for degradation. Thus, the dsRNA eye pathology screen should be extended to investigate key regulators of mitochondrial fission/fusion to determine if this process operates upstream of mitophagy to limit dsRNA toxicity.

The glial subtype analyses regarding dsRNA-mediated neuronal dysfunction opens up a number of opportunities for further study, based on the normal functions of glial cells within the *Drosophila* CNS. Disruption to BBB development and/or maintenance appears a likely critical pathogenic event in the observed neuronal pathology, though evidence of BBB disruption needs to be confirmed in the affected flies. Determining whether peripheral inflammatory cells/molecules infiltrate the CNS following dsRNA challenge should be also considered and may provide insight into the role of systemic molecules in disease pathogenesis. Several glial subtypes within the CNS are capable of phagocytic function(s) (160) and it is plausible based on the results described in this study to suggest that they perform this process to remove repeat dsRNA and minimise the resultant neurotoxicity. Challenging these specific glial cells with the repeat dsRNA following inhibition of phagocytosis (for example through *Draper* ablation) will prove insightful in 1) determining if repeat dsRNA is indeed targeted for removal when present within the CNS, and 2)

confirming that glial-mediated phagocytosis is an important process for restricting dsRNA-mediated CNS pathology. Indeed, knowledge regarding glial function in both vertebrate and invertebrate systems is still only beginning to emerge. As such, it is anticipated that future research into glial cell biology will highlight additional glial functions that either suppress or promote dsRNA-mediated neuronal dysfunction.

A final note; the models utilized in this study recapitulate the expanded CAG.CUG repeat product predicted to form via bi-directional transcription across both CAG and CUG repeat loci. As such, the findings described in this study are directly relevant to the disorders caused through the expansion of a CAG or CUG repeat. However, repeat expansion disease can arise through the expansion of a number of other motifs, including 4, 5 and 6 nucleotide sequences (4). Therefore, a key overarching question is whether dsRNA derived from bi-directional transcription of these other repeat sequences leads to pathology in a manner similar to the CAG.CUG repeat described in this study. Indeed, bi-directional transcription has been observed across all repeat loci tested (21), and so the investigation of other RNA repeat sequences in double-stranded form will determine if dsRNA represents a common and targetable pathogenic feature in this devastating group of neurodegenerative disorders.

Appendix

Appendix A

van Eyk, C.L.*, Samaraweera, S.E.*, **Scott, A.*,** Webber, D.L.*, Harvey, D.P., O'Keefe, L.V., Cropley, J.E., Suter, C., Richards, R.I. (2019) **'Non-self' Mutation: Double-stranded RNA elicits antiviral pathogenic response in a *Drosophila* model of expanded CAG repeat neurodegenerative diseases** *Human Molecular Genetics* doi: 10.1093/hmg/ddz096

*Joint first authors.

The following submitted manuscript contains work described in Chapter 3. The work is described in full in the chapter, however the manuscript is included for reference and to place this work in the broader context of findings from the Richard's Lab. <u>The work included in this thesis was performed by A.S.</u>

Statement of Authorship

Title of Paper	'Non-self' Mutation: Double-stranded RNA elicits antiviral pathogenic response in a *Drosophila* model of expanded CAG repeat neurodegenerative diseases
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	-

Name of Co-Author (Candidate)	Andrew Scott		
Contribution to the Paper	Performed experiments with the CrPV1A full length and truncated constructs, sequenced CrPV1A constructs, verified genotypes of *Drosophila* lines via PCR, commented on the manuscript.		
Signature		Date	28/2/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

   i.   the candidate's stated contribution to the publication is accurate (as detailed above);

   ii.   permission is granted for the candidate in include the publication in the thesis; and

   iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Clare van Eyk		
Signature		Date	28/2/19

Name of Co-Author	Saumya Samaraweera		
Signature		Date	28/2/2019

Name of Co-Author	Dani Webber		
Signature		Date	28/2/19

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GENERAL ARTICLE

Non-self mutation: double-stranded RNA elicits antiviral pathogenic response in a *Drosophila* model of expanded CAG repeat neurodegenerative diseases

Clare L. van Eyk[1,†], Saumya E. Samaraweera[1,†], Andrew Scott[1,†], Dani L. Webber[1,†], David P. Harvey[1], Olivia Mecinger[1], Louise V. O'Keefe[1], Jennifer E. Cropley[2,3], Paul Young[2,3], Joshua Ho[2,3,4], Catherine Suter[2,3] and Robert I. Richards[1,*]

[1]Department of Molecular and Biomedical Science, School of Biological Sciences, The University of Adelaide, Adelaide, South Australia 5000, Australia, [2]Victor Chang Cardiac Research Institute, Lowy Packer Building, Liverpool St, Darlinghurst, Sydney 2010, Australia, [3]Faculty of Medicine, University of New South Wales, Kensington, New South Wales 2042, Australia and [4]School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong

*To whom correspondence should be addressed at: Department of Molecular and Biomedical Sciences, The University of Adelaide, Adelaide, South Australia 5000, Australia. Tel: +61 8 8313 7541; Fax: +61 8 8313 4362; Email: robert.richards@adelaide.edu.au

Abstract

Inflammation is activated prior to symptoms in neurodegenerative diseases, providing a plausible pathogenic mechanism. Indeed, genetic and pharmacological ablation studies in animal models of several neurodegenerative diseases demonstrate that inflammation is required for pathology. However, while there is growing evidence that inflammation-mediated pathology may be the common mechanism underlying neurodegenerative diseases, including those due to dominantly inherited expanded repeats, the proximal causal agent is unknown. Expanded CAG.CUG repeat double-stranded RNA causes inflammation-mediated pathology when expressed in *Drosophila*. Repeat dsRNA is recognized by *Dicer-2* as a foreign or 'non-self' molecule triggering both antiviral RNA and RNAi pathways. Neither of the RNAi pathway cofactors *R2D2* nor *loquacious* are necessary, indicating antiviral RNA activation. RNA modification enables avoidance of recognition as 'non-self' by the innate inflammatory surveillance system. Human ADAR1 edits RNA conferring 'self' status and when co-expressed with expanded CAG.CUG dsRNA in *Drosophila* the pathology is lost. Cricket Paralysis Virus protein CrPV-1A is a known antagonist of *Argonaute-2* in *Drosophila* antiviral defense. CrPV-1A co-expression also rescues pathogenesis, confirming anti-viral-RNA response. Repeat expansion mutation therefore confers 'non-self' recognition of endogenous RNA, thereby providing a proximal, autoinflammatory trigger for expanded repeat neurodegenerative diseases.

Introduction

Dynamic mutation is the common mechanism of expansion for existing repeat sequences that accounts for about 30 inherited diseases. In some cases the repeat expansion causes loss of gene function, and the resultant phenotype can be attributed to loss of function of the protein normally encoded by the affected gene (1). About 20 expanded repeat diseases are dominantly inherited and occur in otherwise unrelated genes, therefore they must gain some type of common attribute that causes diseases (2–4). These diseases share neurodegeneration as a common symptom, yet are typically distinguished by the neurons first affected and therefore the symptoms that lead to their initial clinical presentation and differential diagnosis. As the diseases progress their pathology tends to spread and so their symptoms converge, as overlapping neurons are affected (5).

Despite their identification more than two decades ago, the mechanism(s) by which these dominant mutations cause symptoms is/are as yet unclear. While most of these diseases only have an expanded repeat as their single mutation mechanism, the recent identification of repeat expansion in the C9orf72 gene has been a particularly notable addition (6,7). Repeat expansion in the C9orf72 gene is but one of numerous genetic causes of amyotrophic lateral sclerosis and/or fronto-temporal dementia (ALS/FTD). ALS/FTD causing mutations in other genes are not expanded repeats and in some cases involve the loss of protein function. Commonalities in the lost functions of the different ALS/FTD genes are therefore likely indicators of common pathways of pathogenesis. It is therefore noteworthy that several of these proteins have functions in RNA Stress Granule metabolism (TDP43, FUS, VCP, CHMP2B) (8–11) and innate inflammatory pathways (sequestosome 1 (p62), progranulin, optineurin, TBK1) (12–15).

Late age-at-onset neurodegenerative diseases are increasingly being found associated with activation of inflammation (16–22). In the past this association has been dismissed on the basis that the inflammatory machinery was just coming in to clean up after the proximal cause. However, where timing has been assessed the inflammatory activation precedes neuronal pathology. Presymptomatic Huntington's Disease (HD) gene mutation carriers have elevated circulating levels of cytokines (16,17). Mouse models of prion disease and Alzheimer's disease reveal in both cases that microglial cell activation precedes nerve cell pathology (23,24). The molecular mechanism for triggering inflammatory activation thereby causing neurodegeneration has not been clear.

Inflammation is the front line cellular defense mechanism that responds to a diverse variety of danger signals (25). Some of these danger signals or trigger molecules are components of pathogens, including RNA (22). In such cases the inflammatory surveillance relies on the molecular architecture to distinguish these 'non-self' molecules from the endogenous molecules in the cell. In addition, cells have resident, endogenous trigger molecules that are normally kept below levels required for inflammatory activation by one or other degradative processes or are masked by ligation to inhibitors. A recently reported example of such an endogenous trigger molecule is double-stranded (ds) *Alu* RNA, which is recognized by the IFIH1 pattern recognition receptor unless modified by the ADAR1 editing enzyme (26–28). Pathogens can therefore also be detected when their presence results in the increase and/or activation of such endogenous trigger molecules. Upon activation the inflammatory response has an early acute phase during which trigger molecules (that threaten the organism) are degraded in order to contain the pathogen and destroy its ability to replicate. If the threat is not eliminated, then a chronic phase is activated in order to alarm neighboring cells and prime their response. Chronic inflammation can include the programmed death of cell foci that are activated during the acute phase. While inflammation is effective in restricting exogenous pathogens, this mechanism is the cause of pathology when mutations lead to the perpetual synthesis of endogenous trigger molecules that lack 'self' architecture, such as repeat RNA. Mutations that impair the cell's degradative capacity or cause constitutive activation of pattern recognition pathways, such as those which increase receptor sensitivity (29), can also give rise to autoinflammatory disease (25).

Cells recognize the presence of viral RNA by means of either the absence of 'self' recognition patterns or the presence of 'non-self' patterns, or both, and in so doing distinguish potentially dangerous RNAs from endogenous RNAs. Specific pattern recognition receptors for RNA include the RIG-I-like receptors (RLRs) (30). The RLRs are members of a larger family of proteins that include *Dicer*, *RIG-I* and *IFIH1* (MDA-5) (Supplementary Material, Fig. S1). *Drosophila* has two members of the RLR family—*Dicer-1* and *Dicer-2*—with *Dicer-2* having been found to act as both a component of the RNAi machinery and a pattern recognition receptor for antiviral RNA response (31). The antiviral RNA response in *Drosophila* is heavily dependent upon *Argonaute-2* (32,33), but independent of *R2D2*, a co-factor in the RNAi pathway (31).

Previously, we and the others explored the hypothesis that one or other forms of expanded repeat RNA are pathogenic and have found that (CAG.CUG)$_{100}$ dsRNA is able to cause pathology when expressed in the *Drosophila* eye (34,35). Furthermore, this pathology is both dependent upon components of innate surveillance (TLRs and TLR signaling) and also activates the inflammatory response (as indicated by elevated *drosomycin* and TNF/*eiger*) (36). Pathology was found to be dependent upon *Dicer* and *Argonaute* proteins, and the (CAG.CUG)$_{100}$ RNA was cleaved down to 21mers of (CAG.CUG)$_7$. This latter finding appeared to indicate the participation of the RNAi machinery (34,35); however, herein we tested this hypothesis and found that key components of RNAi processing (*R2D2* and *loquacious*) are not only unnecessary for (CAG.CUG)$_{100}$ dsRNA pathology, but also appear to constitute an alternative pathology avoidance pathway. We have therefore pursued the hypothesis that (CAG.CUG)$_{100}$ dsRNA is recognized as 'non-self' RNA in the manner typical of viral RNA sequences. The human ADAR1 RNA-editing enzyme controls innate immune responses to RNA and when ectopically expressed in *Drosophila* rescues (CAG.CUG)$_{100}$ dsRNA pathology. Ectopic expression of cricket paralysis virus protein CrPV-1A, which facilitates viral infection by suppressing the antiviral cell death response (37–39), rescues (CAG.CUG)$_{100}$ dsRNA pathology in *Drosophila*.

Results

RNAi co-factors R2D2 and *loquacious* not required for dsRNA pathology

The requirement for (CAG.CUG)$_{100}$ dsRNA pathology on certain components of the RNAi machinery (specifically *Dicer-1*, *Dicer-2*, *Argonaute-2*) has previously been reported (34–36). Given the ability of *Drosophila Dicer* proteins to also act as pattern recognition receptors (32,33,40–46) we assessed the need for other components of the *Drosophila* RNAi machinery in (CAG.CUG)$_{100}$ dsRNA pathology. *R2D2* is an obligate component of the RNAi

pathway in *Drosophila* along with *Dicer-2* and *Argonaute-2* (47,48), therefore the role of *R2D2* in (CAG.CUG)$_{100}$ dsRNA pathology was assessed by its heterozygous deletion or Gal4/UAS over-expression.

RNAi has become a widely used experimental tool in *Drosophila*, as a means to investigate gene function in a particular pathway, by lowering levels of targeted RNAs. There is, however, substantial overlap between the RNAi pathway and the antiviral response mechanism in *Drosophila* (31,49). The common roles in these pathways played by *Dicer-2* and *Argonaute-2*, render the RNAi targeted reduction of candidate modifiers of the antiviral response problematic. The studies herein have therefore been restricted to the analyses of loss-of-function mutations (as heterozygotes) or Gal4-UAS directed ectopic over-expression of the relevant protein.

While *Drosophila* carrying a loss-of-function mutation in *Dicer-1* have reduced (CAG.CUG)$_{100}$ dsRNA pathology, those with loss of *R2D2* have enhanced pathology (Fig. 1A'–D'; Supplementary Material, Fig. S2). Furthermore, over-expression of *R2D2* rescues the (CAG.CUG)$_{100}$ dsRNA pathology (Fig. 1I' and J'; Supplementary Material, Fig. S2). The reciprocal effects of a more severe phenotype caused by reduction in *R2D2* and rescued phenotype with *R2D2* over-expression suggest that the pathway in which *R2D2* participates is actually in competition with that which causes (CAG.CUG)$_{100}$ dsRNA pathology. *Loquacious* also acts as an RNAi co-factor. Loquacious protein function is complicated by alternative splicing that gives rise to isoforms with distinct roles in the processing of different small RNA populations. *Loquacious* mutations can therefore differentially affect isoform functions (50,51). Nevertheless, neither of two independent *loquacious* mutations (*loqs^{KO}* and *loqs^{f00791}*) exacerbated expanded CAG.CUG repeat pathology (Fig. 1E' and F'), consistent with RNAi pathways being not required for dsRNA pathology but instead being a competing pathway to that which causes the pathology. Furthermore, miRNA sequence profiles were assessed in (CAG.CUG)$_{100}$ dsRNA expressing *Drosophila* to determine whether small RNA processing is altered. This analysis revealed minimal differences (+/− 1.2-fold change) in a small number of miRNAs, between flies expressing (CAG.CUG)$_{100}$ dsRNA versus control (Supplementary Material, Table S1). Those miRNAs (*miR-184, miR-263b, miR-274, miR-932, miR-1017*) showing altered levels were tested for the ability of increased or decreased levels of these miRNAs to alter pathology in *Drosophila* lines also expressing (CAG.CUG)$_{100}$ dsRNA and none were found to impact eye phenotype (Supplementary Material, Fig. S3).

Since both *Dicer* and *Argonaute* proteins are required for (CAG.CUG)$_{100}$ dsRNA pathology (Supplementary Material, Fig. S4) this suggests that these proteins work in concert to recognize (CAG.CUG)$_{100}$ dsRNA as a viral RNA and give rise to the resultant pathology through pattern recognition pathway instigated antiviral RNA response rather than the RNAi pathway in which *R2D2* and *loquacious* normally participate.

Human ADAR1 (but not human ADAR2) editing rescues dsRNA pathology

RNA modification is a major form of distinguishing 'self' from 'non-self' RNAs. RNA modifying enzymes typically recognize distinct sequence and/or structural features in the RNA. The expanded CAG repeat contains the normal recognition sequence of adenosine deaminase of RNA (ADAR) enzymes. These enzymes are also typically affected by the secondary structure of the RNA. The ds structure of (CAG.CUG)$_{100}$ dsRNA therefore represents a normal target for ADARs. The ADAR enzymes are also of great interest for their relationship with known RNA-binding pattern recognition receptors (IFIH1 in humans). Loss-of-function mutations in IFIH1 are able to rescue the phenotype caused by loss of ADAR1 in mice (51,52). Furthermore, loss-of-function mutation in human ADAR1 is found to be one genetic cause of the congenital neurodegenerative disease Aicardi-Goutieres Syndrome (AGS) (53). Gain-of-function mutations in the IFIH1 pattern recognition receptor are another cause of AGS (29), strongly implicating RNA modification in AGS pathogenesis.

ADAR1 mutations in some individuals give rise to bilateral striatal necrosis (54), an intriguing link to HD given that this brain region is typically the first to exhibit pathology in HD. Based on the finding that mutations in ADAR1 cause AGS and the substrate specificity of the ADAR enzymes it has been proposed that ADAR1 may limit the cytoplasmic accumulation of the dsRNA generated from genomic repetitive elements (55). The finding (56) of interferon-stimulated gene expression signatures in the heterozygous ADAR1 mutation carrier parents of affected offspring with both ADAR1 alleles mutated, suggests that ADAR1 is a rate-limiting determinant of interferon induction and innate inflammatory response. Therefore, given the role of ADAR1 in human pathology and CAG RNA sequence specificity of ADAR proteins, human and *Drosophila* ADAR enzymes were assessed herein for their impact on *Drosophila* (CAG.CUG)$_{100}$ dsRNA pathology.

RNAseq analysis of CAG$_7$mer from *Drosophila* expressing (CAG.CUG)$_{100}$ dsRNA alone revealed the activity of endogenous *Drosophila* ADAR on ∼6% of CAG$_7$mer RNAs, with most having a single edited A>I in the final CAG repeat (Fig. 2E). Thus, while CAG.CUG is a substrate for *Drosophila* ADAR this low level of editing suggests that it is either a poor substrate or the level of endogenous enzyme is rate-limiting. The *Drosophila* genome has a single *ADAR* gene, while three human genes encode *ADAR* proteins. Human *ADAR1* and *ADAR2* are well characterized and expressed, whereas *ADAR3* is not known to have catalytic function (57). Human *ADAR1* is differentially expressed with alternative splicing giving rise to constitutive (*hADAR1c*) and interferon-induced (*hADAR1i*) forms (58). We therefore tested the ability of both human *hADAR1c* and *hADAR1i* as well as *hADAR2* and the *Drosophila* isoform *dADAR* to affect the (CAG.CUG)$_{100}$ dsRNA pathology.

Co-expression with either human *hADAR1c* or *hADAR1i* along with (CAG.CUG)$_{100}$ dsRNA saw a complete rescue of pathology (Fig. 2B' and C'). Little effect was seen with human *ADAR2* (Fig. 2F'), while alteration in *Drosophila* ADAR levels also had minimal impact (Fig. 2G'), consistent with *dADAR* being more closely related to *hADAR2* than *hADAR1* (59). Co-expression of *Dicer-2* with (CAG.CUG)$_{100}$ dsRNA causes lethality in the S1 line; however, lethality and eye phenotype were completely rescued by co-expression of *hADAR1i* (Supplementary Material, Fig. S5). Steady state levels of (CAG.CUG)$_{100}$ dsRNA were determined by quantitative RT-PCR on triplicate RNA samples from flies expressing (CAG.CUG)$_{100}$ dsRNA and either human ADAR1 or ADAR2 or *Drosophila* ADAR (Supplementary Material, Fig. S6). While all gave lower levels than flies expressing (CAG.CUG)$_{100}$ dsRNA alone, the comparable level for flies also expressing hADAR1, hADAR2 or dADAR indicates that lower steady-state RNA levels alone do not account for phenotype rescue seen with hADAR1, as hADAR2 and dADAR have no phenotype rescue. ADAR1 is therefore specifically capable of masking 'non-self' status upon (CAG.CUG)$_{100}$ dsRNA such that it no longer induces inflammatory pathology.
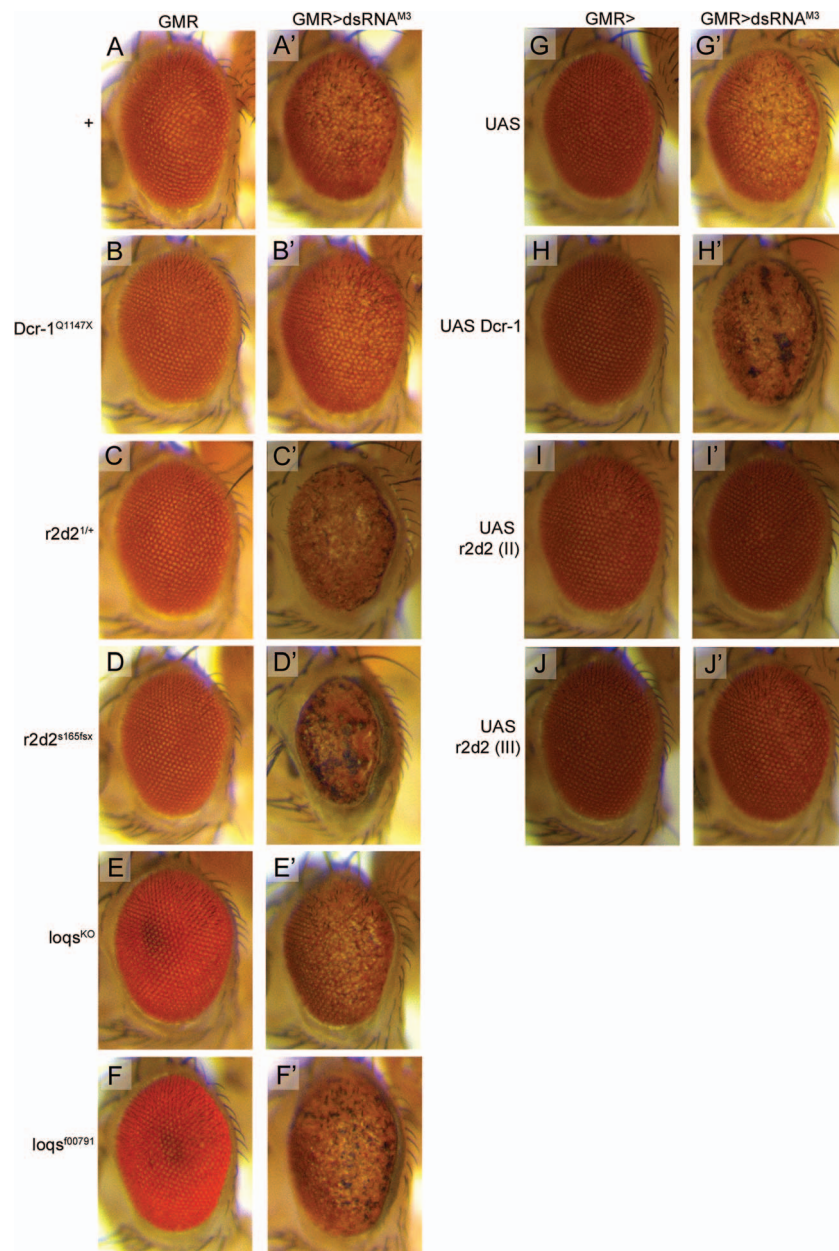
Figure 1. *R2D2* is not required for the dsRNA phenotype in the *Drosophila melanogaster* eye. In each case the *GMR-GAL4* driver is used to express the control or candidate alone (**A–L**) and together with rCAG.rCUG$_{\sim 100}$ M3 dsRNA line (**A'–L'**). (A) *GMR-GAL4* does not have an obvious phenotype alone. (A') Expression of rCAG.rCUG$_{\sim 100}$ dsRNA results in disruption to ommatidial patterning and loss of pigmentation. (B) Reducing *Dicer-1* levels using *Dcr1*^{Q1147X}/+ does not alter the phenotype of the eye. (B') Reducing *Dicer-1* levels while co-expressing dsRNA results in a suppression of the dsRNA rough eye phenotype (when compared to A'). (C and D) Reducing *r2d2* levels using *r2d2*^{1} or *r2d2*^{s165fsx} do not alter the phenotype of the eye. (C' and D') Heterozygous loss-of-function mutants *r2d2*^{1} or *r2d2*^{s165fsx} flies with *GMR-GAL4* driven expression of rCAG.rCUG$_{\sim 100}$ dsRNA results in an enhanced eye phenotype with increased regions of necrosis (when compared to A'). (E and F) Heterozygous loqs^{KO} and loqs^{f00791} have no eye phenotype alone. (E' and F') The dsRNA phenotype is enhanced by each of the loqs mutants (when compared to A'). (G) *GMR-GAL4* does not have an obvious phenotype alone. (G') Expression of rCAG.rCUG$_{\sim 100}$ dsRNA results in disruption to ommatidial patterning and loss of pigmentation. (H) *GMR-GAL4* driven expression of *UAS-Dcr-1* has no eye phenotype alone but (H') enhances rCAG.rCUG$_{\sim 100}$ dsRNA eye phenotype (when compared to G'). (I and J) Over-expression of *r2d2* with either of two independent *UAS-r2d2* lines (II or III) does not affect the normal patterning of the eye. (I' & J') Co-expression of either these *UAS-r2d2* over-expression constructs together with dsRNA rCAG.rCUG$_{\sim 100}$ suppresses the dsRNA phenotype, restoring both regular patterning and pigmentation to the eye (when compared to G').

Copy number requirement for expanded repeat (CAG.CUG) dsRNA pathology

The antiviral RNA defense mechanism involves the binding of multiple RIG-I-like pattern recognition receptors along single molecules of RNA to form a filament structure that is then bound by mitochondrial antiviral signaling protein (MAVS) (60,61). A length requirement for the RNA molecule appears related to the need for such filaments to exceed a threshold for MAVS binding and/or activation (61,62). Therefore, experiments were undertaken to assess the length dependence of the expanded CAG.CUG repeat to cause antiviral-mediated pathology.
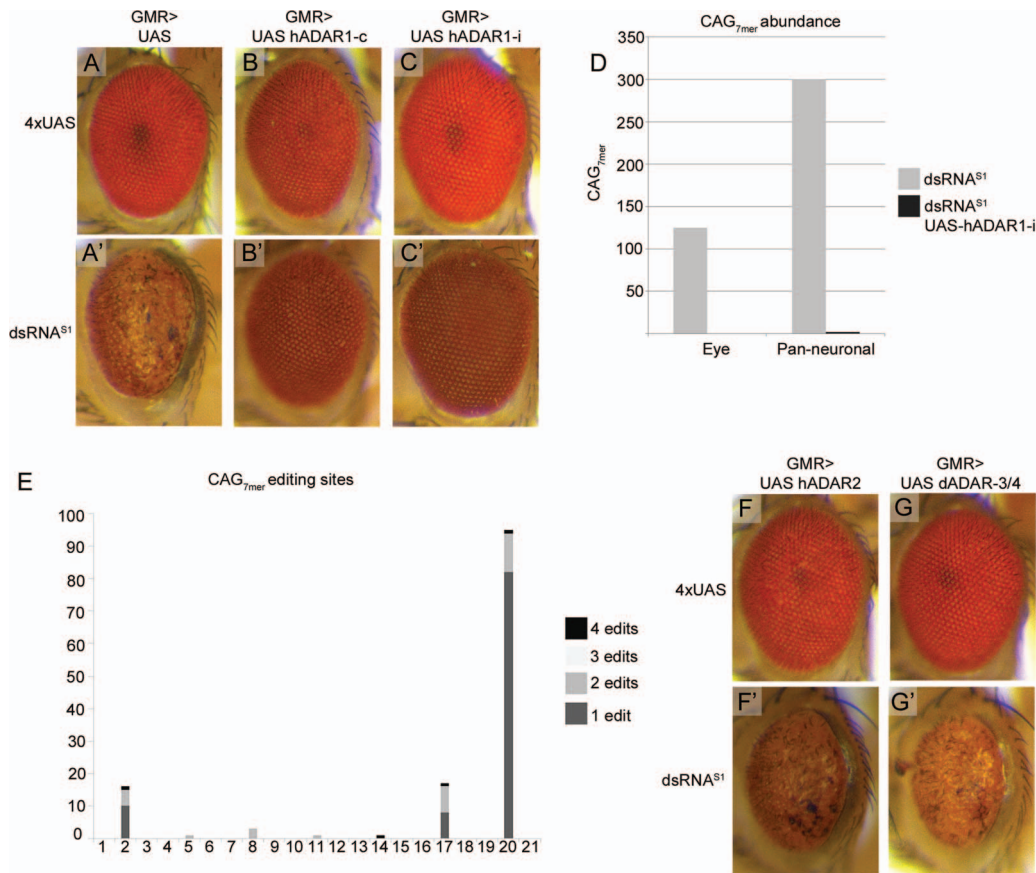
Figure 2. ds RNA pathology is dependent on editing by *ADAR*. GMR-GAL4 was used to express constructs in the eye or ELAV(II)-GAL4 to drive expression in all neurons. Ectopic-expression of either hADAR1-c or hADAR1-i reduce the rCAG.rCUG~100 dsRNA eye phenotype. (**A**) *GMR-GAL4* driven expression of 4xUAS with no repeat construct has no phenotype. (**A'**) *GMR-GAL4* driven expression of rCAG.rCUG~100 causes disruption to patterning of the eye and loss of pigmentation (line S1). (**B** and **C**) *GMR-GAL4* driven expression ectopic-expression of either constitutive (*hADAR1-c*) or interferon inducible (*hADAR1-i*) human *ADAR1* isoforms does not disrupt the patterning of the eye. (**B'** and **C'**) *GMR-GAL4* driven co-expression of either *hADAR1-c* or *hADAR1-i* together with rCAG.rCUG$_{\sim 100}$ rescues the rCAG.rCUG$_{\sim 100}$ eye phenotype (when compared to A'). (**D**) RNAseq of hADAR1 rescued eyes/neurons (GMR/Elav). CAG cleavage products (CAG$_7$mer) are detected following *GAL4* driven expression of rCAG.rCUG$_{\sim 100}$ in the either the eye (GMR) or in all neurons (pan neuronal, Elav). CAG$_7$mer abundance is significantly reduced in either tissue following *GAL4* driven co-expression of *hADAR1-i* with rCAG.rCUG$_{\sim 100}$. (**E**) RNAseq of CAG$_7$mer showing editing of final A in 6% RNAs. CAG$_7$mer is A>I edited in 6% of RNAs predominantly at position 20 (the last CAG). In the minority of CAG cleavage products that are not 21mers that are edited, it is usually the most 3' A residue that is edited, suggesting endogenous *dADAR* editing is targeted to the last A in the sequence. (**F** and **G**) Rescue is specific to hADAR1. *GMR-GAL4*-driven expression ectopic-expression of either human *hADAR2* or *Drosophila dADAR-3/4* does not disrupt the patterning of the eye. (**F'** and **G'**) *GMR-GAL4* driven expression ectopic-expression of either human *hADAR2* or *Drosophila dADAR-3/4* does not reduce the eye phenotype caused by co-expression of rCAG.rCUG$_{\sim 100}$ (when compared to A').

The *Drosophila* model of expanded CAG repeat disease utilized herein has previously been found to exhibit expanded dsRNA pathology for 100 copies of the repeat (34). Using a similar *Drosophila* model, Yu *et al.* (35) have also found that (CAG)$_{250}$ and (CUG)$_{250}$ RNAs cause pathology when co-expressed in the eye, whereas the (CAG)$_{34}$.(CUG)$_{19}$ combination of RNAs (below the human disease threshold) do not. Therefore, the ability of shorter length (CAG.CUG) repeats of 50 copies to cause pathology was assessed. This copy number is well below the putative disease threshold (~100 copies) (63) but above the DNA instability threshold (~36 copies) for HD and related expanded CAG repeat diseases. Fifty CAG.CUG repeats (150 bases) is also well above that required for RNAi processing, as a ds repeat length of 19–29 base pairs is sufficient for *Dicer*-mediated cleavage (64). Multiple lines of *Drosophila* were established that express four UAS-transgenes, two encoding (CAG)$_{50}$ RNA and the other two (CUG)$_{50}$ RNA. None of these (CAG.CUG)$_{50}$ expressing lines exhibited pathology even though comparable levels of (CAG.CUG) RNA were expressed (Fig. 3). The (CAG.CUG)$_{100}$ lines exhibited much greater pathology when *Drosophila Dicer* proteins were also ectopically over-expressed (25), so *Dicer-2* was also ectopically over-expressed in (CAG.CUG)$_{50}$ lines. Even in the presence of excess *Dicer-2* in no case was pathology observed for (CAG.CUG)$_{50}$, in clear contrast to *Dicer-2* increasing pathology in (CAG.CUG)$_{100}$ lines (Fig. 3B'–E'). Therefore, the dsRNA pathology that we have observed in this *Drosophila* model exhibits length dependence typical of that required for the antiviral RNA response, as well as copy number dependence consistent with that in the human expanded CAG.CUG repeat diseases (63).

Cricket paralysis virus CrPV-1A suppressor of *Argonaute* 2 rescues (CAG.CUG)$_{100}$ dsRNA pathology

Length dependence for expanded repeat dsRNA pathology is consistent with the antiviral recognition mechanism where multiple pattern recognition receptor molecules bind to long ds RNA molecules, forming a filament structure. This filament assembly takes place in RNA stress granules and if unresolved (by granulophagy) brings about MAVS-mediated inflammatory activation,
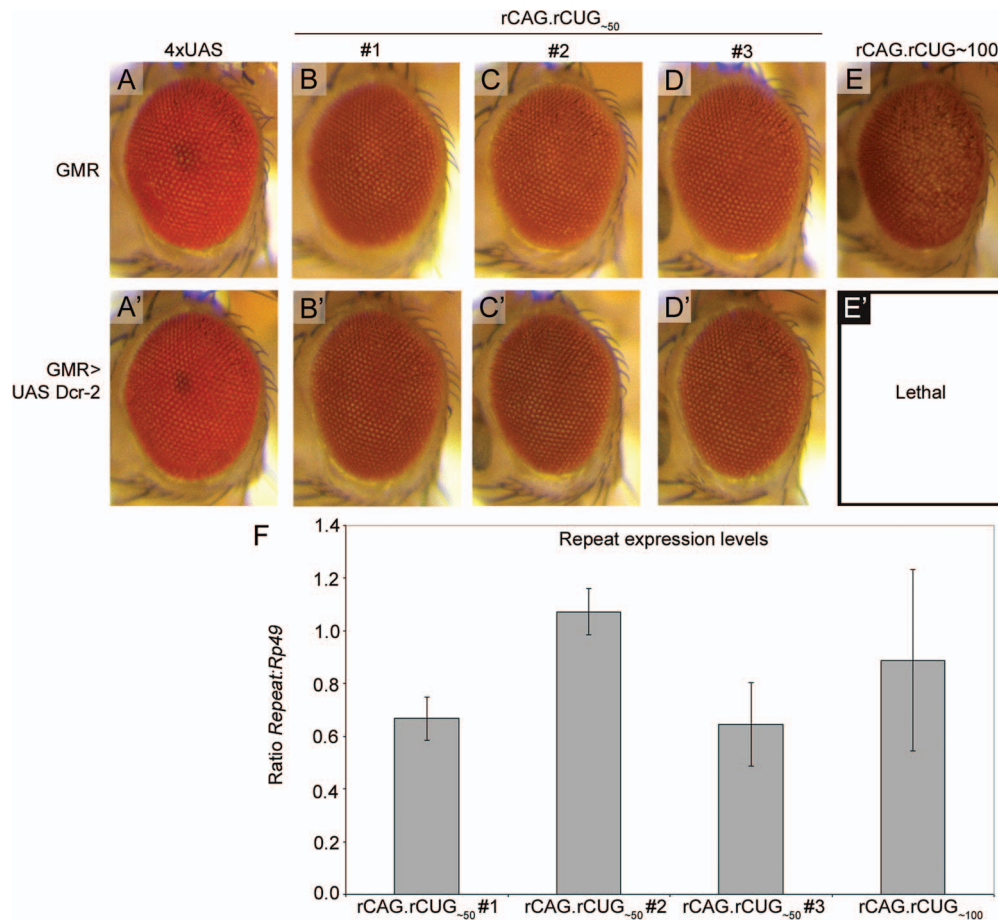
Figure 3. Fifty copies of CAG.CUG are insufficient to cause pathology, even in the presence of increased levels of *Dicer-2*/ ds RNA pathology is length dependent. In each case the *GMR-GAL4* driver is used to express the UAS constructs in the eye. (**A**) Expression of 4xUAS with no repeat construct has no obvious phenotype. (**A'**) Ectopic over-expression of *Dicer-2* also has no phenotype in the eye. (**B–D**) Three independent lines expressing rCAG.rCUG$_{\sim50}$ dsRNA do not affect the patterning of the eye (when compared to A). Line #1 – rCAG$_{\sim50}$ [M1,M7]; rCUG$_{\sim50}$ [M5,M9], line #2 – rCUG$_{\sim50}$ [M4,M8]; rCAG$_{\sim50}$ [M2,M4] and line #3 – rCAG$_{\sim50}$ [M3,M9]; rCUG$_{\sim50}$ [M2,M3]. (**B'–D'**) Co-expression of *Dicer-2* together with each of the three independent lines of rCAG.rCUG~50 dsRNA also does not affect the patterning of the eye (when compared to A'). (**E**) Expression of a mild line of rCAG.rCUG$_{\sim100}$ (M5) results in loss of patterning and pigmentation. (**E'**) Co-expression of *Dicer-2* together with rCAG.rCUG$_{\sim100}$ (line M5), results in lethality indicating an enhancement of the phenotype. (**F**) qRT-PCR levels of *GMR-GAL4* driven expression of repeat RNA transcripts in independent rCAG.rCUG$_{\sim50}$ lines compared to rCAG.rCUG$_{\sim100}$ (M5 line). Steady-state rCAG.rCUG repeat RNA levels measured relative to *Rp49*. PCR primers sequences are given in Supplementary Material, Table S2.

including cell death. The hypothesis that the antiviral cell death mechanism is responsible for expanded repeat dsRNA pathology was therefore tested by co-expression with a viral protein that inhibits this mechanism (38).

Insect viruses have evolved an effective ablation of the host antiviral defense mechanism. RNA silencing endonuclease *Argonaute-2* mediates specific antiviral immunity in *Drosophila melanogaster* (32). CrPV escapes the antiviral response by encoding an inhibitor of *Argonaute-2*. The CrPV suppressor (CrPV-1A) interacts with the endonuclease *Argonaute-2* and inhibits its activity without affecting microRNA (miRNA)-Ago1-mediated silencing (37). We therefore crossed two independent lines of flies ectopically expressing CrPV-1A with fly lines expressing (CAG.CUG)$_{100}$ dsRNA—one exhibiting moderate (M3) and two exhibiting strong (S1 and S2) eye phenotype. In each case (CAG.CUG)$_{100}$ dsRNA pathology was completely rescued (Fig. 4; Supplementary Material, Fig. S7) consistent with the antiviral RNA functions of *Argonaute-2* (and *Dicer-2*) being required for (CAG.CUG)$_{100}$ dsRNA pathology.

Discussion

The pathogenic mechanism by which expansion of existing DNA repeat sequences brings about dominantly inherited neurodegenerative diseases has been elusive. Indeed, it has been unclear whether there are multiple such pathways, distinct for each expanded repeat and its cognate gene, or whether there is a common mechanism that the different diseases share in a common chain of causality. These expanded repeat diseases have diverse repeat motifs located in unrelated genes. However, in all cases the repeat is transcribed, therefore RNA could be a common causal agent.

Expanded repeat RNA pathology has been modeled in *Drosophila* and ds expanded repeat RNA has indeed been found to induce pathology (34,35). For all expanded repeat disease loci tested, transcription is found on both strands, raising the possibility that ds expanded repeat RNA could be the culprit in these diseases (65). Indeed, human cells normally contain dsRNA formed from co-expressed sense and antisense transcripts (66).
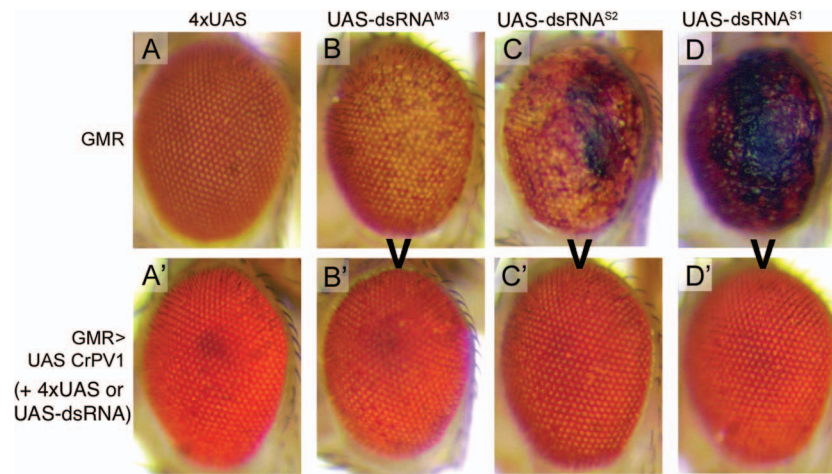
Figure 4. Cricket paralysis viral protein (*CrPV-1A*) antagonist of *Argonaute* ablates rCAG.rCUG$_{\sim100}$ dsRNA pathology when co-expressed in the *Drosophila* eye. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. (**A**) Expression of 4xUAS with no repeat construct has no obvious phenotype. (**A'**) Ectopic expression of *CrPV-1A* alone has no phenotype in the eye. (**B–D**) Lines of *Drosophila* expressing rCAG.rCUG$_{\sim100}$ dsRNA exhibit variable eye phenotype with one mild (B line M3) and two strong lines of flies (C, line S1; D, line S2) chosen to assess *CrVP-1A* impact. (**B'–D'**) in all three lines expressing rCAG.rCUG$_{\sim100}$ dsRNA, together with the co-expression of *CrVP-1A* exhibit complete inhibition of rough eye phenotype.

Furthermore, cytoplasmic ds expanded repeat RNAs have been detected in the brains of individuals affected with C9ORF72 repeat expansion caused ALS/FTD (67).

Dicer and *Argonaute* proteins are necessary for *Drosophila* expanded repeat dsRNA pathology (34,35) (Fig. 1; Supplementary Material, Fig. S4). These observations together with cleavage of long RNAs down to (CAG.CUG)$_7$ 21mers, have been interpreted as the RNAi pathway being responsible and the 21mers the causal agent. This hypothesis is tested herein by assessing the requirement for further components of the RNAi pathway, *R2D2* and *loquacious*. In both cases reduction of these co-factors led to an increase in pathology. Indeed, not only does reduction of *R2D2* increase pathology, but also an increase of *R2D2* reduces pathology. These relationships are consistent with the RNAi pathway and cleavage of the (CAG.CUG)$_{100}$ RNA to 21mers being in competition with the responsible pathogenic pathway. Since the *Dicer* and *Argonaute* proteins are also required for antiviral RNA-mediated cell death we hypothesize that this pathway is the one responsible for (CAG.CUG)$_{100}$ RNA pathology.

Both the activation of, and requirement for, the innate inflammatory surveillance system, have been reported in this *Drosophila* model of expanded repeat dsRNA pathology (36). Inflammatory activation correlates with various neurodegenerative diseases but has been typically thought of as a consequence of cell death—coming in to clean up the mess that something else has started. There is, however, increasing evidence that inflammatory activation precedes neurodegeneration, consistent with a causal role both in the human diseases (16,17) and in experimental animal disease models (23,24).

Nucleic acids are the common means of encoding genetic information for both hosts and pathogens, therefore innate host defense requires a mechanism to distinguish harmless endogenous nucleic acids from those that are foreign and potentially harmful. A large number and variety of modification reactions alter specific nucleotides in host nucleic acids to essentially 'barcode' those that are 'self' and allow their distinction from those that are 'non-self' or foreign. RNA editing is a form of modification in which the coding potential of the target nucleotide is changed, most notably the sequence specific conversion of adenosine to inosine in RNA sequences, catalyzed by adenosine deaminase of RNA (ADAR). Editing of endogenous RNAs by ADAR1 is required to prevent activation of the cytosolic innate immune system (51–54,68).

In the expanded CAG repeat diseases, ADAR editing is of particular interest as the recognition motif/modification site is contained in the expanded CAG repeat. Furthermore, ADAR enzymes have a general preference for dsRNA. Therefore, expanded CAG.CUG dsRNA is an ideal target for ADAR editing. The ability of human ADAR1, but neither human ADAR2 nor *Drosophila* ADAR, to confer 'self' status on expanded CAG.CUG dsRNA is noteworthy as ADAR1 is a known component of inflammatory activation (51–54,68). The human ADAR1 gene has two promoters, one for constitutive expression and the other interferon-inducible (69). The acute inflammatory response produces a pulse of interferon signaling as one of its 'feed-forward' responses aimed at resolving the trigger molecules/danger signals that activate inflammation. Interferon-induced increase in ADAR1 levels therefore enables the editing of RNAs, thereby reducing the level of 'non-self' RNA signal. Together with induced increases in RNA turnover processes, this mechanism can degrade RNA trigger molecule/danger signal threats.

ADAR1 also has a functional relationship with the RIG-I-like pattern recognition receptor *IFIH1* (*MDA5*), a member of the same family of proteins as *Dicer* (51,52). Loss-of-function mutations in *ADAR1* cause early lethality in mice that can be rescued by deletion of *IFIH1*. *ADAR1* is responsible for the editing of endogenous RNAs to enable their recognition as 'self' (27,28,70). Absence of *ADAR1* sees the 'non-self' forms of these RNAs recognized by *IFIH1* that then activates inflammation (26,28). The endogenous RNAs recognized by *IFIH1* include not only *Alu* dsRNA but also products of the unfolded protein response (UPR) pathway. One of the UPR response proteins, IRE-1, cleaves RNAs in a manner that renders them inducers of *IFIH1*. Loss-of-function mutations in ADAR1 and gain-of-function mutations in IFIH1 are both causes of AGS, a congenital disorder that includes neurodegeneration, with affected newborns having the appearance of being subjected to *in utero* inflammatory injury (53,71). The gain-of-function mutations in *IFIH1* that cause AGS also increase the affinity of this PRR for its RNA ligands (29).

The sensitivity to ADAR1 editing of the pathology caused by (CAG.CUG)$_{100}$ dsRNA in this *Drosophila* model is therefore consistent with the recognition of the product of repeat expansion mutation as a 'non-self' RNA trigger molecule/danger signal that then activates inflammation. 'Non-self' driven antiviral RNA recognition is an important process for virus removal as evident by the finding that HIV evades this process by recruiting the RNA 2′-O-methyltransferase FTSJ3 to avoid innate immune sensing (72).

A growing body of evidence is consistent with there being a distinction between repeat instability threshold and a greater copy number threshold for pathology (1,63,73–80). Genetic variations in mismatch repair enzymes have been shown to be major modifiers of age-at-onset of Huntington's Disease (75) and other polyglutamne diseases (76), as well as mouse models of these diseases (74,77–80). These enzymes control the rate of increase by somatic mutation from its inherited (germ-line) copy number and imply the existence of a higher copy number disease threshold of approximately 100 copies (63). Copy numbers above 36 are unstable, with the shorter unstable repeats (e.g. 40 copies) taking longer to reach the disease threshold than inherited 80 copy alleles (typically associated with juvenile HD) (1,63). Such a mechanism explains *genetic anticipation*: the inverse relationship between inherited repeat copy number and age-at-onset of disease symptoms (1,81). The expanded (CAG.CUG) dsRNA pathology in this *Drosophila* model exhibits length dependence consistent with the antiviral RNA response mechanism being responsible. In addition, the observed copy number dependence is consistent with distinct thresholds for DNA instability and pathology in the human expanded CAG repeat diseases (63).

In order to be infectious, viruses must evade detection and/or response mechanisms of the innate surveillance system. This response can include the programmed death of cells if the viral threat is not degraded. Cell death is orchestrated in both the cell in which the threat is detected and in adjacent sensitized cells to give a focal 'scorched earth' response that denies the virus both the opportunity to replicate and to infect adjacent cells. CrPV has evolved a mechanism of specific interference with the anti-viral RNA response. The CrPV-1A protein is a specific inhibitor of *Argonaute* and RNA stress granule formation (37,39). Stress granules are the location of filament formation between viral RNA and the RIG-I-like receptors. In vertebrates these filaments are then bound by the protein MAVS on the mitochondrial outer surface that in turn activates the antiviral response (60,61). Co-expression of CrPV-1A with expanded CAG.CUG repeat dsRNA completely rescues the pathology otherwise caused by this dsRNA. Therefore, the same cellular defense pathway of cell death that is normally utilized against viruses (and evaded by CrPV) is responsible for the pathology caused by the presence of expanded CAG.CUG repeat dsRNA, indicating its recognition by the cell as a viral 'non-self' RNA, a gain-of-function that accounts for dominant inheritance.

Inflammatory activation, with increased circulating cytokines (16) and microglial activation (17) precedes neuronal pathology in premanifest HD carriers. Inflammatory genes are activated in cells that are destined to undergo neurodegeneration. These include the induction of interferon-regulated genes in the cataracts of patients with myotonic dystrophy due to expansion of either CUG (DM1) or CCUG (DM2) (18). Increased inflammatory gene expression is evident in HD brain (19) and inflammatory gene signatures found in ALS motor neurons (20). Inflammation is activated at the right time and place to be the causal mechanism of disease.

In addition to the requirement for inflammatory activation in the expanded CAG.CUG model described herein (36), other animal models of dominantly inherited neurodegenerative diseases caused by the expansion of various repeat motifs have shown dependence on components of inflammation for pathology. Loss of MyD88 attenuates early Purkinje cell loss in a spinocerebellar ataxia type-6 mouse model (82), while p62 plays a protective role in polyglutamine disease model flies (83). Furthermore, deficiency of toll-like receptors 2, 3 or 4 extends life expectancy in HD mice (84). Genetic ablation of inflammation typically results in rescue of disease phenotypes—indicating that inflammatory activation is required for pathogenesis. While the agent causing inflammatory activation has not been identified in these latter cases, a zebrafish model for C9orf72 ALS/FTD reveals RNA as a pathogenic agent (85). In addition, many of the genes mutated in non-repeat expanded cases of ALS have roles in inflammatory activation pathways and RNA metabolism. Furthermore, their mutation typically leads to an increase in inflammatory activation (12,15).

Repeat expansions are therefore 'non-self' mutations that convert expanded repeat RNA from a harmless molecule when in low copy number to a nucleic acid that lacks the molecular architecture to be distinguished as 'self'. Expanded repeat RNA that exceeds a copy-number threshold is a persistent trigger of pattern recognition receptor signaling and inflammatory responses. Preventing the detection of the proximal repeat RNA trigger by either masking the 'non-self' RNA (with overexpression of human ADAR1) or blocking viral-detection pathways (with co-expression of a viral antagonist) both prevent pathology. Elongated repeat sequences are conducive to multimerisation of repeat structures that are known to trigger chronic inflammatory activation. These characteristics not only account for autoinflammatory cell death being the pathogenic mechanism for neurodegeneration, they are also consistent with the age-related somatic mutation of inherited (but sub-pathogenic) unstable copy numbers being the rate-limiting determinant of disease onset.

While studies herein describe the acquisition 'non-self' recognition by RNA, this principle can also apply to other gene products. For example, structures adopted by prion proteins activate inflammation (23) and glycated forms of proteins are preferential activators of the RAGE pattern recognition receptor (86). 'Non-self' mutation can include alteration in structure or post-translational modification that confers pattern receptor recognition. 'Non-self' mutation therefore provides a specific mechanism for pathogenic activation of inflammation that can account for numerous neurodegenerative diseases.

Materials and Methods

Fly husbandry

All crosses were performed at 25°C with 70% humidity unless otherwise indicated. *Drosophila melanogaster* stocks were maintained on fortified (F1) medium composed of 1% (w/v) agar, 18.75% compressed yeast, 10% treacle, 10% polenta, 1.5% acid mix (47% propionic acid, 4.7% orthophosphoric acid) and 2.5% tegosept (10% para-hydroxybenzoate in ethanol).

Fly stocks

Controls stocks used were *4xUAS* (empty vector) as we have previously described (34), *UAS-LacZ*[RNAi] was kindly provided by Richard Carthew (87) and *UAS-mCherry* [RNAi] was obtained from

Bloomington Drosophila Stock Centre (BDSC). Driver lines used were *GMR-GAL4* (76) and *P{GAL4-elav.L}2/CyO* (referred to as *elavII-GAL4*) (88). Pathogenic dsRNA *rCAG~100~.rCUG100~100~* lines used are as previously described (34). *Dcr-1^Q1147X^* and *Dcr-2^L811fsx^* (89) as well as *UAS-Dcr1, UAS-Dcr2* and *UAS-r2d2* (90) were kindly provided by Richard Carthew, as were *UAS-AGO2* (II) and *UAS-AGO2* (III) (90) lines. *AGO2^414^* (91) was obtained from the Kyoto stock centre. *AGO2^V966M^* (92) was obtained from the BDSC. *r2d2^1^* (47), *r2d2^s165fsx^* (93) and *loqs^f00791^* (94) were obtained from BDSC. *loqs^KO^* was kindly provided by Qinghua Liu and Dennis McKearin (95). *UAS-miR-184* and *ΔmiR-184* were kindly provided by Ulrike Gaul (96). *UAS-miR-263b and ΔmiR-263b* were kindly provided by Stephen Cohen (97). *UAS-miR-1017* was kindly provided by Eric Lai (98). D*f(3L)BSC577* (including *miR-274*) and D*f(3L)BSC577* (including *miR-932*) were obtained from BDSC. *UAS-hADAR-1c, UAS-hADAR1i* and *UAS-hADAR2* (99) and *UAS-dADAR* (100) were kindly provided by Liam Keegan. *ADAR^1F1^* was kindly provided by Michael Palladino (101). CrPV1A lines (39) were obtained from Christophe Antoniewski. Genotypes of fly lines were confirmed by PCR (Supplementary Materials, Fig. S8 and Table S2 of oligo PCR primers).

Generation of untranslated CAG~50~ and CUG~50~ repeat constructs

Generation of (rCAG.rCUG)~100~ lines is described in Lawlor *et al.* (34). CAG~50~ repeats were digested from pBluescript CAG~50~ (102) using flanking Hind III. This fragment was then ligated into the Hind III site of pENTR/D-TOPO IVM2 MYC FLAG. The presence of the insert was confirmed by restriction digestion with Acc65I and XbaI. Plasmids were also sequenced and screened for insert orientation and integrity. Constructs containing either CAG~50~ or CTG~50~ were subcloned into pDEST-UAST by LR clonase recombination. Constructs were confirmed by digestions with the restriction enzymes BsrG I and BamH I and by sequencing.

Generation of transgenic lines

Microinjections to generate transgenic lines were performed by BestGene Inc., Chino Hills, CA, USA. Transformants were mapped to determine the chromosome of insertion and balanced stocks were generated by standard genetic crosses. Single insertion lines for either the CAG~50~ or CTG~50~ constructs were labeled rCAG~50~ M1-10 and rCUG~50~ M1-10.

Generating recombinant lines

Recombinants carrying two independent insertions of the same transgene (either rCAG~50~ or rCTG~50~) on the same chromosome were generated by crossing trans-heterozygous virgin females to male *w^1118^* flies. Standard genetic crosses were then performed to generate three lines each carrying four transgenes, i.e. two expressing rCAG.2 and two expressing rCUG~50~, as follows:

rCAG.rCUG~50~#1 – rCAG~50~ [M1,M7]; rCUG~50~ [M5,M9]
rCAG.rCUG~50~#2 – rCUG~50~ [M4,M8]; rCUG~50~ [M2,M4]
rCAG.rCUG~50~#3 – rCAG~50~ [M3,M9]; rCUG~50~ [M2,M3].

RNA isolation and quantitative real-time PCR

Expression in the fly eye enables qualitative assessment of the phenotypic consequences of transgene expression, however the

GMR promoter only drives RNA expression in a small proportion of cells (even though some of these are nerve cells) with the majority of non-expressing cells of the fly head effectively diluting out responses. Therefore, as in previous studies in this model (34,36), RNA was isolated from the whole heads of either *GMR-Gal4* (eye) or *ElavII-Gal4* (pan-neuronal) expressing fly lines of various UAS-transgene genotypes and subjected to quantitative reverse transcriptase real-time PCR (qRT-PCR) as described (34,36). Approximately 100 male *Drosophila* heads from newly eclosed flies were collected and snap frozen for each genotype, before homogenization in Trizol (Invitrogen). Total RNA was extracted using chloroform and precipitated with isopropanol, then further purified using the RNeasy mini kit (Qiagen). RNA to be used for RNAseq was precipitated in sodium acetate and ethanol and shipped under ethanol on ice. 1 µg of total RNA per sample was treated with DNAse I (Invitrogen) and reverse-transcribed with oligo(dT)18 and SuperScript III (Invitrogen). Quantitative real-time PCR was performed in a LightCycler (Roche Molecular Biochemicals) using Power SYBR green mix (Applied Biosystems) and either GAL4-specific primers (forward: 5′-CACTGACCCCGTCTGCTTTG-3′, and reverse: 5′-GGTTCGGACCGTTGCTACTG-3′) or primers specific for the repeat-containing transcript. The transgene expression level was quantified using the delta Ct method for relative quantification and expressed relative to the level of GAL4 transcript for each line.

The quantity of amplified cDNA product for each primer pair was normalized to the quantity of product with primers for the housekeeping gene, ribosomal protein 49 (Rp49), for the same sample. The average of triplicate reactions for each sample was obtained and the standard deviation of the mean was calculated. The significance between samples was determined by using a two-tailed Student's *t*-test. The significance threshold was set at $P < 0.05$.

RNAseq identification of CAG 21-mer repeat editing and changes in miRNA profiles

RNAseq was as previously described in Samaraweera *et al.* (36). Barcoded small RNA libraries were prepared from 1 µg total RNA isolated from neurons and eyes using the NEBnext Small RNA Library Prep set for Illumina (NEB). 2S RNA was depleted at the RT primer hybridization step using a terminator oligo blocking strategy, as described in http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4077022/. Libraries were sequenced on an Illumina HiSeq 2500 in Rapid Run mode. Fastq files were trimmed with Cutadapt (v1.6) to lengths of 18–35 nt, with 10% adapter sequence error allowed. CAG repeats were identified in the sequencing data by searching for exact matches to 18–35 nt strings of CAG repeating units. Edited repeats were identified by allowing replacement of any to all CAG units with CGG.

Eye photos

Eyes of at least 15 flies were viewed and representative eyes imaged. Flies were photographed at 24–48 h post-eclosion. Light photos were taken with an Olympus SZX7 dissection microscope fitted with an SZX-AS aperture. Images were captured with a Colorview IIIu camera and AnalysisRuler image acquisition software. In all cases, anterior is to the left. Image preparation was performed using Adobe Photoshop 6.0.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Author contributions

C.L.vE., S.E.S., A.S., D.L.W., D.L.H., O.M. and L.V.O conducted and analyzed *Drosophila* experiments. J.E.C., P.Y., J.H. and C.S. conducted and analyzed RNAseq experiments. L.V.O and R.I.R. designed the experiments. R.I.R wrote the first and final drafts of the paper and its revision.

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Supplementary Data:

Table of contents

Figures

Tables

Figure S1 –

Homology between Dicer proteins and other members of the RIG-I family (IFIH1 and RIG-I)

- Homology determined using Clustal alignment in MegAlign Pro program from DNASTAR (Lasergene)

A – overview alignment (human IFIH1, RIG-I, Dicer, *Drosophila* Dicer1, Dicer2)
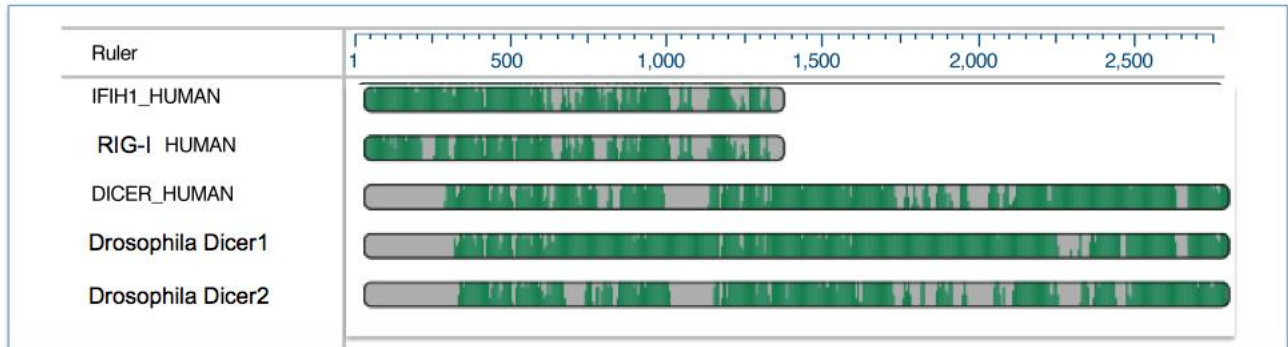
B – sequence homology alignment (consensus, human IFIH1, RIG-I, Dicer, *Drosophila* Dicer1, Dicer2, logo)

Figure S2 – [relates to Figure 1]

Variable severity eye phenotype *Drosophila* rCAG.rCUG~100 lines (Weak, Medium and Strong) expressed with altered levels of RNAi co-factor *R2D2* at 25°C.

A, E and I) EV51C is the empty vector control exhibiting no eye phenotype when crossed with GMR-Gal4.
B) Weak, **C)** Medium and **D)** Strong rCAG.rCUG~100 lines eye phenotypes exhibited when crossed with GMR-Gal4.
E – H) When crossed in to heterozygous loss-of-function *r2d2[1]* mutation line, increased severity of eye phenotype is evident in the Medium rCAG.rCUG~100 line **(G)** and lethality for the Strong rCAG.rCUG~100 line **(H).**
I – L) When additional *r2d2* was ectopically co-expressed from the UAS-*r2d2* transgene, reduction in the severity of the Medium **(K)** and Severe **(L)** lines is evident.
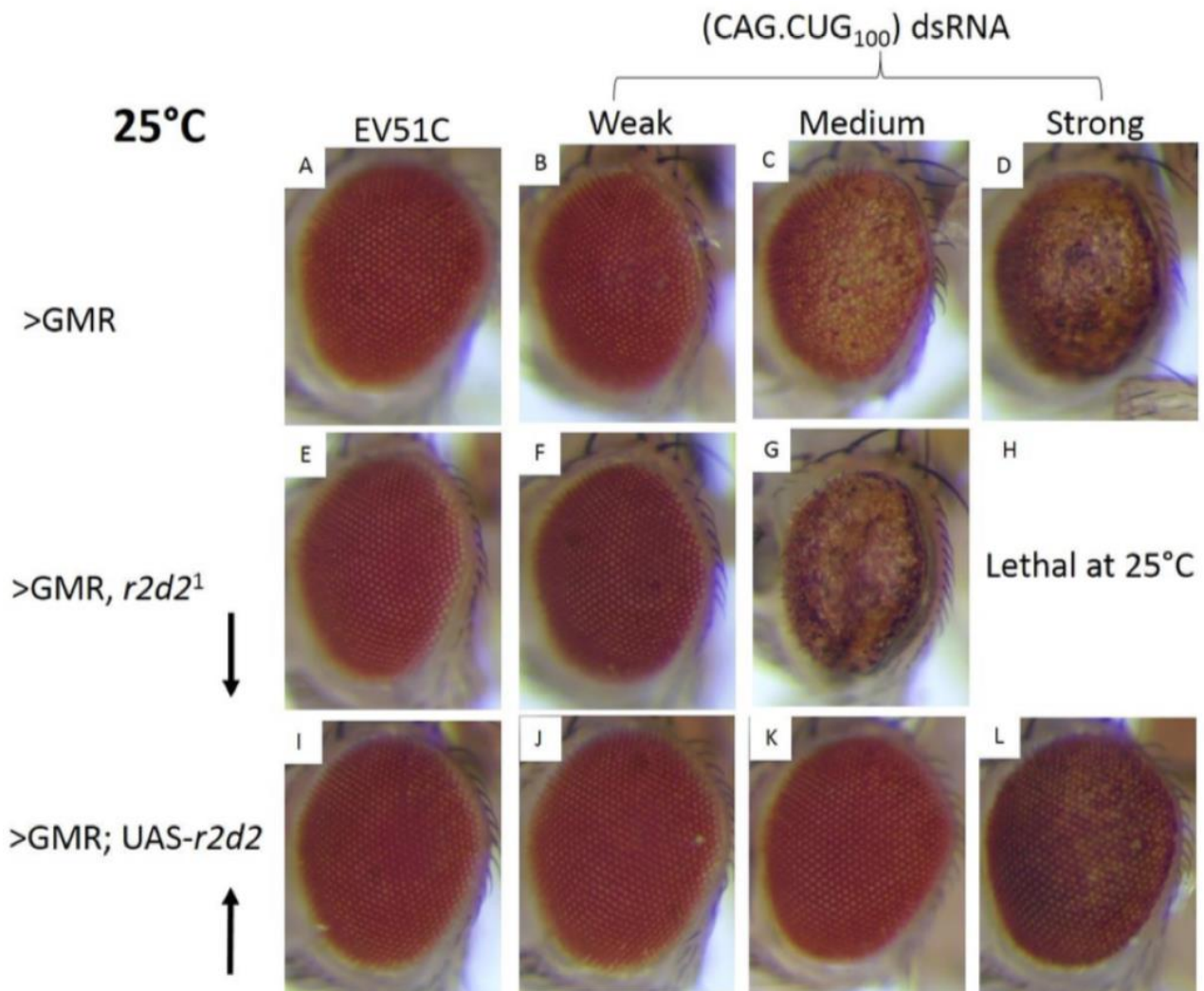
Figure S3 [relates to Figure 1]

Altered miRNAs have a minimal effect on double stranded meditated toxicity

In each case the *GMR-GAL4* driver is used to express the constructs in the eye. A) The *GMR-GAL4* driver alone has no phenotype in the eye. A') Expression of *dsRNA* causes disruption to patterning of the eye and loss of pigmentation. Heterozygous mutation of B) *miR-184* using the *miR-184$^\Delta$* allele, C) *miR-263b* using the *miR-263b$^\Delta$* allele D) *miR-274* using the deficiency *Df(3L)BSC577* and E) *miR-932* using the deficiency *Df(3L)BSC577* does not affect the patterning of the eye alone and B'-E') does not have a significant effect on the dsRNA phenotype. F) *GMR-GAL4* driven expression of a *UAS* construct alone has no phenotype in the eye. F') Expression of the *dsRNA* together with *UAS* alone causes mild disruption to patterning and loss of pigmentation. G) *UAS-miR-184* causes a significant phenotype alone showing disruption to patterning, loss of pigmentation and necrotic spots and G') is lethal when expressed together with dsRNA. H) Expression of *UAS-miR-263b* alone causes a change in the pigmentation of the eye that is H') also seen together with dsRNA. I) Expression of *UAS-miR-1017* does not have a phenotype alone and I') does not have a significant effect together with dsRNA. This experiment was performed at 25°C. The dsRNA used in this experiment is rCAG.rCUG$_{\sim100}$^{M3}.
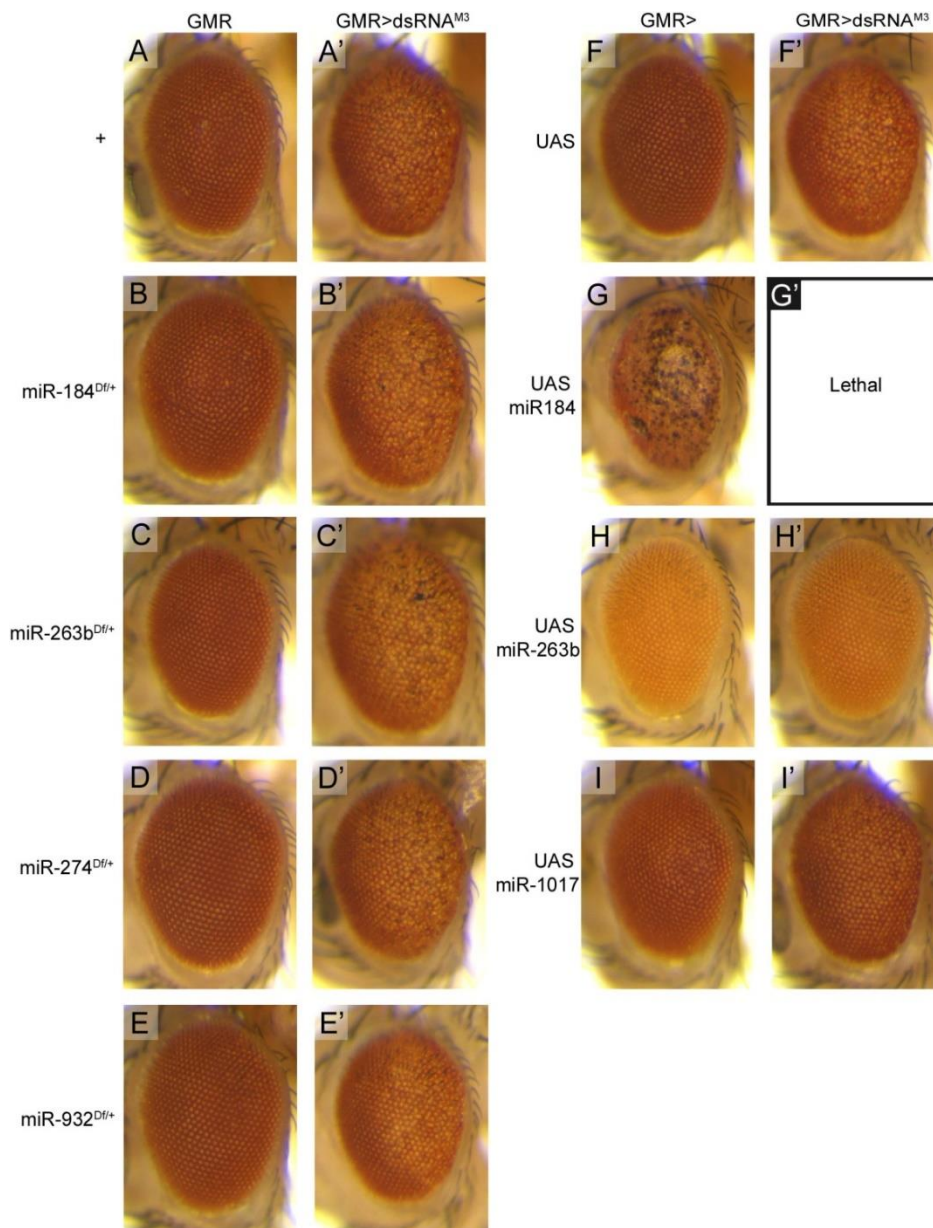
Figure S4 - [relates to Figure 1]

Argonaute-2 is a rate-limiting factor in double-stranded RNA mediated toxicity.

In each case the *GMR-GAL4* driver is used to express the constructs in the eye. The M3 line of rCAG.rCUG$_{\sim100}$ dsRNA *Drosophila* is utilized. This experiment was performed at 25 C.
A) GMR-GAL4 does not have an obvious phenotype alone.
B & C) Heterozygous *AGO2^{414}* and *AGO2^{V966M}* mutants have no effect on the patterning of the eye.
A') Expression of *dsRNA* results in disruption to ommatidial patterning and loss of pigmentation.
B' & C') Expression of the *dsRNA* together with these Argonaute-2 mutants suppresses the rough eye and loss of pigmentation of dsRNA toxicity.
D) *GMR- GAL4* driving a single *UAS* transgene with no insert does not show a phenotype.
E & F) Over-expression of Argonaute-2 with two independent *UAS-AGO2* lines does not have a phenotype alone.
D') Expression of rCAG.rCUG$_{\sim100}$ dsRNA causes loss of ommatidial patterning and pigment loss.
E' & F') Expression of these *UAS-AGO2* contructs along with dsRNA has no effect.
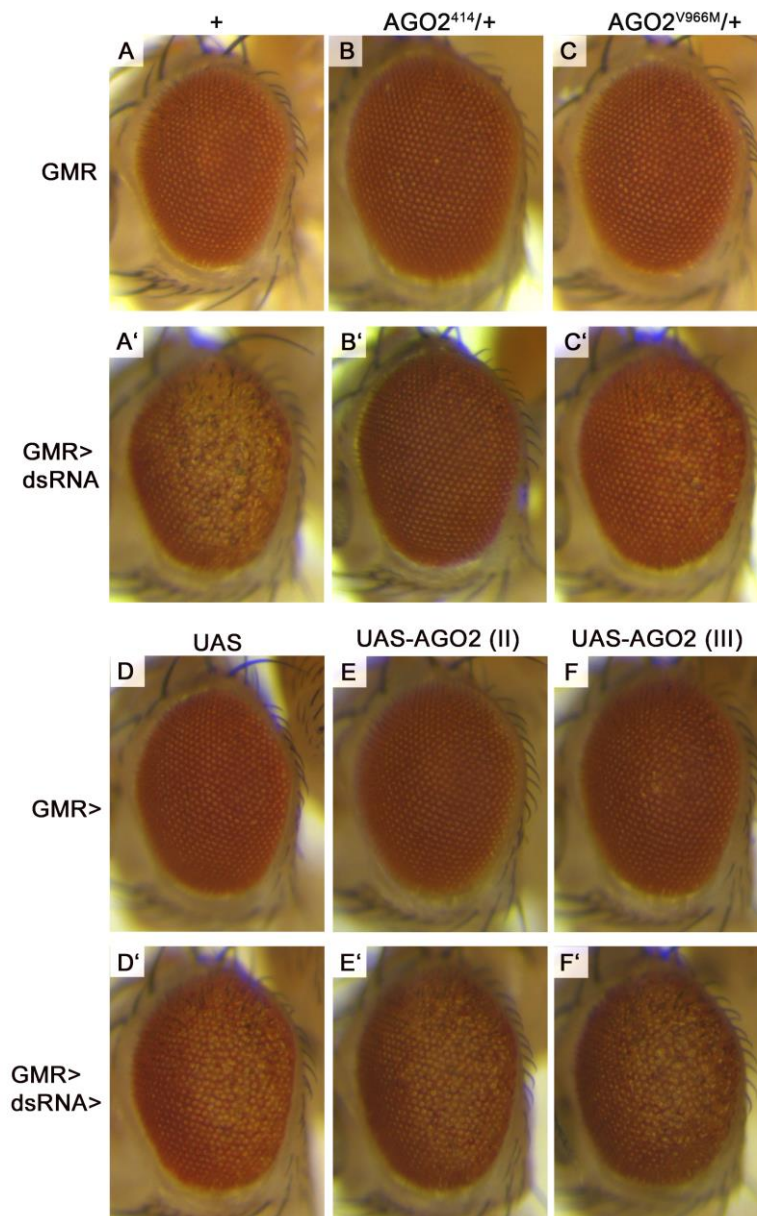
Figure S5 – [relates to Fig 2]

hADAR-1 **function overrides that of** *dicer2* **in the rCAG.rCUG~100 dsRNA phenotype in the** *Drosophila melanogaster* **eye.**

rCAG.rCUG~100 toxicity in the eye is not restored by over-expressing *dicer2* in flies also over-expressing hADAR1 FL. The *GMR-GAL4* driver was used to express all constructs.
A) Ectopic expression of *dicer2* (together with empty UAS vector) has no phenotype in the eye.
A') Co-expression of *dicer2* and rCAG.rCUG$_{\sim100}$ dsRNA is lethal in the S1 line.
B) Co-expression of *dicer2* together with hADAR1-i does not affect the patterning of the eye.
B') hADAR1-i rescues lethality and eye phenotype in S1 flies co-expressing rCAG.rCUG$_{\sim100}$ dsRNA and *dicer2* in the eye.
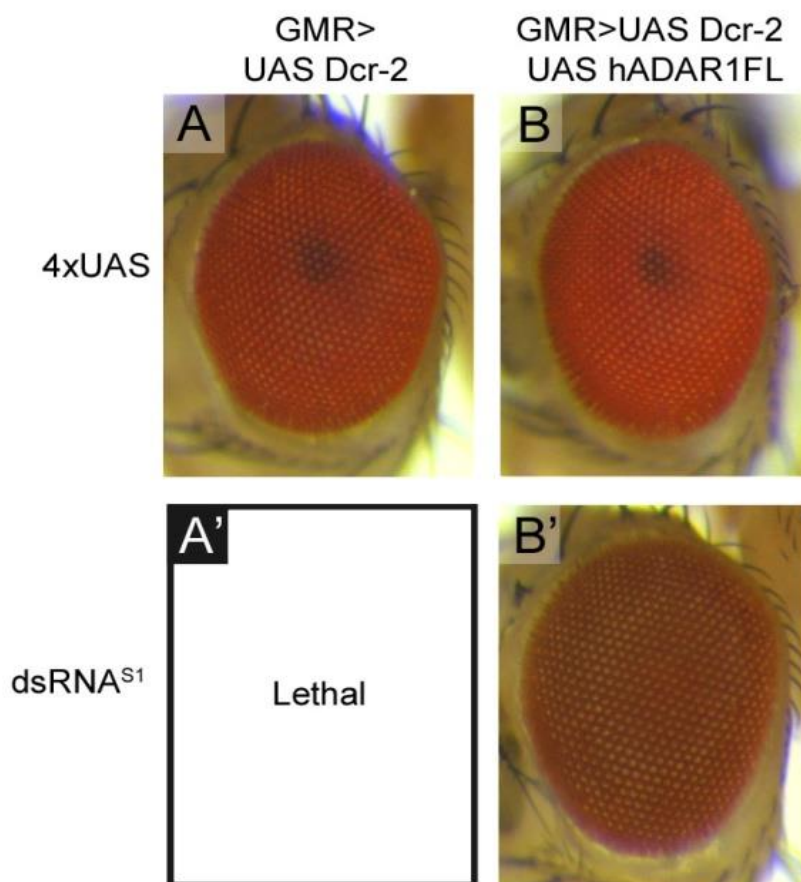
Figure S6 [relates to Fig 3]

qRT-PCR levels of uncleaved repeat transcripts in rCAG.rCUG~100 dsRNA flies with co-over-expression of *Drosophila* or human ADAR proteins.

qRT-PCR levels of *GMR-GAL4* driven expression levels of repeat transcript in (**A**) three independent rCAG.rCUG~100 dsRNA lines compared to rCAG.rCUG~100 dsRNA lines co-expressing either (**B**) *Drosophila dADAR-3/4* or (**C**) human *hADAR1-i* or (**D**) *hADAR2* (samples as per **Figure 2 A', C', F'** and **G'**). Repeat expression is measured relative to *Rp49*.

Figure S7 - **[relates to Figure 4]**

Figure S7A: Ectopic expression of *CrPV1A* completely rescues dsRNA-mediated pathology. In each case the *GMR-GAL4* driver is used to express the constructs in the eye. **A)** Expression of a single UAS transgene with no insert as a control eye. **B-C)** Expression of two independent full-length (FL) *UAS-CrPV1A* constructs alone. **D-E)** Expression of two independent truncated *UAS-CrPV1A* mutant constructs alone. **A')** Co-expression of repeat dsRNA together with a single UAS transgene. **B'-C')** Co-expression of repeat dsRNA together with two independent full-length (FL) *UAS-CrPV1A* constructs. **D'-E')** Co-expression of repeat dsRNA together with two independent truncated *UAS-CrPV1A* mutant constructs. The full length *CrPV1A* encodes a 148 amino acid long protein. The truncated *CrPV1A* encodes a 108 amino acid long protein. The experiment was performed at 25°C using the CAG.CUG$_{\sim 100}$^{M3} dsRNA line.
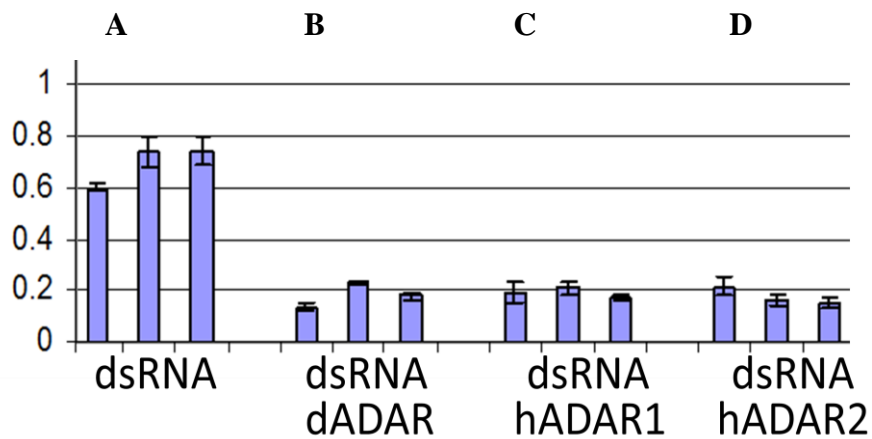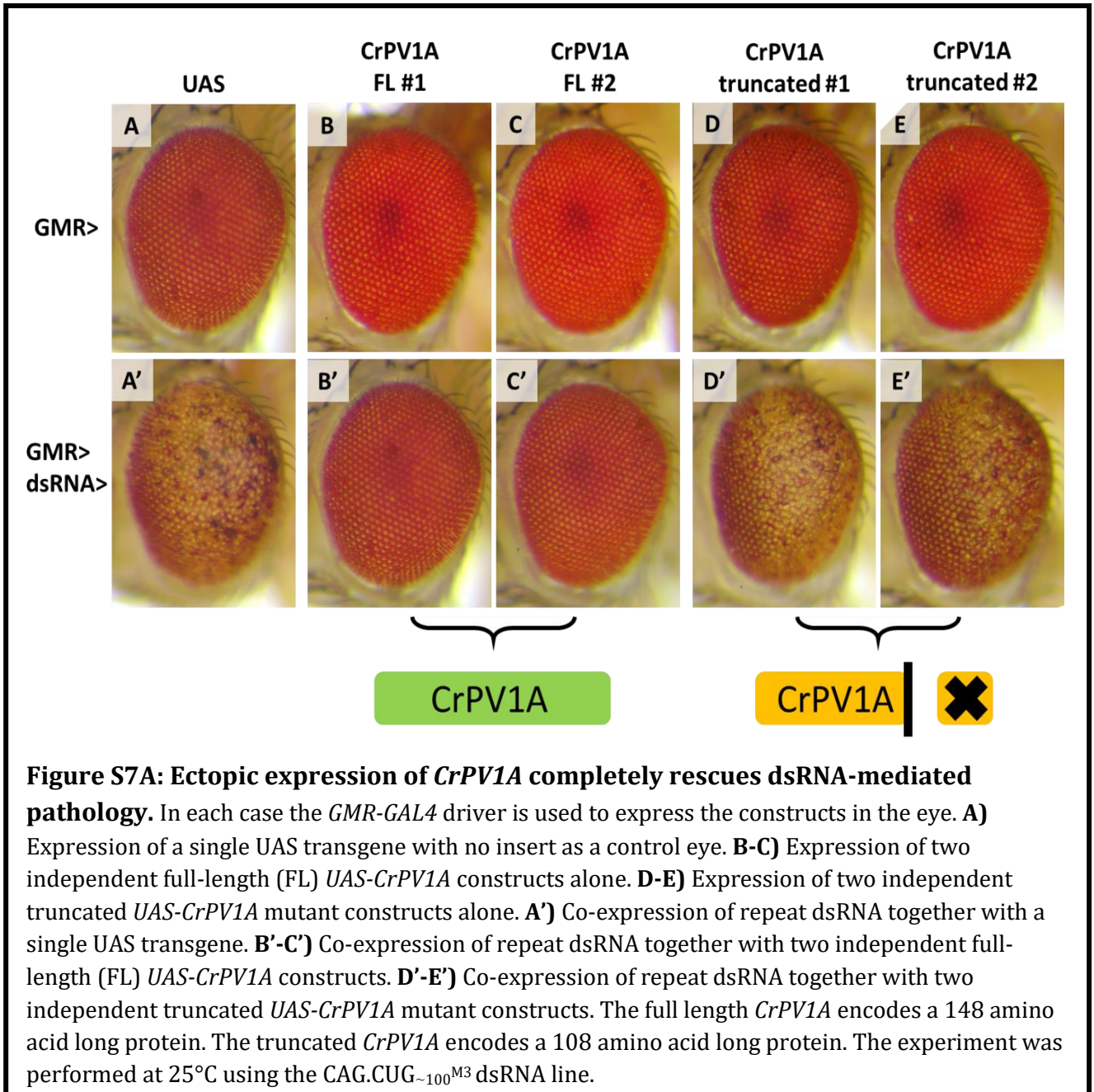
Figure S7B – Sequence alignment of Cricket Paralysis Virus 1A coding sequences

Alignment of the PCR generated sequence from the constructs encoding full length 148 amino acid protein (BB17F_G05) and truncated 108 amino acid protein (BB20F_G07) against the annotated sequence (NC_003924.1).

```
NC_003924.1    TCCCTTTGATGTGAGCTAATAAGGAGTAACCGATTCTTACAATGTGATCATGTCTTTTCA
BB17F_G05      CCCCCT-------------------------------------TCACCATGTCTTTTCA
BB20F_G07      CCCCCT-------------------------------------TCACCATGTCTTTTCA
               *** *                                      * * **********

NC_003924.1    ACAAACAAACAACAACGCAACCAACAACATCAACTCCCTTGAGGAGCTTGCTGCTCAAGA
BB17F_G05      ACAAACAAACAACAACGCAACCAACAACATCAACTCCCTTGAGGAGCTTGCTGCTCAAGA
BB20F_G07      ACAAACAAACAACAACGCAACCAACAACATCAACTCCCTTGAGGAGCTTGCTGCTCAAGA
               ************************************************************

NC_003924.1    ACTAATAGCAGCACAATTTGAAGGAAATCTTGATGGTTTCTTTTGCACTTTTTATGTGCA
BB17F_G05      ACTAATAGCAGCACAATTTGAAGGAAATCTTGATGGTTTCTTTTGCACTTTTTATGTGCA
BB20F_G07      ACTAATAGCAGCACAATTTGAAGGAAATCTTGATGGTTTCTTTTGCACTTTTTATGTGCA
               ************************************************************

NC_003924.1    GTCCAAACCACAACTATTGGACTTAGAGAGTGAATGTTATTGTATGGATGATTTTGATTG
BB17F_G05      GTCCAAACCACAACTATTGGACTTAGAGAGTGAATGTTATTGTATGGATGATTTTGATTG
BB20F_G07      GTCCAAACCACAACTATTGGACTTAGAGAGTGAATGTTATTGTATGGATGATTTTGATTG
               ************************************************************

NC_003924.1    TGGGTGTGATAGGATCAAGAGAGAAGAAGAATTACGTAAACTGATTTTCTTAACATCGGA
BB17F_G05      TGGGTGTGATAGGATCAAGAGAGAAGAAGAATTACGTAAACTGATTTTCTTAACATCGGA
BB20F_G07      TGGGTGTGATAGGATCAAGAGAGAAGAAGAATTACGTAAACTGATTTTCTTAACATCGGA
               ************************************************************

NC_003924.1    CGTTTATGGATATAACTTTGAAGAGTGGAAAGGATTAGTTTGGAAATTTGTTCAAAATTA
BB17F_G05      CGTTTATGGATATAACTTTGAAGAGTGGAAAGGATTAGTTTGGAAATTTGTTCAAAATTA
BB20F_G07      CGTTTATGGATATAACTTTGAAGAGTGGAAAGGATTAGTTTGGAAATTTGTTCAAAATTA
               ************************************************************

NC_003924.1    TTGCCCAGAACATCGATATGGATCAACTTTTGGTAATGGATTATTAATTGTGAGTCCCCG
BB17F_G05      TTGCCCAGAACATCGATATGGATCAACTTTTGGTAATGGATTATTAATTGTGAGTCCCCG
BB20F_G07      TTGCCCAGAACAT-----------------------------------------------
               *************

NC_003924.1    TTTCTTTATGGATCATCTTGACTGGTTTCAGCAATGGAAACTTGTTTCAAGTAATGATGA
BB17F_G05      TTTCTTTATGGATCATCTTGACTGGTTTCAGCAATGGAAACTTGTTTCAAGTAATGATGA
BB20F_G07      ------------------------------------------------------------

NC_003924.1    ATGCAGAGCCTTCTTGAGAAAGAGAACGCAACTTTTGATGAGTGGTGATGTCGAATCTAA
BB17F_G05      ATGCAGAGCCTTC----------------------AAGGGTGG---------------
BB20F_G07      ------------------------------------AAGGGTGG---------------
```

Figure S8 – Verification of genotypes of *Drosophila* lines by PCR

Gel electrophoresis of PCR products using oligodeoxynucleotide primers specific for the genes indicated (see Table S2 for oligodeoxynucleotide sequences). All PCR products (indicated by vertical red arrows) are of the anticipated size as predicted by the distance apart of oligodeoxynucleotide primers.
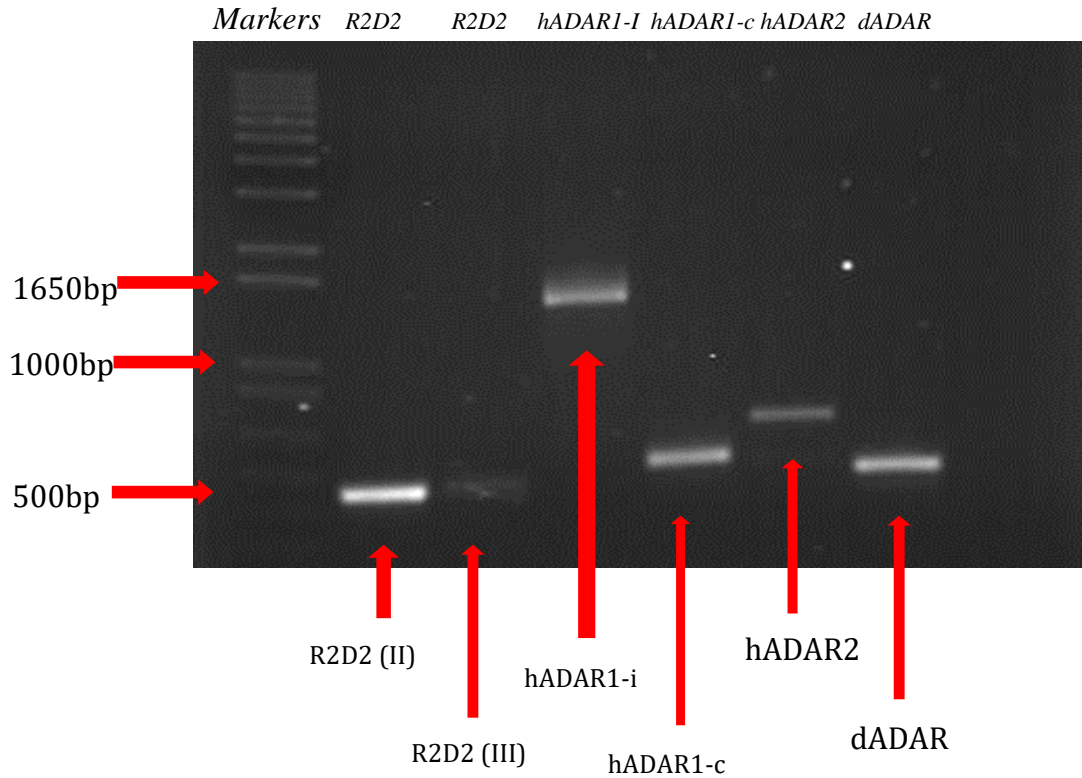
Figure S9 – Graphical Abstract of 'Non-Self' Mutation and Experiments in the *Drosophila* model of expanded CAG repeat diseases.
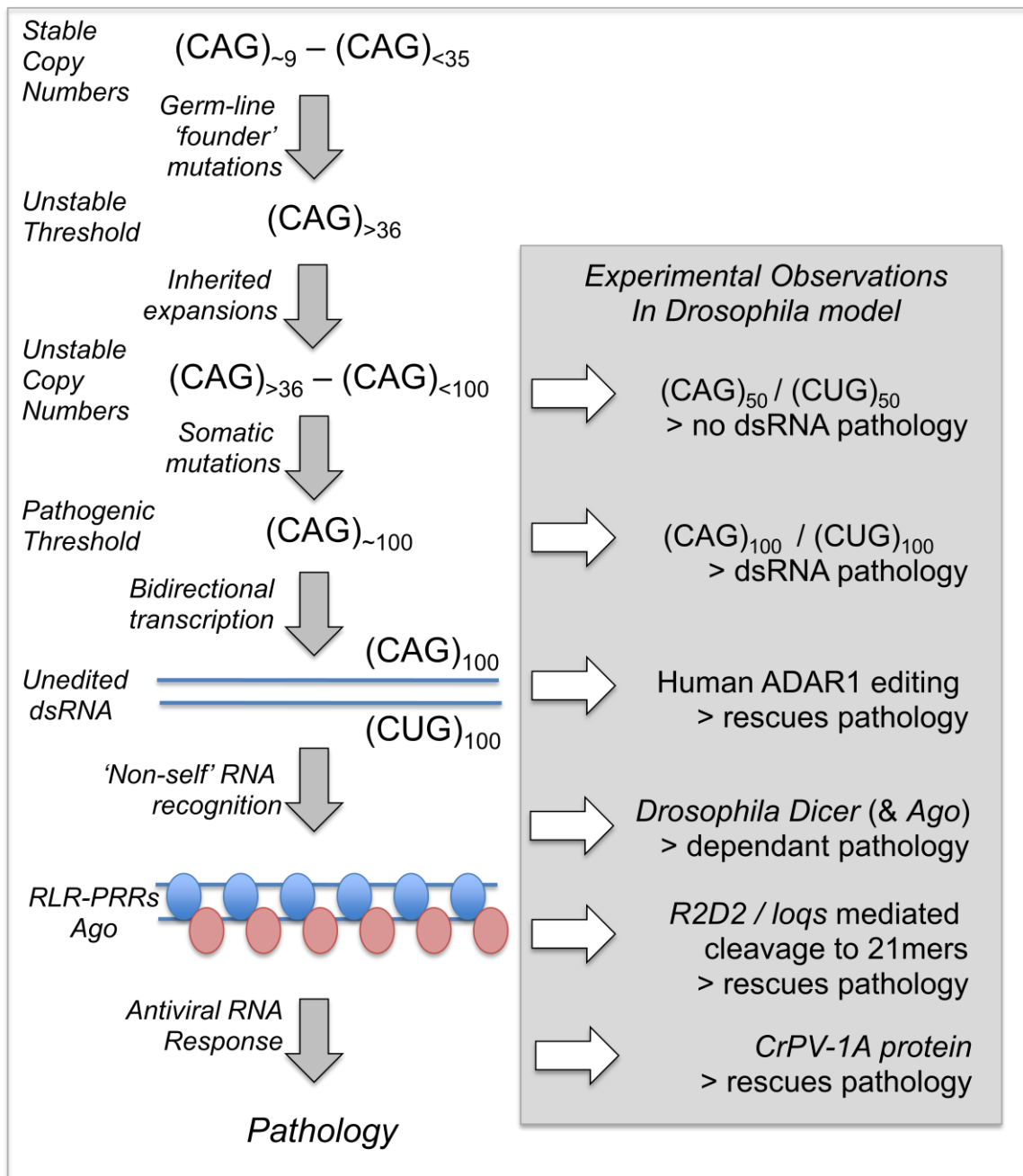
Abbreviations

RLR-PRRs	= RNA binding RIG-I-like pattern recognition receptors
Dicer, Ago	= components of RNAi and antiviral RNA recognition and defense pathways
R2D2, loqs	= components of RNAi pathway
21mers	= RNA comprised of 7 copies of the CAG repeat (typical RNAi product)
ADAR1	= human adenosine deaminase of RNA
CrPV-1A	= Cricket Paralysis Virus 1A protein

Table S1 *Drosophila* miRNAs changes with rCAG.rCUG~100 dsRNA expression **[relates to Fig 1]**

miRNA	Control Reads per million	dsRNA Reads per million	log2 (ratio) dsRNA vs control
Experiment 1 elavII-GAL4			
miR-932	**4732.415**	**317.347**	**-3.898**
miR-7	17414.671	2742.788	-2.667
miR-34	1153.333	238.882	-2.271
miR-988	3894.429	1272.877	-1.613
miR-317	4897.807	1802.952	-1.442
miR-100	10042.600	4238.855	-1.244
miR-8	15573.307	7551.822	-1.044
miR-125	4245.060	2476.007	-0.778
miR-184	**4963.964**	**3145.576**	**-0.658**
miR-274	**3391.638**	**2174.353**	**-0.641**
miR-34*	1503.964	985.172	-0.610
miR-279	26039.310	17696.478	-0.557
let-7	4099.515	2915.411	-0.492
miR-133	1874.442	1340.880	-0.483
bantam	36736.862	27408.704	-0.423
miR-970	8893.677	7046.159	-0.336
miR-263b	**2059.681**	**1644.278**	**-0.325**
miR-263a	9436.163	11567.488	0.294
pre-miR-34	14594.186	19869.087	0.445
pre-miR-193	6538.495	9705.251	0.570
pre-miR-263a	793.881	1332.162	0.747
miR-1017	**2002.345**	**3658.214**	**0.869**
Experiment 2 elav^c155-GAL4			
miR-184	**48388.914**	**9845.824**	**-2.297**
miR-274	**5763.316**	**2064.591**	**-1.481**
pre-miR-34	16768.186	9640.060	-0.799
miR-957	4220.718	2613.293	-0.692
miR-278	4210.636	3063.587	-0.459
miR-263b	**2973.029**	**2317.074**	**-0.360**
miR-995	1406.486	1111.321	-0.340
pre-miR-193	5060.073	3998.965	-0.340
miR-932	**2283.649**	**1820.061**	**-0.327**
miR-285	12319.355	10111.228	-0.285
miR-1003	7356.325	6058.586	-0.280
miR-193	3916.988	3247.482	-0.270
miR-1017	**917.493**	**1204.759**	**0.393**
miR-987	986.809	1305.156	0.403
miR-87	1616.955	2585.461	0.677
miR-317	9278.270	15641.987	0.753
miR-133	1305.663	2667.965	1.031
let-7	12081.160	26889.387	1.154
pre-miR-210	142.413	1010.924	2.828
pre-miR-284	148.714	1475.134	3.310
miR-210	1192.236	22028.601	4.208
bantam	520.501	15582.345	4.904
miR-34	37.809	14031.665	8.536

The miRNA with common changes in both experiments are shown in bold. ie. Those with a ±1.2 fold change, between dsRNA and the control sample, and a minimum of 1000 reads per million in at least one of the samples (control or dsRNA within an experiment). Control= 4xUAS, dsRNA in Experiment 1= rCAG.rCUG~100^S1, dsRNA in Experiment 2= rCAG.rCUG~100^S2.

Methods for Table S1

Alterations of miRNA profiles were analysed using the data sets generated by deep sequencing of flies expressing expanded repeats throughout the nervous system. Briefly, in Experiment 1 the *elavII-GAL4* driver was used to express *rCAG.rCUG~100^S1* (dsRNA^S1), *rCAG~100^#1* and *rCUG~100^#1* whereas in Experiment 2, *rCAG.rCUG~100^S2* (dsRNA^S2) was expressed using the *elav^C155-GAL4* driver. In each experiment the small RNA population was sequenced in each of the repeat expressing samples using *Illumina* small RNA sequencing and was compared to each driver with no repeat expression (*4xUAS*-control). Independent pan-neuronal drivers together with independent rCAG.rCUG~100 lines were used with the expectation of identifying miRNA changes that can be replicated using different genetic backgrounds to exclude biological and experimental artefacts in order to obtain robust changes caused by dsRNA.

The sequencing analysis produced between 1.5-2.6 million reads that were uniquely mapped to the *Drosophila* genome (BDGP 5.0, 2006). These reads were then mapped to the annotated miRNA precursors (*D.melanogaster* miRBase version 14). Reads mapping to multiple precursors (pre-miR) were further analysed to identify the 'best match' based on the longest alignment. These reads were further matched to the annotated mature miRNA sequence or predicted star sequence (miRNA*). Reads that did not map to miRNA precursors were removed from the analysis. The total number of reads mapping to miRBase was normalised across the samples to allow comparison.

Significant changes in miRNA abundance were identified based on two parameters. Firstly the complete list was filtered to include miRNAs that recorded a minimum of 1000 reads in at least one of the samples (control or dsRNA within an experiment). This list was then filtered based on fold change between the samples. Additionally, in Experiment 1 miRNAs that were significantly changed in either *rCAG~100^#1* or *rCUG~100^#1* compared to the control were excluded, in order to generate a list of altered miRNAs that are unique to double-stranded RNA expression.

In order to identify common changes to the miRNA profile between the two experiments, these analyses were expanded to include miRNAs with a log2 (ratio) of >+0.26 or <-0.26; that is a ±1.2 fold change, between dsRNA and the control sample, and a minimum of 1000 reads in at least one of the samples (control or dsRNA within an experiment) was included. This generated a list of 22 and 23 miRNAs with a significant change in abundance in the two experiments respectively. *miR-184*, *miR-263b*, *miR-274* and *miR-932* are decreased and *miR-1017* is increased in dsRNA compared to the control in both Experiment 1 and 2, indicating the potential for a functionally relevant effect, independent of the different genetic backgrounds used in the two experiments.

Table S2

Oligodeoxynucleotide primers utilized for verification of genotypes of *Drosophila* lines.

A common (pUAST forward) primer was used for each PCR in conjunction with a unique reverse primer as indicated.

Primer name	Sequence (5'-3')
pUAST forward	GAAGAGAACTCTGAATAGGG
R2D2 reverse	GGACGCAGTAGTCACGCAG
hADAR1 reverse	GCCATTGTAATGAACAGGTGGTT
hADAR2 reverse	CCCACGTAAAAGGGAGGCTC
dADAR reverse	AGAACTGCTCCATCCTTAAACTG
pUASp forward	GGCAAGGGTCGAGTCGATAG
pUASp reverse	AGGTTTAACCAGGGGATGCT
pUASp sequencing forward	CAAGGGTCGAGTCGATAG
pUASp sequencing reverse	TAACAAGTATGAATGTCAGGTT

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