A Role for Metabotropic Glutamate Receptors in Healthy Ageing

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Thesis Abstract

The proportion of older individuals globally is increasing. This results in an increase in socioeconomic costs as more people are living long enough to suffer from the detrimental effects of ageing such as chronic age-related diseases. Thus, there is an urgent need to understand the mechanisms that drive ageing and promote healthy ageing. Ageing research using simple model organisms has shown that ageing can be modulated by genetic and environmental factors and these interventions extend lifespan and improve health in evolutionarily distant organisms.

In this thesis, the fruit fly, *Drosophila melanogaster* was used to study the role of metabotropic glutamate receptors (mGluR) in biological ageing. mGluRs are highly conserved G-protein coupled receptors activated by the amino acid, L-glutamate to modulate intracellular signal transduction cascades, including the PI3K and MAP/ERK signalling pathways which play evolutionary conserved roles in animal ageing. By utilising a null mutation of the gene encoding the single fly mGluR we have found that loss of mGluR activity in flies causes sex-specific differences on longevity. This sex-specific effect was found to be strain-specific and may be partly attributed to differences in *DmGluRA* mRNA levels.

This study also aimed to elucidate the mechanism(s) by which loss of mGluR extends lifespan. Phenotyping studies revealed that the long-lived mutants were associated with several phenotypes including increased resistance to stress, body weight and differences in triglyceride usage under starvation conditions. The studies also appear to rule out some possible mechanisms for the lifespan extension associated with loss of mGluR in *Drosophila* such as dietary restriction and improvements in gut physiology.

RNA-Seq analysis also identified several genes which change in expression as a response to loss of mGluR signalling, many of which may explain the phenotypes observed and so are potential mechanisms of longevity. Further studies are required to fully understand the mechanisms of lifespan extension but taken together, the results form a case for mGluR as a novel ageing regulator.

Key words: *Drosophila*, lifespan, single gene mutation, healthspan, ageing, metabotropic glutamate receptors

Dedication

To my dear mum, thank you for pushing me, encouraging me when I couldn't see the end of the tunnel. Thank you for laying a solid foundation for us, for your constant sacrifices and showing us there is no limits to what we can achieve.

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First of all, I would like to express my sincere gratitude to my supervisor, Dr Cathy Slack. Thank you for being so supportive and extremely patient with me, for always being available to help or to answer my confusing questions and for always reminding me that I can do it - especially in the middle of a global pandemic! You've inspired me to do well and are the best supervisor I could ask for.

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To all my friends and family here and aboard, so many people cheered me on, prayed for me, helped me out and told me I could do it. This thesis wouldn't be possible without you all.

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List of Abbreviations

4-EBP – Eukaryotic initiation factor 4E-binding protein 1

AC – adenylyl cyclase

AchyL1 – S-adenosylhomocysteine hydrolase-like protein 1

AchyL2 – S-adenosylhomocysteine hydrolase-like protein 2

Alk– Anaplastic lymphoma kinase

AL– ad libitum

AMPK – AMP-activated protein kinase

AOP – Anterior open

Bmm – Brummer

CAFE – CApillary FEeder

CAMKII- calcium/calmodulin-dependent protein kinase II

cAMP– cyclic AMP

ChIP – Chromatin immunoprecipitation

CnC – Cap 'n' collar

CNS – Central Nervous System

DAG – Diacylglycerol

D-GADD45 – growth arrest and DNA damage- Inducible gene 45

DEGs – Differentially expressed genes

dFak – Drosophila focal adhesion kinase

dFMRP – Drosophila fragile X mental retardation protein

DR – Dietary Restriction

DHPG – 3,5-Dihydroxyphenylglycine

DILPs – Drosophila Insulin-Like Peptides

DmGluRA- *Drosophila* metabotropic glutamate receptor A

dNGLY1 – Drosophila N-Glycanase 1

ETS – E-twenty six

FIRKO – fat-specific insulin receptor knockout

FOXO – Forkhead box

FXS – Fragile X Syndrome

GABA – Gamma aminobutyric acid

GDP – Guanosine diphosphate

GEF – Glucose-enriched food

GI – Gastrointestinal tract

GO – Gene Ontology

GPCR – G protein-coupled receptor

Grb2 – Growth factor receptor-bound protein 2

GTP – Guanosine-5'-triphosphate

GWAS – Genome-Wide Association

H_2O_2 – Hydrogen peroxide

H3K9me3 – Histone 3 lysine 9 trimethylation

HP1 – Heterochromatin protein 1

Hsp83 –83kDA heat-shock protein

hUCP2 – human uncoupling protein 2

IGF-1– Insulin-like growth factor 1

IGF1R–

IGFBP7– Insulin Growth Factor Binding Protein 7

IIS– Insulin/insulin-like growth factor signalling

IMP-L2– Imaginal morphogenesis protein-Late

Indy– I'm not dead yet

InR– Insulin-like receptor

IP3– inositol 1,4,5-trisphosphate

IPCs – insulin-producing cells

IRS– insulin receptor substrate

ISCs –intestinal stem cells

Jou- Jouvence

KRG- Korean red ginseng

L-Glu – L-Glutamate

LTD – Long-term depression

LTP– Long-term potentiation

MAPKKK – MAP Kinase kinase kinase

mGluR– Metabotropic glutamate receptor

mNSCs – median neurosecretory cells

Mn-SOD – Manganese Superoxide Dismutase

MPEP–2-Methyl-6-(phenylethynyl)pyridine

Mth – Methuselah

mTORC1 – mTOR complex 1

mTORC2 – mTOR complex 2

mTOR– Mechanistic target of rapamycin

NGS – Next-generation sequencing

NIA – National Institute on Ageing

NMJ – Neuromuscular junction

O/A interneurons – oriens/alveus interneurons

PBS – Phosphate-buffered saline

PBST – 1X Phosphate-Buffered Saline, 0.1% Tween® 20 Detergent

PCR- Polymerase chain reaction

PDK – Phosphoinositide-dependent kinase

PGC-1 – Peroxisome proliferator-activated receptor gamma coactivator 1

PI3K– Phosphoinositide 3-kinase

PI – Performance index

PIP_2 –Phosphatidylinositol 4,5-bisphosphate

PIP_3 –Phosphatidylinositol 3,4,5-trisphosphate

Pol I - RNA polymerase I

Pol III- RNA polymerase III

PTEN – Phosphatase and Tensin Homolog

qRT-PCR – Real-Time Quantitative Reverse Transcription PCR

RNAi– RNA interference

RNA-seq – RNA Sequencing

ROS – Reactive oxygen species

rRNA – ribosomal RNA

Rut – rutabaga

S6k1 – S6 kinase beta-1

SAGE –Serial Analysis of Gene Expression

SIRT-1 – NAD-dependent deacetylase

SLC22 – solute carrier 22

SNPs – single nucleotide polymorphisms

SOD – superoxide dismutase

STR-2 – serpentine receptor

Sun – stunted

SYA – sugar/yeast/agar

TAG – Triacelgylceride

TSC1- tuberous sclerosis complex genes 1

TSC2 – tuberous sclerosis complex genes 2

TIF-IA – transcription initiation factor-1A

Tot– Turandot

TMD – transmembrane domain

w^{1118} – white 118

W^{CS10} – white Canton-S

w^{Dah} – White Dahomey

w^{DahT} – white DahomeyT

UW – University of Wisconsin

VFD – Venus fly trap domain

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Chapter 1 : Introduction

1.1 Biological Mechanisms of Ageing

Average life expectancy in human populations continues to rise in both developed and developing countries. It is predicted that by 2030, in many countries average life expectancy will have risen to 90 years (Kontis et al., 2017). While this increase in life expectancy reflects great advances in modern medicine, it also poses serious socioeconomic concerns because increased age is the major risk factor for many chronic and debilitating diseases such as cancer, cardiovascular disease and neurodegenerative disorders (Niccoli and Partridge, 2012). Therefore, as the number of older individuals (>60 years) in the population increases, these diseases become much more prevalent. For example, Alzheimer's Disease, an age-related neurodegenerative disorder, was the 7th highest cause of mortality in 2019 while coronary heart disease continues to be the highest cause of death globally (WHO, 2019).

As research continues to find treatments and cures for these age-related diseases, a more radical approach has been suggested in that to collectively treat these age-related diseases, it may be possible to target their common underlying cause, the biological mechanism of ageing itself. Ageing can be defined as the decline of physiological function, over time, increasing the risk of mortality (Katic and Kahn, 2005). It is now widely accepted that this process is not fixed but can be influenced by both genetic and environmental factors (Passarino et al., 2016).

1.1.1 Evolutionary theories of ageing

From an evolutionary point of view, ageing should not occur. Ageing is disadvantageous for an organism as it reduces both survival and fecundity (referred to as senescence) therefore contradicting natural selection. Natural selection favours heritable traits that increase an organism's chance of survival and reproduction. In contrast, genetic traits which do not provide an advantage for survival will be removed. This raises an important question: why does the ageing process exist? Ageing is a universal trait, observed across almost all organisms with a few exceptions. For example, fresh water *Hydra* show no obvious signs of ageing (Martinez, 1998) and *Turritopsis dohrnii* termed "the immortal jellyfish" which reverses its life cycle by returning to its juvenile stage (Piraino et al., 1996) . That evolution would favour a process that causes disease and death and would be considered deleterious is therefore somewhat intriguing. Several theories have been proposed that attempt to explain how and why ageing has evolved.

The mutation accumulation theory was developed by Medawar (1952) and proposes that deleterious mutations that manifest themselves after reproduction, when genes have already been passed to the next generation, are missed by natural selection because at later ages the force of natural selection declines. These mutations accumulate over time and their combined effects lead to ageing. On the other hand, the forces of natural selection are stronger early in life and thus deleterious alleles which have an early-life onset are removed. In a hypothetical environment where ageing is absent, death still occurs from extrinsic hazards such as predation, disease and starvation. Therefore, fewer animals survive to experience the deleterious effects of any late-acting mutations and so these mutations persist into the following generations (Flatt and Schmidt, 2009).

The antagonistic pleiotropy theory of ageing was developed from the mutation accumulation theory by Williams (1957). Antagonistic pleiotropy proposes that ageing is caused by the selection of genes that have pleiotropic effects at different ages, that is the same gene can have multiple effects at different stages of the life history which may have opposite effects on fitness. Due to natural selection, these genes are selected as they may be beneficial to survival during early life even if the same genes have deleterious effects late in life. However, these deleterious effects may not become apparent until post-reproductive stages of the life history and so are therefore not removed by natural selection. A classic example of antagonistic pleiotropy in humans, is the p53 gene. p53 encodes a tumour suppressor, which stops damaged cells from proliferating and so helps avert cancer early in life. But the same protein also suppresses the proliferation of stem cells which prevents the renewal and replacement of deteriorating cells leading to ageing (Rodier et al., 2007).

The third theory, the Disposable Soma theory, was first proposed by Kirkwood (1977) and is based on the idea that organisms have a limited amount of nutritional or metabolic resources that are divided to provide energy for either reproduction or maintenance of the body, i.e. somatic maintenance. The theory suggests that there is a trade-off between allocating energy to reproduction and maintaining or repairing the somatic tissues of the body. Natural selection will favour resource allocation that will promote reproduction. As such, more energy will be invested in reproduction and so there will be fewer resources available for somatic maintenance leading to somatic deterioration with ageing resulting in a trade-off between reproduction and longevity. A clear example of this trade-off is the sex hormone, oestrogen, which increases

female fertility during early life but increases the risk of several cancers during older ages (Velarde, 2013).

However recently some problems have been identified with the disposable soma theory. The theory only explains why we live to a certain age - after reproduction, the soma is no longer maintained as this is too costly and therefore the soma is 'disposable' (Weinert and Timiras, 2003). The theory however fails to explain why females live longer in most species. Since females use their energy for reproduction, according to the theory they should rather be short-lived because they have less resources for maintenance and repair (Blagosklonny, 2010). The disposable soma theory does not explain why organisms with limited resources or nutrients can often live longer than organisms with plenty of resources for example dietary restriction in most organisms extends lifespan and improves health (Fontana and Partridge, 2015, Kapahi et al., 2017) (see 1.2.1: Dietary Restriction). According to the disposable soma theory, less food means there are less resources available for anti-aging repair and therefore should accelerate ageing.

A relatively new theory has been proposed by Mikhail Blagosklonny, which is known as the hyperfunction theory (Blagosklonny, 2008, Gems and de la Guardia, 2013, Gems and Partridge, 2013). Based on the antagonistic pleiotropy theory, the hyperfunction theory proposes that ageing is not a programmed process but a quasi-program i.e. ageing is a result of continued activity of processes that drive growth and development early in life, which fail to be terminated. The unwanted and excessive continuation of these processes during adulthood leads to hyperfunction of tissues and subsequent tissue damage and age-related pathologies (Blagosklonny, 2008). In contrast to Kirkwood's disposable soma theory, the hyperfunction theory proposes that ageing is not a result of an accumulation of molecular damage caused by failure of somatic maintenance. Rather, molecular damage is a consequence of ageing and not the cause or driving force behind ageing (Blagosklonny, 2012) .

An example of such growth promoting processes needed for development is the target of rapamycin or TOR pathway (see 1.3.2: The mechanistic target of rapamycin (mTOR) pathway) (Blagosklonny, 2008, Blagosklonny, 2013). The TOR pathway during development promotes growth by increasing protein synthesis and inhibiting autophagy (Jacinto and Hall, 2003, Papadopoli et al., 2019). During ageing, the TOR pathway becomes overactive as it is not terminated and is not required at such high levels later in life. Thus, the hyperfunction of TOR

can lead to age-related diseases such as cancer, arteriosclerosis and type II diabetes (Blagosklonny, 2008).

1.1.2 Lifespan vs Healthspan

A major challenge caused by the continuing increase in human life expectancy is that people are now living long enough to suffer from various age-related diseases. Therefore, the ultimate goal of ageing studies is not to merely extend lifespan but to increase healthspan. Healthspan is commonly defined as the length of time spent in good health i.e. physiologically functional without any age-related chronic disease (Kaeberlein, 2018).

According to the Office of National Statistics, life expectancy at birth in the UK in 2016 to 2018 was 79.3 years for males but estimated healthy life expectancy was only 63.1 years. This means a male born in the UK between 2016 and 2018 is expected to spend 20% of his life (16.2 years) in poor health (Ons.gov.uk, 2019). For females born in the same period, they had a life expectancy of 82.9 years but are expected to spend 23% of their life (19.3 years) in poor health (Ons.gov.uk, 2019). This gender difference, of women living longer that males is seen in most countries and even across species, and though different hypotheses have been proposed it remains one of the unanswered questions of biology (Marais et al., 2018). Yet despite females living longer on average than males (+ 3.6 years), they will spend a greater period of their life in poor health (Figure 1.1).

As people live longer and therefore spend longer time in poor health, this highlights the need to promote healthspan in humans. Importantly, lifespan extending interventions in model organisms have proven capable of also extending healthspan. For example, administration of the biguanide drug Metformin, used in the treatment of Type 2 diabetes, can not only prolong lifespan in rodents but also prevent multiple deleterious effects of ageing including improvements in physical performance and reduction of age-related cataracts in the eye (Martin-Montalvo et al., 2013).

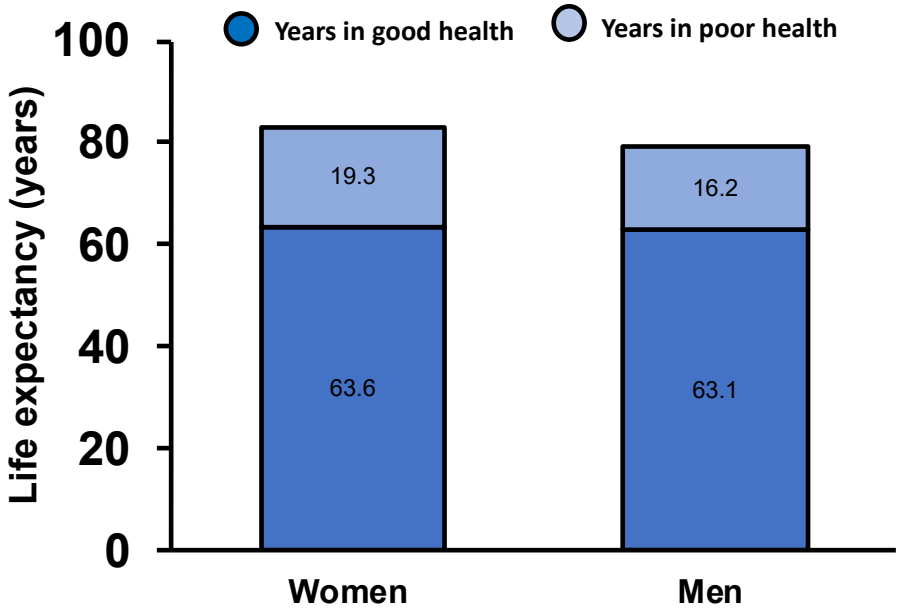


Figure 1.1 Healthy life expectancy and life expectancy at birth for men and women in the UK, 2016-2018.

Figure shows the life expectancy for males and females born in the UK between 2016-2018 and the proportion of their lives that are predicted to be spent in good or poor health. Data from the Office of National Statistics (Ons.gov.uk (2019).

1.1.3 Hallmarks of Ageing

It has been proposed that ageing is characterised by nine hallmarks, key biological processes that are associated with ageing and which are therefore thought to contribute to its associated functional decline (Lopez-Otin et al., 2013). These hallmarks are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Figure 1.2). Together, they have been proposed to function as key drivers of ageing by promoting physiological dysfunction. These nine hallmarks can be further grouped into three main classes: primary, antagonistic and integrative. Primary hallmarks which include genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis are thought to be the key sources of cellular damage. Antagonistic hallmarks include deregulated nutrient sensing, altered mitochondrial function and cellular senescence, and occur in response to the cellular damage caused by the primary hallmarks. Integrative hallmarks including stem cell exhaustion and altered intercellular communication result from the combined effects of the

other classes of hallmarks. Interventions that extend lifespan can be categorized as belonging to one or several of these hallmarks.

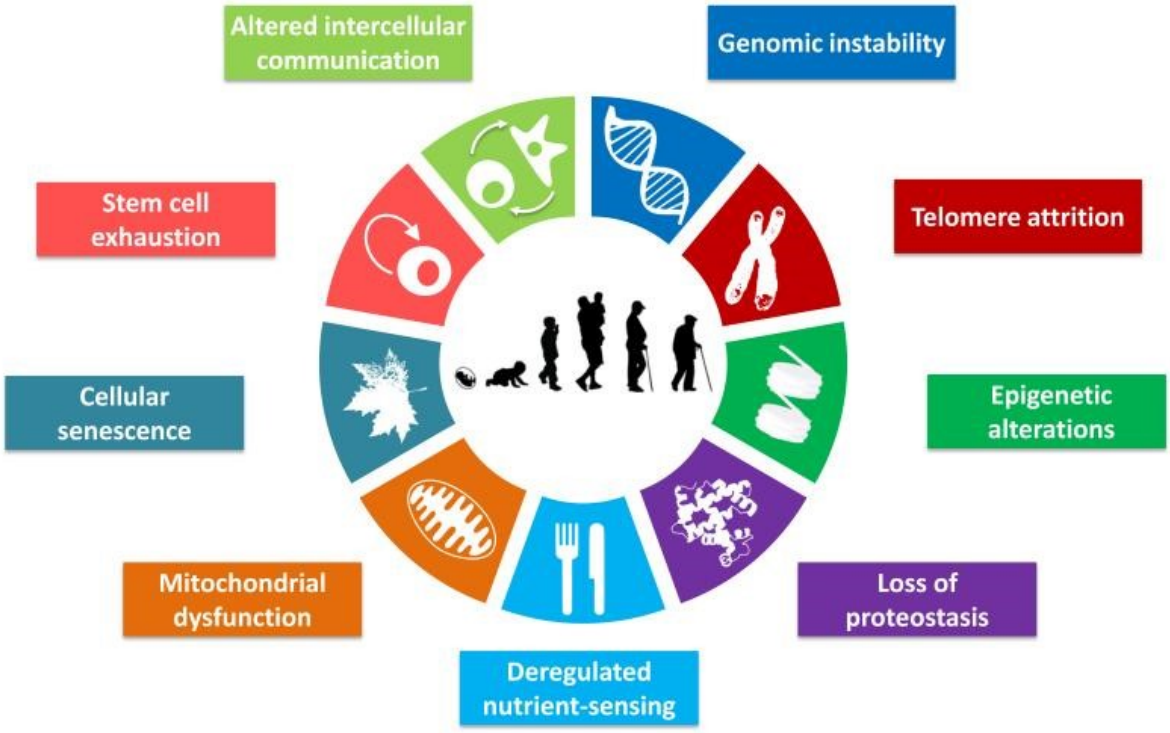


Figure 1.2 The nine hallmarks of ageing.
Nine common denominators of ageing across different organisms have been identified. These hallmarks represent dysfunction in key biological processes that are associated with increased age including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered cell signalling (taken from Lopez-Otin et al. (2013)).

1.2 Lifespan-extending interventions

1.2.1 Dietary restriction

Dietary restriction (DR) is the most robust environmental intervention known to extend lifespan across a wide range of organisms (Grandison et al., 2009a). It is commonly defined as a reduction of food intake without causing malnutrition and was first reported to extend lifespan in rodents over 80 years ago (McCay et al., 1935). This study showed that by restricting food intake by 40% of that provided by a normal *ad libitum* diet, the diet restricted animals lived

longer. Since these early findings in rodents, lifespan extension by DR has been shown in many species including those commonly used in the laboratory such as yeast (*Saccharomyces cerevisiae*), worms (*Caenorhabditis elegans)* and fruit flies (*Drosophila* melanogaster) (Partridge, 2010).

There is also evidence of DR effects in more complex, non-human primates (rhesus monkeys) (Bodkin et al., 2003, Mattison et al., 2012, Colman et al., 2014), however there is some controversy over these studies. The two main studies initiated in the 1980s, focused on the effect of DR on survival and health of healthy male and female rhesus monkeys; one study was based at the National Institute on Aging (NIA) (Mattison et al., 2012)and the other at the University of Wisconsin (UW) (Colman et al., 2014). Prior to this, Bodkin et al. (2003) at the University of Maryland provided preliminary data when comparing a small number (only 8 individuals) of DR monkeys to controls (109 monkeys which included insulin resistant and diabetic monkeys), showing risk of death was higher in controls than DR (2.6 fold increase).

The UW study involved 76 adult monkeys and reported that the DR monkeys had increased survival and fewer incidences of age-related diseases such as cancer and cardiovascular disease compared to non-DR controls (Colman et al., 2009). In contrast, the NIA study, which involved 121 monkeys between the ages of 1 and 23 years, did not show any significant improvements in survival and the improvements in health observed did not reach significance.
Mattison et al. (2017) has since compared the two studies and suggested possible reasons for the conflicting results. They found that the two studies had notable methodological differences including timing of DR, food intake and dietary composition. In the NIA study, some monkeys were introduced to DR as juveniles and some at older age whereas the UW study induced DR to adults. This affects lifespan as restricted diet is not beneficial in juveniles but rather has detrimental effects when applied to young monkeys.

NIA control animals also ate less than the control group of the UW study as they were allowed to eat as much as they wanted whereas UW control monkeys had lower food intake. This suggests the NIA controls were already dietary restricted, to an extent which may result in less differences between their DR group and controls. Small differences in food intake could therefore significantly affect the ageing process. Mattison et al. (2017) reported another key difference between the two studies in their dietary compositions. The monkeys in the UW study were fed on diets with more calories i.e. a semi-purified diet with higher sugar content whereas

NIA animals were fed naturally sourced foods. As a result, the controls in the UW study had increased bodyweight compared to controls in the NIA study. The naturally sourced diet contains phytochemicals and minerals which can also provide health and lifespan benefits. In contrast, the diet used in the UW study was more defined in terms of its specific components. Taken all these differences in consideration, Mattison et al. (2017) suggest that DR was sufficient to extend lifespan in rhesus monkeys.

Studies looking at other ageing parameters have also shown short term DR has potential benefits in humans (Fontana et al., 2004, Redman et al., 2018). Fontana et al. (2004) studied individuals who had voluntarily practiced DR for 3-15 years and found that DR lowered several of the main risk factors associated with atherosclerosis that usually increase with age. This included improvements in systolic and diastolic blood pressure, serum total cholesterol, and triglyceride levels. Also, in the first clinical trial testing DR effects in 53 adults over a two-year period, DR proved effective in decreasing energy metabolism and markers of oxidative stress (such as Urinary F2-isoprostane excretion) which can contribute to ageing (Redman et al., 2018). The DR group had sustained weight loss (on average individuals lost 8.7kg) and energy expenditure was decreased (including sleeping metabolic rate) compared to controls who rather gained weight.

Importantly, the lifespan extension associated with DR is also accompanied by improved health during ageing. For instance, in rodents and non-human primates, there is existing evidence that DR delays the onset of diabetes and other age-related diseases (Balasubramanian et al., 2017). For example, cancer is the main cause of death in laboratory mice. Alongside extended lifespan, mice subjected to DR showed a decrease in tumour incidence from 80% to 67% compared to fully-fed controls when DR was introduced in older animals (Dhahbi et al., 2004). This effect on survival and cancer incidence appears to occur within 2 months of DR induction, suggesting DR acts quickly to induce its beneficial effects. However, it is worth noting, there is evidence that different cancers respond differently to DR. For instance, the incidences of plasma cell neoplasm is actually higher in middle-aged DR mice (66%) than controls (41%) (Pugh et al., 1999). In non-human primates, imposing DR has also been shown to prevent cancers, as there were no cases of cancer in any of the young-onset DR monkeys (Mattison et al., 2012). The onset of diabetes is also delayed in young-onset DR monkeys (by 50%) and no cases of diabetes were seen in the old-onset DR group when compared to controls.

Interestingly, the Japanese island of Okinawa has the largest proportion of centenarians in the world and these older individuals are often in exceptional health, with much lower incidences of age-related diseases such as cardiovascular disease, cancer and diabetes compared to Western countries and even other regions of Japan (Willcox et al., 2008). The Okinawan diet is traditionally low in calories and fat and studies have attributed this to the longevity and age-related health of the local population (Willcox et al., 2006).

Long-term DR studies in humans are difficult because most people are reluctant to adhere to long-term restriction of their diet. It is therefore important to find DR mimetics, which are essentially drugs or compounds that can mimic the beneficial effects of DR without restricting food intake. Several existing drugs have been identified as potential DR mimetics (Baur, 2010, Mouchiroud et al., 2010) such as resveratrol, metformin and rapamycin.

Resveratrol is a natural phenol, found in red grapes which has been shown to extend lifespan in yeast, worms and flies (Wood et al., 2004, Howitz et al., 2003). It has been demonstrated that this lifespan extension is dependent on the Sirtuin, SIR2 (ortholog of mammalian SIRT-1) (Lin et al., 2000). It is thought to be a DR mimetic, as DR activates Sirtuins (Guarente and Picard, 2005). There is also evidence that SIRT-1 can extend lifespan, for example mice overexpressing SIRT-1 in the brain are long-lived and show a delay in age-associated physiological decline (Satoh et al., 2013). In *Drosophila*, Resveratrol does not increase lifespan in SIR2 mutants that lack or show reduced SIR2 expression (Wood et al., 2004). Also, Resveratrol treatment in flies did not extend lifespan any further in DR flies suggesting that the Resveratrol and DR mediate their effects on lifespan via similar mechanisms (Wang et al., 2013).

Likewise, Metformin, a drug used in the treatment of type II diabetes, may also function as a potential DR mimetic. It works by activating adenosine monophosphate-activated protein kinase (AMPK). As a major energy sensor, AMPK is activated in response to reduced cellular energy levels and so could be a possible mechanism via which DR extends lifespan (Greer et al., 2007) . Greer et al. (2007) found that in *C. elegans*, AMPK is required for lifespan extension by DR. They suggested that AMPK is activated in response to DR resulting in the increase of Daf-16 (the *C. elegans* homolog of the FOXO transcription factor) dependent transcription. This then promotes longevity and also increased resistance to oxidative stress. Worms overexpressing AAK-2 (the worm AMPK homolog) have increased lifespan of 13% (Apfeld et al., 2004) and similar increases in lifespan have been reported in *Drosophila* with increased AMPK expression

(Stenesen et al., 2013). Stenesen et al. (2013) demonstrated that overexpression of AMPK in the adult fat body or muscle is sufficient to extend lifespan whereas knockdown of *AMPK* via RNAi specifically in these tissues shortens lifespan.

Metformin has been reported to extend lifespan in worms and mice (Martin-Montalvo et al., 2013, Cabreiro et al., 2013). Moveover, in mice the lifespan extension (5.83% extension) observed in mice treated with 0.1% metformin was associated with improved physical performance and enhanced insulin sensitivity, which are also beneficial effects of DR (Martin-Montalvo et al., 2013). However the lifespan-extending effects of metformin do not appear to be conserved in *Drosophila* as metformin does not extend *Drosophila* lifespan despite activating AMPK (Slack et al., 2012).

Rapamycin is an immunosuppressive drug, which targets the mechanistic target of rapamycin (mTOR) pathway (see 1.3.2: The mechanistic target of rapamycin (mTOR) pathway). mTOR, like AMPK, is also a nutrient sensor and mTOR signalling is reduced upon DR in worms, flies and mice (Santos et al., 2016). mTOR inhibition has been shown to extend lifespan in worms, flies and mice (Weichhart, 2018) and DR does not further increase the lifespan of worms or flies when mTOR signalling is reduced, suggesting that overlapping mechanisms may be causing the lifespan effects of both interventions (Grandison et al., 2009a). However, Rapamycin may not be a true mimetic of DR, as the drug can further increase lifespan of DR flies (Bjedov et al., 2010).

It is important to note here that there are various forms of DR including calorie restriction or restricting individual dietary components such as proteins or amino acids (Mair et al., 2005, Min and Tatar, 2006b). Studies have indicated that the determinant of the lifespan extension via DR may not be calorie intake but the specific nutritional components, specifically proteins (Mair et al., 2005). Additionally, (Grandison et al., 2009a) further identified the amino acids responsible for the lifespan effects by DR and showed that it is a balance of these amino which are essential for the DR-induced lifespan response. This study found that the addition of ten essential amino acids to the DR diet enhanced fecundity, which is normally decreased in response to DR, and shortened lifespan. The effect on lifespan was similar to that of the fully fed condition. Supplementing the diet with vitamins, carbohydrates or lipids did not affect lifespan or fecundity. Also, the addition of non-essential amino acids reduced lifespan only slightly and had no effect on fecundity.

One essential amino acid, methionine, when supplemented back into the diet of DR flies individually, was capable of increasing fecundity. Adding methionine however, failed to decrease lifespan. This is an important point as reduced fecundity is often associated with DR in *Drosophila* Grandison et al. (2009a) and long-lived DR females show a reduction in egg laying. It is generally thought that the reduction in lifespan observed during full feeding is a result of reallocating nutrients from somatic maintenance to reproduction, which is a more energy costly process. However, these findings show that increased lifespan does not necessarily corelate with decrease fecundity. In addition, reduced fecundity is not required for longevity. Sterile females respond normally to DR and therefore show lifespan extension without reducing fecundity (Mair et al., 2004). Restricting particular amino acids have also been shown to be sufficient to extend lifespan in rodents. For example, low methionine and tryptophan diets not only extend lifespan but improve health in mice (De Marte and Enesco, 1986, Miller et al., 2005). Mice under methionine restriction show increased stress resistance and delayed the age-related decline of the immune system and liver function (Miller et al., 2005).

Due to the conserved effects across species, DR has become the centre of extensive research, however its biological mechanisms are yet to be fully understood. As the studies mentioned above show, there are several signalling pathways that are altered in response to DR and therefore appear to play a part in the lifespan and health-promoting effects of DR. What remains unclear is whether the same mechanisms by which DR induces lifespan extension are the same in different species. This is because there is variability in how DR is applied in different species. For example, in *C. elegans*, DR is achieved either by diluting the *Escherichia coli* bacterial food source or use of *Eat* mutants, a genetic mutation which reduces food intake due to pharyngeal dysfunction (Lakowski and Hekimi, 1998, Houthoofd et al., 2003). However, in *Drosophila,* DR is typically induced by diluting the concentration of nutrients, particularly dietary yeast in the food media(Chapman and Partridge, 1996, Mair et al., 2005).

There are also various DR protocols even within the same species, this makes it difficult to compare studies across laboratories and introduces confusion in the field, making it difficult to find the DR mechanism. In *C. elegans*, there are at least eight different ways of inducing DR, which have been shown to extend lifespan to varying degrees (Greer and Brunet, 2009). *Eat-2* mutation increases lifespan of worms by 0-57% whereas diluting the bacteria in liquid medium (bDR) extended worm lifespan by 60-73% (Greer and Brunet, 2009).

Furthermore, extension of lifespan by different methods of DR in the same organism may operate through both overlapping and non-overlapping mechanisms. For instance, in worms, the transcription factor DAF-16 (FOXO) is not required to extend lifespan in *eat-2* mutants or bDR (Lakowski and Hekimi, 1998).On the other hand, DAF-16 is essential for lifespan extension induced by dilution of peptone in bacterial plates which restricts bacterial growth (Greer and Brunet, 2009). Greer et al. (2007) also reported that another form of bacterial dilution, where *E. coli* is serially diluted (sDR) requires DAF-16.

There are also differences in the way DR is performed in invertebrates compared to rodents. For example, in *Drosophila,* the flies are constantly exposed to the food, that is they have ad libitum access to food (Chapman and Partridge, 1996). In contrast, in rodents DR is typically applied by feeding the DR group with a reduced amount of the chow diet (approximately a 40% reduction) compared to fully fed rodents (Weindruch et al., 1986). There is some evidence that rodents when supplied with the restricted food consume it within 2 hours of receiving the food and fast for the remaining 24 hours till the food is supplied again (Acosta-Rodriguez et al., 2017). Therefore, DR in rodents is more of an intermittent fasting or a cycle of periods of starvation, rather than a constant restricted diet.

There is also some criticism for the DR regimen in invertebrates. Studies have suggested that flies increase their food intake under DR to compensate for the lower nutrient intake. Carvalho et al. (2005) used radio-labelled food to directly measure feeding rate in flies and reported that at lower nutrient concentrations flies consumed a higher volume of food compared to flies at higher concentrations. However, there are some studies which have provided evidence against such compensation, suggesting compensatory feeding does not occur (Mair et al., 2005, Min and Tatar, 2006a). For instance, Mair et al. (2005) measured feeding rate indirectly by measuring the time flies spent feeding on the food and found the time flies spent feeding on DR diet did not differ from the time spent on a fully fed diet. In addition another study suggested that flies do not compensate by increasing food intake but by eating less on DR (Min and Tatar, 2006a). There is also evidence that shows that worms do not compensate by eating more when given a diluted food source (Mair et al., 2009). Due to such conflicting results, some confusion exists as it is difficult to reproduce DR effects and so the precise mechanism by which DR extends lifespan remains unknown.

The timing of DR is a factor that should be considered when studying the effects of DR on lifespan. As seen in the Rhesus monkey studies, DR was beneficial to lifespan when imposed in monkeys later in life (16-23 years) but not with juvenile onset (1-2years) (Mattison et al., 2012). These results were actually surprising as in previous DR studies in rodents, restricting diet earlier in mice still led to lifespan extension (Masoro, 2005, Cameron et al., 2012)

Recently, Hahn et al. (2019) showed that when DR is introduced at an old age, it had little to no effect on mice lifespan. In their study of 800 female mice, animals were either allowed to eat as much as they wanted *ad libitum* (AL) or were introduced to DR at 12 weeks of age. At 24 months of age, some of the DR mice were switched to an AL diet and some AL diet mice were switched to DR. The mice switched to DR, showed no improvements in mortality for the first 7 months after the switch.

Similar DR switch experiments in *Drosophila* have yielded different findings. Flies were switched from DR to control food or vice versa at 14 and 22 days of age (Mair et al., 2003). The fully-fed flies switched to DR showed reduced mortality as if they had been on the DR treatment throughout their lifespan. This study suggests that in flies, switching to DR reduces the risk of death, and any physiological damage experienced before switch can be reversed. Moreover, this response was rapid as the changes in mortality happened only two days after the switch.

Taken together, these studies suggest that the effects of timing of DR implementation may be different depending on the species studied.

1.2.2 Single gene mutations

The discovery that numerous single-gene mutations can increase lifespan has been a major breakthrough in the ageing field. Importantly, many gene mutations that alter lifespan appear to do so across homologues in different species. The first single-gene mutation shown to extend lifespan was the *age-1* mutation in *C. elegans* (Friedman and Johnson, 1988). The study showed that *age-1* loss-of-function mutants had a 65% increase in mean lifespan. The *age-1* gene encodes a catalytic subunit of phosphatidylinositol 3-kinase (PI3K), a key component of the insulin/insulin-like growth factor signalling (IIS) pathway (Morris et al., 1996). Following this initial discovery, another single-gene mutation in the *daf-2* gene was found to extend lifespan in *C. elegans.* Daf-2 represents the *C. elegans* orthologue of an insulin receptor-like protein

(Kenyon et al., 1993). Further genetic epistasis experiments identified that the longevity effects of *daf*-2 mutation were dependent on the activity of DAF-16, orthologue of the forkhead box (FOXO) transcription factor, a key downstream transcriptional effector of IIS (Kenyon et al., 1993, Larsen et al., 1995) .

Since these initial discoveries in *C. elegans*, single-gene mutations in genes encoding key IIS molecules have been shown to extend lifespan in other species including mammals, as will be discussed below. Identification of the evolutionary conserved functions of IIS in ageing by analysing the effects of single-gene mutations highlights the important role that such an approach provides to understand the mechanisms underlying the ageing process.

1.3 Evolutionary conserved nutrient signalling pathways

1.3.1 The insulin/insulin-like growth factor signalling (IIS) pathway

Perhaps the most widely studied biological pathway that has been shown to affect the rate of ageing is the evolutionarily-conserved, insulin/insulin-like growth factor signalling (IIS) pathway (van Heemst, 2010). Signalling through this pathway is activated by the binding of insulin or insulin-like peptides (ILPs) to the cell surface insulin receptor, a member of the receptor tyrosine kinase family (Figure 1.3). On activation of the receptor, insulin receptor substrate (IRS) proteins are recruited to the intracellular cytoplasmic tail of the receptor where they act as scaffolds to recruit other proteins to the activated receptor. The two major downstream pathways through which the insulin signal is transduced are via PI3K/AKT and Ras/MAPK.

Recruitment of PI3K to the activated receptor through its interactions with IRS proteins results in its phosphorylation and activation by the tyrosine kinase activity of the activated receptor. In flies and worms, the insulin signal can also bypass the IRS protein and be transduced directly from the activated receptor to PI3K (Broughton & Partridge, 2009). Activated PI3K then phosphorylates the membrane-associated PIP_2 (phosphatidylinositol (4,5)-bisphosphate), which is converted to PIP_3 (phosphatidylinositol (1,4,5)-trisphosphate). This pathway is negatively regulated by PTEN (Phosphatase and tensin homolog) which converts PIP_3 to PIP_2 and therefore blocking PI3K function. PIP_3 recruits AKT and its activators PDK1(Phosphoinositide-dependent kinase 1) and mTORC2 to the cell membrane resulting in the phosphorylation and activation of AKT and AKT is then released from the membrane.

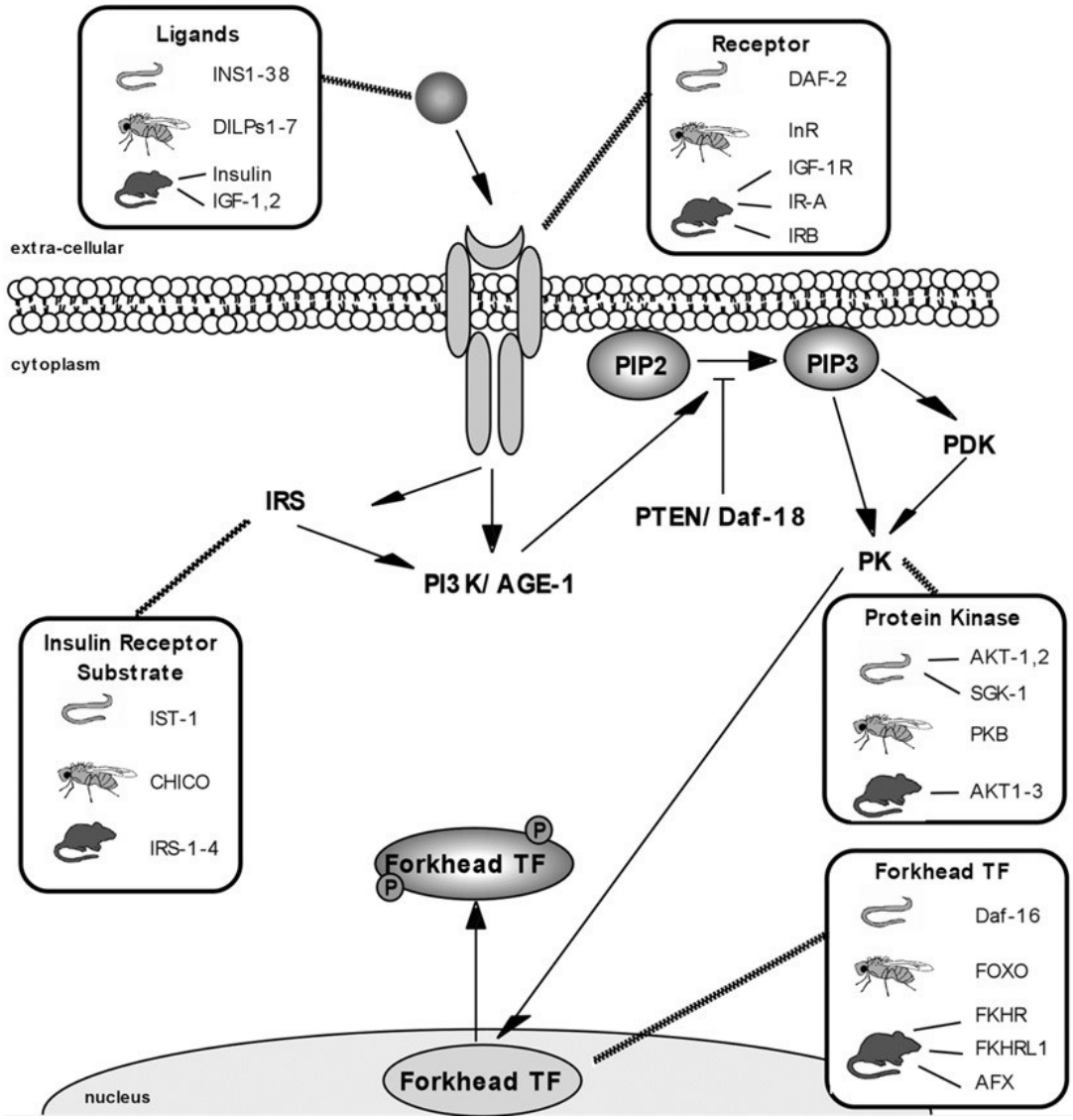


Figure 1.3 Schematic diagram of evolutionary conserved insulin/insulin-like growth factor signalling (IIS).

The different homologs in worms, flies and mice/mammals are shown in the various boxes. Extracellular ligands bind to receptor, and PI3K (in mammals and flies) or Age-1 (in worms) is recruited to the cell membrane and activated. PI3K/AGE-1 can be activated via IRS, when ligand and receptor bind. PIP_2 is converted to PIP_3 and the signal to passed to protein kinases, PDK and PK (AKT/PKB). This leads to the phosphorylation and inhibition of the transcription factor FOXO as it is excluded from the nucleus. (Taken from Broughton & Partridge, (2009))**.**

A key target of activated AKT is the FOXO transcription factor. Phosphorylation of FOXO by AKT results in its nuclear exclusion thereby preventing FOXO-dependent gene expression.

Multiple studies across several species, including worms, flies and mouse models, have shown that genetic inhibition of signalling through this pathway by single-gene mutations within any of the core signalling components extends lifespan and improves multiple aspects of age-related health (van Heemst et al., 2005, Altintas et al., 2016, Pan and Finkel, 2017). As described for mutations in *age-1 and daf-2* (Kenyon et al., 1993), similar effects on longevity have been observed in flies expressing a dominant negative form of the catalytic subunit of dPI3K, Dp110 (Slack et al., 2011) and also mutants of the *Drosophila* insulin-like receptor (dInR) (Tatar et al., 2001). Loss of the IRS protein, encoded by a gene named *chico*, has also been showed to extend lifespan (Clancy et al., 2001). Clancy et al. (2001) reported homozygous null mutant females exhibited a 48% increase in lifespan and 36% in heterozygotes. In *Drosophila,* overexpression of PI3K/AKT negative regulator, PTEN, in the adult fat body is also sufficient to extend lifespan (Hwangbo et al., 2004). Inactivation of DAF-18, the *C. elegans* PTEN homolog, has also been shown to lead to reduced lifespan, further suggesting reduced IIS increases lifespan (Mihaylova et al., 1999).

In addition, genetic ablation of the insulin-producing cells (IPCs) in the *Drosophila* brain extends lifespan (Broughton et al., 2005). The IPCs are responsible for the production of three out of the eight *Drosophila* insulin-like peptide (DILPs), DILP2, 3 and 5. Broughton et al. (2005) reported that adult flies with ablation of these cells, which results in reduced DILP2, 3 and 5 expression and reduced systemic IIS, live longer than their controls. Triple knockout of the genes encoding these three DILPs are also long lived, indicating that reduced IIS even at the ligand level can promote longevity (Gronke et al., 2010).

Reduced IIS can also increase lifespan in mice, suggesting that the evolutionary conserved effects of this pathway on lifespan also extends to mammals. Bluher et al. (2003) identified that deletion of the insulin receptor in the adipose tissue of mice (FIRKO) can extend lifespan. Both male and female FIRKO mice showed increased average lifespan of approximately 18% in addition to reduced fat mass. Mice with a heterozygous null mutation of the gene encoding the insulin-like growth factor receptor type 1 (IGF-1) receptor (IGF-1R$^{+/-}$) lived longer than controls (Holzenberger et al., 2003). The mutant mice presented a ubiquitous reduction of IGF1-R (50% reduction) and males and females lived 16% and 33% longer than controls, respectively.

Moreover, similar to long-lived *chico* mutants in flies, lifespan effects of the insulin substrate, IRS1 (insulin receptor substrate protein 1) has also been reported in mice. Ubiquitous deletion of IRS1 in both sexes show extended lifespan and also show improvements in health as they show delayed onset of many age-related pathologies including dermatitis, bone disease and decline in motor function (Selman et al., 2008, Selman et al., 2011). Taguchi et al. (2007) previously found that heterozygous deletion of another insulin substrate IRS2(insulin receptor substrate protein 2) in the mouse brain could also extend lifespan but this has yet been disputed by Selman et al. (2008) that found they are rather short-lived.

Both male and female transgenic mice overexpressing PTEN ($PTEN^{tg}$) showed significant lifespan extensions of 12% and 16%, respectively (Ortega-Molina et al., 2012). Ortega-Molina et al. (2012) also show in their studies that though PTEN is a tumour suppressor in addition to its role as an PI3K antagonist, its longevity effects are independent of cancer protection and directly due to PI3K activity. This is because $PTEN^{tg}$ mice which were cancer free at the time of death (no malignant tumours detected) were also long lived compared to cancer free controls.

The effects of reduced IIS on ageing in worms and flies require the activity of the Daf-16/FOXO transcription factor as removal of Daf-16/FOXO activity blocks the ability of reduced IIS to extend lifespan ((Kenyon et al., 1993, Slack et al., 2011) Moreover, over-expression of Daf-16/FOXO is sufficient to induce longevity in these model systems (Giannakou et al., 2004, Hwangbo et al., 2004, Kwon et al., 2010). Giannakou et al. (2004) overexpressed FOXO in the adult *Drosophila* equivalent of the adipose tissue, the fat body, and (Hwangbo et al., 2004) specifically overexpressed dFOXO in the fat body of the head, which both extends lifespan. Kwon et al. (2010) found that two isoforms of Daf-16, *daf-16a* and *daf16d/f* in worms are required to promote longevity and ubiquitous overexpression of *daf16d/f* in worms promotes longevity.

Interestingly, human genome wide association studies (GWAS) studies have also linked allelic variants of FOXO3A, to exceptional longevity in human populations (Willcox et.al.,2008; Flachsbart et al., 2009). Wilcox compared long-lived Japanese men (>95 years old) to men of average lifespan who died before 81 years of age and found reported associations of three single-nucleotide polymorphisms (SNPs) in one of the human FOXO genes (humans have four FOXO proteins: FOXO1a, FOXO3a, FOXO4 and FOXO6), FOXO3A with the longevity group. This was also replicated in independent populations such as German centenarians (Flachsbart et al.,

2009). FOXO therefore provides a good example of how the work carried out in lower organisms such as worms and flies can give an insight into human ageing.

Manipulating IIS in both systemically and in specific tissues can extend lifespan (Mathew et al., 2017). In addition, manipulating downstream transcription factors of IIS such as FOXO is sufficient to extend lifespan, even when restricted to certain tissues (Hwangbo et al., 2004, Giannakou et al., 2004, Demontis and Perrimon, 2010). For instance, in *Drosophila*, overexpression of FOXO in the adult fat body (equivalent to mammalian adipose tissue/liver) extends lifespan (Giannakou et al., 2004, Hwangbo et al., 2004). Specifically overexpressing FOXO in the muscles in flies during adulthood extends lifespan and improves the age-related decline in muscle function (Demontis and Perrimon, 2010). Also, reducing IIS by overexpressing dPTEN in muscle is also sufficient to extend lifespan of flies. These examples implicate a role for these tissues in regulation of longevity and that ageing of these tissues may be life-limiting. An alternative view is that upregulation of FOXO may extend lifespan in a cell non-autonomous manner. This means it may involve FOXO in one specific organ can alter the general physiology of the whole organism. Alic et al. (2014) showed that lifespan extension observed by activating dFOXO in the fat body in *Drosophila* does not require FOXO to be present elsewhere in the body. This suggests FOXO does not promote longevity by signalling to FOXO in other distal tissues but by signalling to other unidentified factors. Therefore, the effects of FOXO on lifespan occurs in a cell non-autonomous manner.

In various animal models, the CNS is also important for lifespan extension as reduction of IIS in the CNS has also been shown to extend lifespan (Ismail et al., 2015, Alcedo and Kenyon, 2004, Azpurua et al., 2013). Despite this, lowered IIS in the CNS may have negative effects on neuronal health as IIS is a neuroprotective and plays a role in the growth and survival of neurons (Broughton and Partridge, 2009). A role for the CNS in longevity has been indicated in *Drosophila* (Broughton et al., 2005) as the ablation of the mNSCs in the CNS extends lifespan. In worms, there is evidence that loss-of-function of the sensory neurons (which recognize environmental cues) extends lifespan and is thought to do so via reducing IIS (Alcedo and Kenyon, 2004)- In particular some gustatory neurons promotes lifespan. Considering all this evidence, reducing IIS in specific tissues achieves enhanced longevity and improved healthspan. Hence, it is not essential to reduce IIS in every tissue to observe the effects on ageing (Broughton and Partridge, 2009).

The insulin/insulin-like peptide signal can also be transduced via activation of Ras/MAPK signalling. Activation of the insulin receptor tyrosine kinase leads to the recruitment of the adapter proteins, Grb2 and Shc (Figure 1.4). Grb2 then recruits SOS, a Ras-GTPase exchange factor (Ras-GEF) which leads to the activation of Ras, a small GTPase. This stimulates a phosphorylation cascade via Raf, MEK and ERK. Upon its activation, ERK can enter the nucleus and activate members of the E-twenty-six (ETS) transcription factor family to induce ETS-dependent gene expression.

Recent studies have shown that lifespan extension by reduced IIS requires inhibition of signalling via Ras/MAPK (Slack et al., 2015). The study also used the MEK kinase inhibitor, trametinib, to inhibit Ras/MAPK activity directly and showed that this also extends lifespan in *Drosophila* and requires the activity of the ETS transcription factor, Anterior open (AOP) (Slack et al., 2015). Activation of AOP itself is also sufficient to extend lifespan in flies and recent reports suggest that AOP and FOXO may share a subset of target genes (Alic et al., 2014).

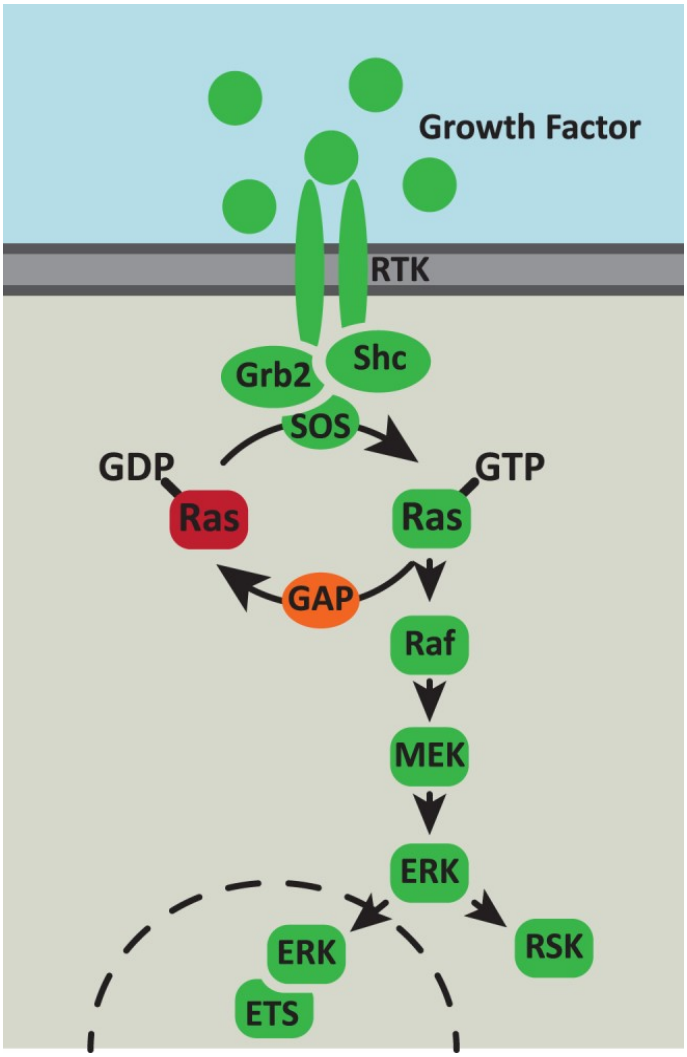


Figure 1.4 Ras/MAPK signalling.
Receptor tyrosine kinase (RTK) is activated by insulin/growth factor binding. This causes Grb2 and Shc adaptor proteins to bind to RTK recruiting the Ras-GEF, SOS, which induces the exchange of GDP to GTP, activating Ras. This then initiates a phosphorylation cascade via Raf, MEK and ERK. Activated ERK phosphorylates ribosomal S6 kinase (RSK) or it can enter the nucleus and activate transcription factors of the E-twenty-six (ETS) transcription factor family. (Taken from Slack (2017)).

1.3.2 The mechanistic target of rapamycin pathway (mTOR)

Cell signalling via the IIS pathway is intricately linked to a second conserved cell signalling pathway, the mechanistic Target of Rapamycin (mTOR) pathway (Figure 1.5). mTOR exists as part of two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). PI3K/AKT signalling activates mTORC1 and mTORC1 can downregulate IIS, via the activation of S6 kinase beta-1 (S6K1) which leads to the phosphorylation and inhibition of insulin receptor substrate 1 (IRS-1) (Takano et al., 2001).

Again, genetic manipulations that reduce signalling via mTOR have been shown to have evolutionary-conserved effects on animal lifespan. For example, in worm mutation of *raptor/daf-15,* which encodes a component of mTORC1 extends lifespan (Jia et al., 2004). *Daf-15* heterozygotes lived 30% longer than controls and they also reported that the reduction in expression of *mTOR* itself increases lifespan as RNAi knockdown of *let-363* (the gene that encodes the *C. elegans* mTOR protein) extended lifespan. Similarly, RNAi knockdown of the worm S6K1, *rsks-1,* also show increased lifespan associated with reduced fecundity and increased stress resistance relative to controls. This indicates that inhibiting a key downstream effector of mTOR signalling is also sufficient for lifespan effects.

Similarly, in flies, genetic manipulations that reduce mTOR activity extend lifespan (Kapahi et al., 2004; Luong et al., 2006). Luong et al. (2006) reported that hypomorphic mutation of the *Drosophila tor* gene extended lifespan as flies heterozygous for this mutation lived 20% longer than controls. Luong et al. (2006) also found that old and young *tor* mutants had fewer heart failures than controls and therefore were protected from age-related cardiac decline. Also, reduced mTOR activity through the overexpression of *dTSc1* (tuberous 1) and *dTSc2* (tuberous 2) extends *Drosophila* lifespan (Kapahi et al., 2004). dTSc1 and dTSc2, together form a complex that function as an upstream inhibitor of mTOR. Transgenic flies overexpressing *dTSc1* or *dTSc2* were 14% and 12% longer lived that controls, respectively. Kapahi et al. (2004) also showed that overexpression of a dominant negative form of *Drosophila* mTOR ($dTOR^{FRB}$) or flies overexpressing a dominant negative form of *Drosophila* S6K have increased lifespan.

Lifespan extension by inhibition of mTOR has subsequently been demonstrated in mice (Selman et al., 2009). Female mice with a genetic deletion of S6K1 ($S6K1^{-/-}$) live longer than controls (9%) and show improvements in a number of age-related markers of ageing (Selman et al., 2009). $S6K1^{-/-}$ also show improvements in bone, immune, motor function which all decline with age.

Similar to flies and worms, mice hypomorphic for mTOR, with a reduction in mTOR levels of around 25% compared to wild type levels and less mTORC1 and mTORC2 activity, live longer compared to controls (Wu et al., 2013). Older mice with mTOR reduction also show improvements in spatial learning and memory, which declines with age, and decreased indicators of oxidative stress in ageing tissues including the brain (Wu et al., 2013).

Importantly, pharmacological inhibition of mTOR activity using the highly specific inhibitor, rapamycin, produces similar effects on longevity and age-related function as genetic inhibition in worms, flies and mice (Robida-Stubbs et al., 2012, Bjedov et al., 2010, Harrison et al., 2009). In *Drosophila*, rapamycin-treated flies showed extended lifespan and these flies displayed increased stress resistance, reduced fecundity and increased lipid levels compared to controls (Bjedov et al., 2010). They also found that the effects of rapamycin was due to mTOR inhibition specifically as they treated flies overexpressing a constitutively active form of S6K and 4E-BP null mutant flies with rapamycin and found rapamycin had no effect on their lifespan. Therefore, downregulation of S6K activity and activation of 4E-BP may both mediate the lifespan extension by rapamycin. Rapamycin fed to mice at 270 days of age show extended lifespan compared to controls (Harrison et al., 2009). Harrison et al. (2009) further showed that when rapamycin is administered late in life at 600 days of age, it still extended the lifespan of males by 9% and females by 14%.

Thus, these studies and others such as Ras inhibition by trametinib (Slack et al., 2015) suggest that pharmacological manipulation of the biological pathways that influence ageing can also increase lifespan in model organisms and maintain health well into old age.

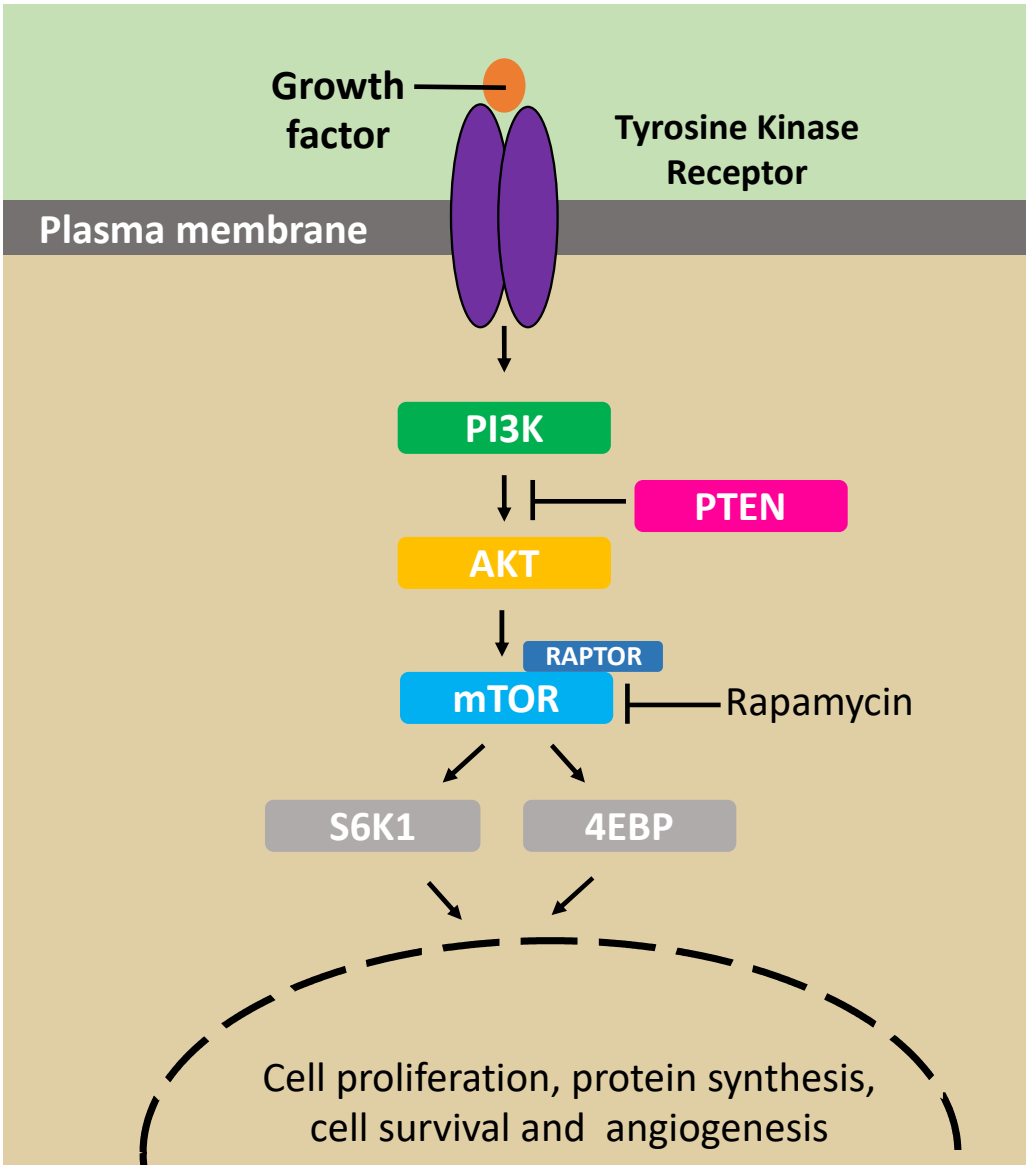


Figure 1.5 Schematic diagram of the mTOR pathway.
Growth factors bind to the tyrosine kinase receptor which activates PI3K, leading to the activation of AKT and downstream mTOR (and raptor). mTOR promotes cell proliferation, protein synthesis, cell survival and angiogenesis via the phosphorylation 4E-BP or S6K1. PI3K activation of AKT can be inhibited by PTEN, preventing mTOR signalling. mTOR can be inhibited by the drug Rapamycin.

1.4 *Drosophila* as a model for ageing

The fruit fly, *Drosophila melanogaster,* has been extensively used as a tool to study the mechanisms underlying ageing for over thirty years. There are several features of this species that makes them a useful model for ageing studies. The *Drosophila* life cycle consists of four stages: egg, larva, pupa and adult fly (Figure 1.6). At 25°C, the developmental time from egg to adult fly takes 10 days (Jennings, 2011). The adult fly can then live between 2-3 months (Zwaan et al., 1992). This short lifespan and rapid developmental time make them an ideal model for the experimental analysis of ageing. In addition, the average mated female fly will produce approximately 80 fertilised eggs a day (Markow, 2011). This means a large population of flies can be generated in one round of mating.

Besides the practical laboratory advantages, flies also show other features that make them a useful model for studying the mechanisms that underlie human ageing. Perhaps, most importantly, the *Drosophila* and human genomes share a high degree of homology, sharing approximately 60% sequence similarity. For instance, 77% of the genes that cause disease in humans have direct counterparts in the fly genome (Reiter et al., 2001). Furthermore, many human proteins have direct functional orthologues within *Drosophila,* demonstrating a high degree of evolutionary conservation between the two species at the functional level.

Drosophila have clearly defined tissues such as a heart, brain, fat, gut, muscle and kidneys which can be easily isolated by dissection allowing tissue-specific effects of ageing to be studied in detail. Moreover, as flies age they exhibit multiple aspects of age-related physiological decline within these tissues (Jones et al., 2011) including a deterioration in heart, brain, immune and muscular functions (He and Jasper, 2014). Various markers of age-related loss of function can also observed and measured such as alteration in metabolism, behaviours like feeding, reduced stress resistance, reduced fecundity and altered physical activity (including impairment in negative geotaxis) and decreased intestinal barrier function (Piper and Partridge, 2018).

Another strength of *Drosophila* as a model over other invertebrates, is that they exist as two sexes, males and females, which facilitates the study of sex differences in ageing.

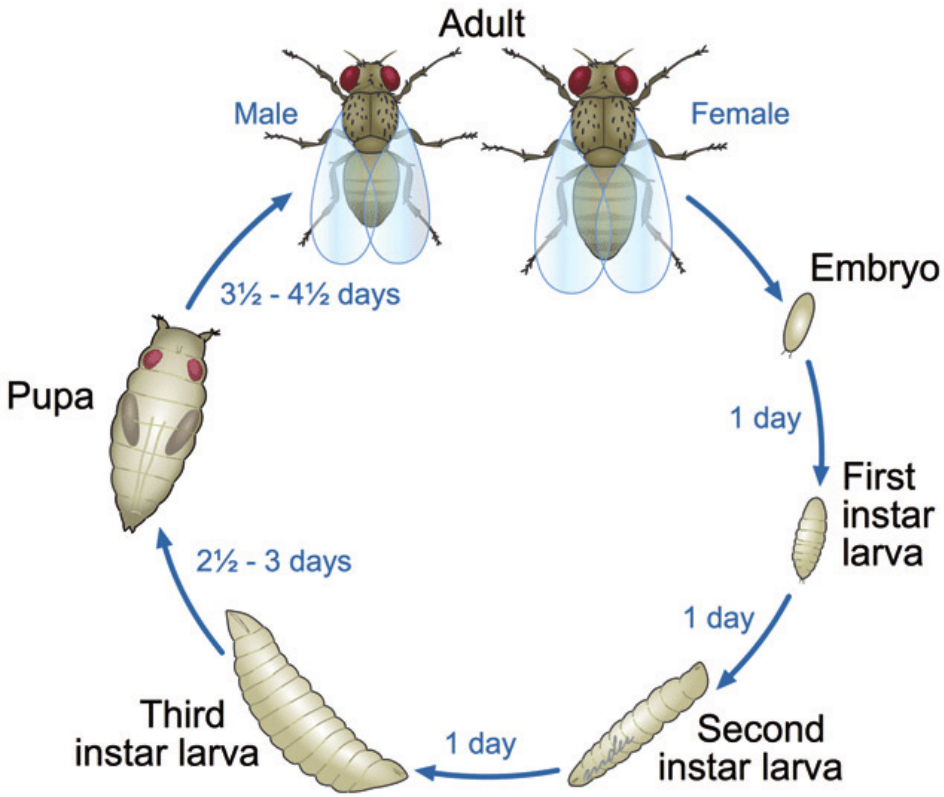


Figure 1.6 Life cycle of *Drosophila.*
The *Drosophila* life cycle consists of four developmental stages from fertilized egg through three larval moults to pupa and adult. Image taken from Ong et al. (2015).

1.5 G protein-coupled receptors and ageing

G protein-coupled receptors (GPCRs) are a superfamily of membrane bound receptors characterised by a seven-transmembrane domain. GPCRS make attractive drug targets because they control a range of important physiological functions including neurological (neurotransmission), endocrine (hormone release) functions and immune responses (Krishnan and Schioth, 2015).

Around 35% of currently marketed drugs work by targeting GPCRs (Basith et al., 2018). Two examples of drugs which target GPCRs are abaloparatide and lixisentide. Both drugs target class B GPCRs: abaloparatide targets the parathyroid hormone-related protein receptor and is used

in the treatment of osteoporosis whereas lixisentideis is a drug used to treat type II diabetes and targets glucagon-like peptide receptor (Hauser et al., 2017).
GPCRs are grouped into six classes based on sequence and function: Class A (rhodopsin-like), Class B (secretin receptors), Class C (metabotropic glutamate receptors), Class D (fungal mating pheromone receptors), Class E (cAMP receptors) and Class F (frizzled and smoothened receptors) (Basith et al., 2018). A primary function of these receptors is to sense extracellular signals and transmit the signals to intracellular targets.

GPCRs are typically associated with G proteins, a heterotrimer made up of three subunits: G_α (which binds the guanine nucleotides GTP and GDP), G_β and G_γ. On activation, conformational changes occur within the receptor that leads to the exchange of GDP for GTP and the dissociation of the G_α subunit from the G_β/G_γ complex. The dissociated subunits can then act on various downstream effectors such as calcium, potassium channels, adenylyl cyclase, phospholipase C (PLC) leading to activation of intracellular signalling cascades (Tuteja, 2009).
One such example of a signalling pathway activated by G proteins is MAPK and also JNK pathways, this involves the βγ subunits(Gutkind, 1998).

Importantly, GPCRs have been shown to play a role in animal ageing. In *Drosophila*, partial loss of function mutations in *methuselah (mth),* a gene encoding a class B GPCR, extends lifespan (Lin et al., 1998) where *mth* mutants have a 35% increase in lifespan compared to control animals. Similarly, females heterozygous for sun^{EM67} and sun^{Y6} mutations show extended lifespan (Cvejic et al., 2004). These mutations delete a copy of the *stunted* (*sun*) gene, which encodes two peptide ligands of *mth,* Sun A *and* Sun B. Lifespan can also be extended by consitutive expression of peptides which act as antagonists of Mth function (Ja et al., 2007b) . These peptides innibit *mth* activity by binding to the N-terminal ectodomain of the receptor which mediates ligand binding. Studies have shown the effects of *mth* on lifespan are mediated through interactions with the mTOR pathway (Wang et al., 2015a). Activation of mTORC1 leads to phosphoyrlation of S6K and 4E-BP and Wang et al. (2015a) showed that *mth* mutants display decreased levels of p-S6K and p-4EBP indicative of reduced mTOR activity. The study also showed similar effects on these two mTOR effectors in flies fed with an antagonist of Mth, 65-A11. (Wang et al., 2015a) also showed mTOR knockdown in flies fed with 65-A11 are long lived compared to controls but this could not further extend the lifespan of *tor* mutants not treated with the antagonist.

In *C. elegans,* the Class A GPCRs (Rhodopsin-like) are subdivided into 4 subclasses: chemosensory, peptidergic, aminergic and muscarinic (acetylcholine). The chemosensory GPCRs are a non-conserved group of receptors that help animals sense external chemosensory information and recent evidence suggests that they may also play a role in ageing in *C. elegans*. For example, it was recently shown that loss of a chemosensory GPCR, STR-2 (serpentine receptor) in olfactory neurons by RNAi extends *C. elegans* lifespan (Alcedo and Kenyon, 2004) with STR-2 mutants living 18% longer than controls.

Together, these data show that inhibition of different classes of GPCRs lead to prolonged lifespan in different animal models.

1.6 Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) are Class C GPCRs which include other GPCRs such as GABA receptors and a calcium-sensing receptor (Fredriksson et al., 2003). In the mammalian Central Nervous System (CNS), L-Glutamate acts as the major excitatory neurotransmitter via ionotropic receptors and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors act fast and produce short lived responses while mGluRs exhibit slow and long lasting responses due to the signalling cascades that occur following their activation (Byrne et al., 2014). In mammals, there are eight mGluRs (mGluR1 to mGluR8) which are subdivided into 3 families: Group I, Group II and Group III, based on their sequence homology, pharmacology and the G protein with which they are associated. Group I (mGluR1 and mGluR5) are coupled to G_q and G_{11} and activate phospholipase C (PLC), which leads to the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), release of Ca^{2+} and activation of protein kinase C (PKC) (Figure 1.7). Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs are coupled to G_i/G_o and inhibit adenylyl cyclase (AC). Activation of these two classes also results in the decrease of the second messenger, cyclic AMP (cAMP), inside the cell.

Although Group I mGluRs predominantly activate G_q, there is evidence that it can also be coupled to other G proteins. For example, Group I receptors have been shown to be associated with both G_s, activating AC and therefore increasing cAMP production (Wang et al., 2015b) and also G_i, suppressing AC activity (Sharon et al., 1997). The association of mGluR with G protein is therefore not rigid and there is promiscuity in the coupling of receptors to the G proteins.

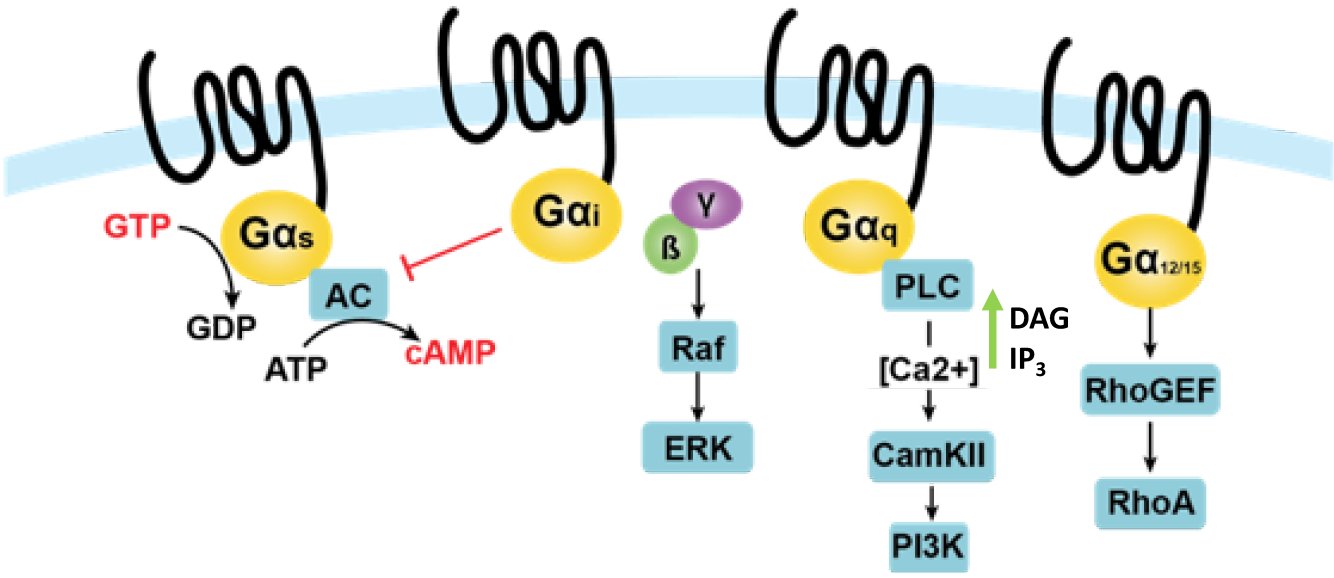


Figure 1.7 G-protein signalling downstream of mGluR activation
As a GPCR, metabotropic glutamate receptors (mGluRs) can activate various downstream signalling pathways based on their G protein association. The different G_{α} subunits G_s, G_i, G_q and $G_{12/15}$ activate distinctive pathways. The G_{β} /G_{γ} subunits can also activate downstream pathways when dissociated from G_{α} subunit, they can activate Raf and Erk.

1.6.1 mGluR Structure

Like all members of the GPCR superfamily, mGluRs possess a seven-transmembrane domain (TMD). They also possess a large N-terminal domain containing the ligand-binding site, which is much larger than that of typical GPCRs (Ji et al., 1998). The ligand binding site is made up of two hinged domains called the Venus flytrap domain (VFD). The cysteine-rich domain links the VFD to the 7-transmembrane domain. Once glutamate binds, the VFD closes together, leading to conformational changes within the TMD that stimulates G protein activation to initiate intracellular signal transduction events via interactions between the C-intracellular C-terminal domain of the receptor and the G protein (Figure 1.8). As Figure 1.8 shows, group I mGluRs can couple to scaffolding proteins such as Homer. The role of scaffolding proteins involves coupling group I mGluRs to intracellular signalling molecules. For example, group I mGluRs are linked to the IP_3 receptor through the C-terminal tail (Tu et al., 1998).

It is apparent from crystal structure data that mGluRs are dimers of two molecules linked by a disulphide bridge, confirming previous findings of mGluRs in cells (Kunishima et al., 2000).

Kunishima et al. (2000) found that mGluR exists in three stable crystal structures: two forms known as free form I and II, with no ligand bound (open) and one form with glutamate bound known as the complex form (closed). The ligand binding domain is made of two domains LB1 and LB2 and in the complex form and free form II, the two LB2 domains are in close proximity. In free form I, the LB2 domains are far from each other. Crystal structure analysis of mGluR1 dimers found that the Venus fly trap domain and cysteine rich domains keep the two 7 -transmembrane domains apart when receptor is unbound (Nicoletti et al., 2011) and only one 7-transmembrane domain is required to be activated to activate the receptor. The secondary structure of the complex form of several mGluR types (mGluR1, mGluR2, mGluR5 and mGluR6) are shown in Figure 1.9.

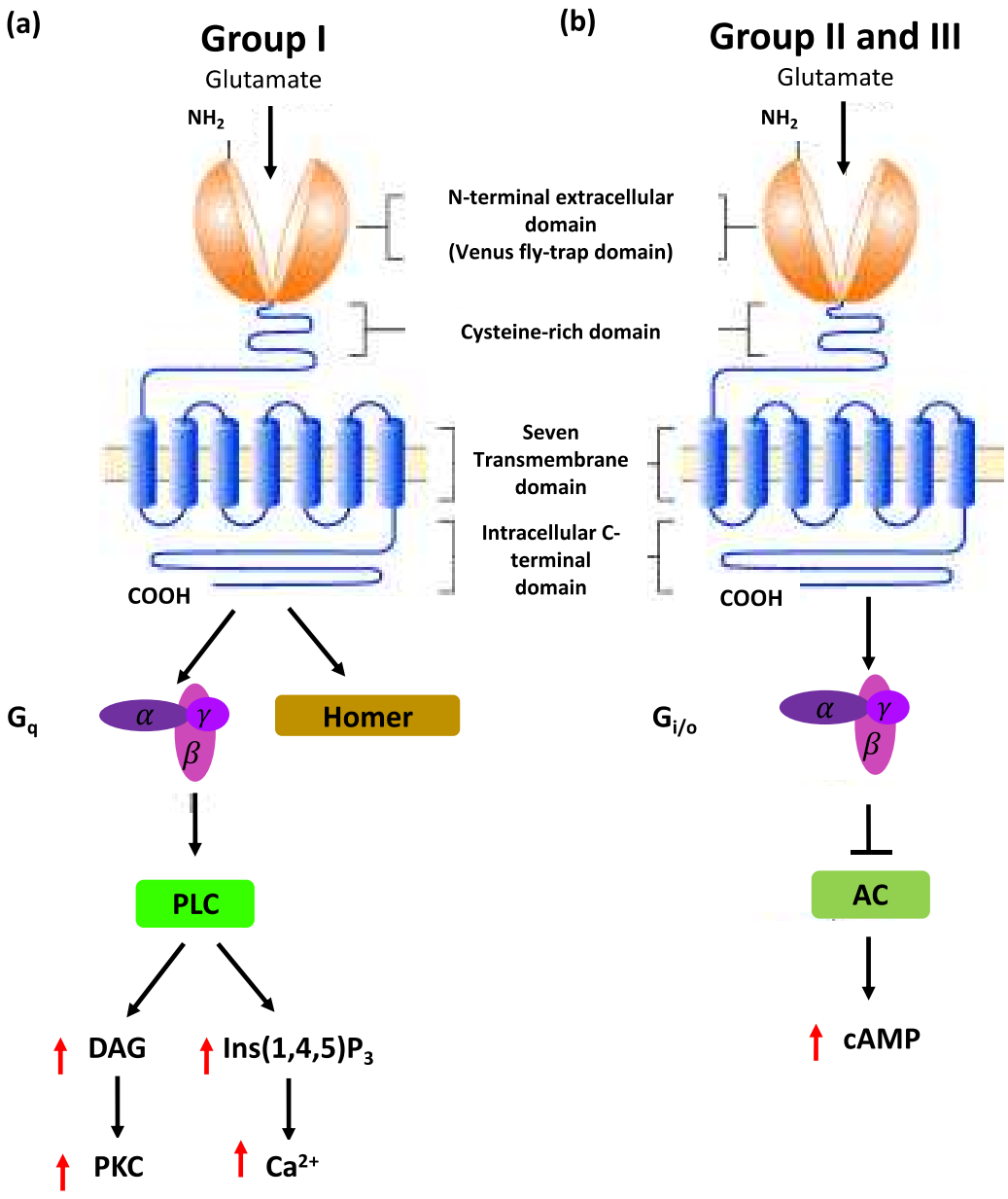


Figure 1.8 Intracellular signalling cascades activated by Group I or Group II/III metabotropic glutamate receptors (mGluRs).

Metabotropic glutamate receptors (mGluRs) are activated by glutamate binding to the ligand binding site within the N-terminal extracellular region of the receptor (Venus fly-trap domain (VFD)). A cysteine-rich domain links the VFD to the seven-transmembrane domain (TMD).

a) For Group I mGluRs, ligand binding leads to activation of the heterotrimeric G_q protein and activation of phospholipase C (PLC). This stimulates diacylglycerol (DAG) formation and activation of protein kinase C (PKC). Group I mGluRs also couple to the scaffolding protein, Homer.

b) For Group II and Group III mGluRs, ligand binding leads to activation of G_i/o. This inhibits adenylyl cyclase (AC) and reduces levels of the secondary messenger, cAMP.

(Adapted from Kenny and Markou (2004)).

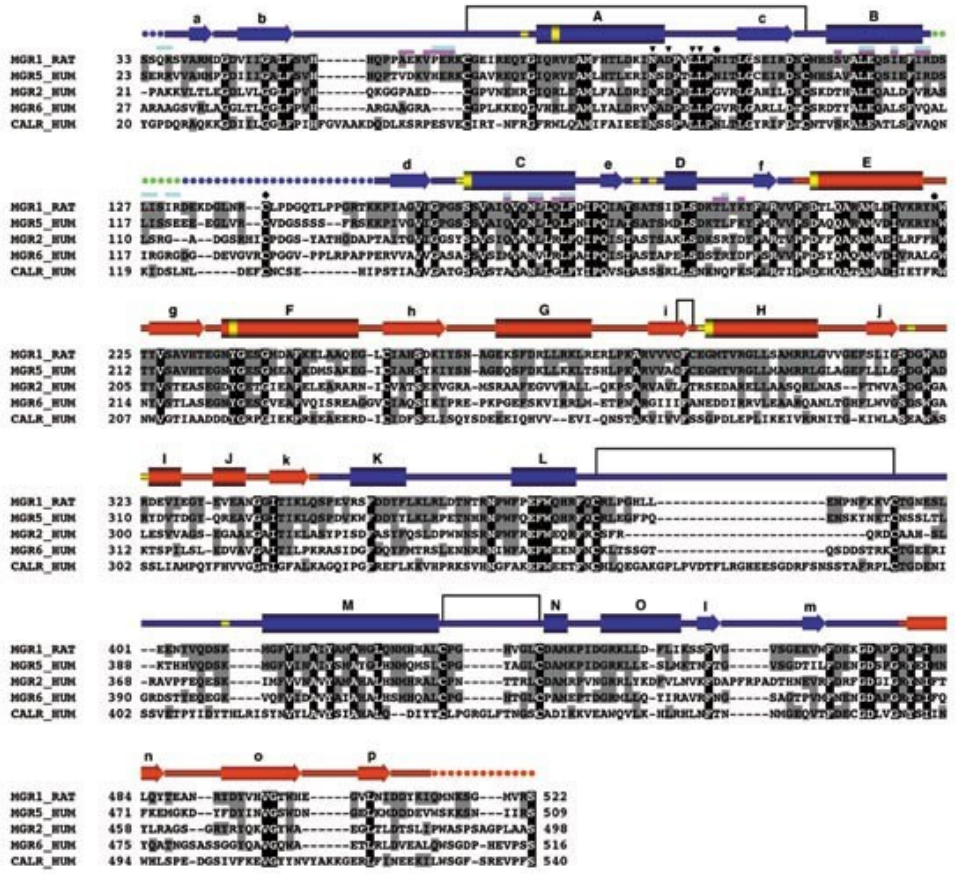


Figure 1.9 Secondary structures of mGluR
Sequence alignment of the closed conformation of mGluR (complex form), with secondary sequence displayed over the sequences. The LB1 and LB2 domains are in blue, the ligand-binding sites red, additionally visible region in the free form is highlighted in green and the disulphide bridges are indicated by thin solid lines connecting cysteines. Helices of the crystal structure are shown as cylinders, arrows represent strands and dots represent disordered residues. Sequences are for mGluR1, mGluR2, mGluR5 and mGluR6 and the calcium receptor, the conserved sequences between these receptors are shaded (Figure taken from Kunishima et al. (2000)).

1.6.2 Distribution of mammalian metabotropic glutamate receptors

In mammals, mGluRs are widely expressed throughout both the central and peripheral nervous systems, apart from mGluR6, which is only expressed in the retina (Nomura et al., 1994). In the CNS, the different mGluR subtypes are found in specific areas where they seem to perform specific functions. mGluRs are also expressed in other tissues outside the nervous system such as the pancreas, reproductive system and adrenal medulla (Durand *et al*., 2012). There is also evidence of expression in the gastrointestinal tract (GI), particularly ligands of mGluR5 have

been found in the GI tract (Ferrigno et al., 2017) . The functions of mGluRs within these tissues is not fully understood.

In the CNS, the expression of the two group I mGluRs in the is mostly complementary. mGluR1 is found in mainly in the cerebellum (Zanotti-Fregonara et al., 2016) and also found in the thalamus olfactory bulb, interneurons of the hippocampus, hippocampal CA1 region, CA3 hippocampal pyramidal neurons and the dendate gyrus (Ferraguti and Shigemoto, 2006). The other class I mGluR, mGluR5, is found in the dendritic fields of CA1 of the hippocampus olfactory bulb, cerebral cortex, striatum and nucleus accumbens (Ferraguti and Shigemoto, 2006).

Group I mGluRs are typically located postsynaptically whereas group II and III mGluRs are found mostly presynaptically (Niswender and Conn, 2010). Group II mGluRs are found in the deeper layers of the cerebral cortex and the Golgi bodies of the cerebellum (Ferraguti and Shigemoto, 2006). Group III mGluRs can be found widely in the brain including the cerebellum, hippocampus and olfactory bulb.

1.6.3 Functions of mammalian metabotropic glutamate receptors

mGluRs have been implicated in synaptic plasticity (Conn and Pin, 1997), which is the ability for synapse strength to be changed i.e. weakened or strengthened over time in response to decreases or increases in their activity. Two processes that underlie synaptic plasticity are long term potentiation (LTP), a persistent strengthening of synapses, and long-term depression (LTD), a long-lasting decrease in synaptic strength. Both processes are widely considered to be the major cellular mechanisms that underlie learning and memory (Howland and Wang, 2008).

Activation of Group I mGluRs can lead to LTD (Palmer et al., 1997). This takes place in the hippocampus, cerebellum and prefrontal cortex and in many other areas in the brain (Lin et al., 2011). mGluR1 plays a role in inducing LTP in the CA1 region of the hippocampus, specifically in the excitatory synapses of *stratum oriens*/*alveus* (O/A) interneurons (Lapointe et al., 2004). LTP is usually induced in this area of the hippocampus (Perez et al., 2001) but was not induced in O/A interneurons in mGluR1 knockout mice. Lapointe et al. (2004) therefore demonstrating that LTP in this area is dependent on mGluR1.

mGluRs also play a role in neuromodulation by influencing neuronal excitability and neurotransmitter release. Group I mGluRs act to increase neuronal excitability and increase the

release of the neurotransmitters glutamate or GABA, and group II and III supress neuronal excitability by inhibiting the release of these neurotransmitters (Niswender and Conn, 2010). NMDA and AMPA receptors, glutamate-gated ion channels, respond to the increase in glutamate by increasing intracellular sodium or calcium, resulting in neuronal excitability.

1.7 *Drosophila* mGluR

The *Drosophila* genome encodes a single functional mGluR, DmGluRA. DmXR, another receptor thought to be similar to the mammalian mGluR does exist but studies have shown that DmXR is incapable of binding glutamate (Mitri et al., 2004). DmGluRA is most similar to the mammalian Group II mGluRs sharing 45% amino acid sequence identity with mGluR3 and 43% with mGluR2 (Parmentier et al., 1996b) (Figure 1.9). Like other class II mGluRs, DmGluRA is coupled to G_i, but also has G_q-coupled signalling activity suggesting that the single *Drosophila* receptor can carry out all mGluR functions (Pan and Broadie, 2007). It has recently been found that *DmGluRA* shares functional homology with Group I mGluRs because of its coupling to Homer proteins, a conserved Group I mGluR function(Ly et al., 2020). Cloning of the *DmGluRA* gene from *Drosophila* confirmed that DmGluRA also shares structural homology with the mammalian mGluR, as all structural features are conserved in the fly orthologue, including the N-terminal extracellular ligand-binding domain, cysteine-rich domain, seven transmembrane domain, intracellular loops and intracellular C-terminal domains (Parmentier et al., 1996b).

1.7.1 Distribution and functions of DmGluRA

Like the mammalian mGluRs, DmGluRA is expressed widely throughout the fly brain. DmGluRA expression was first described to be in the antenatal lobes (AL), optic lobes, the tritocerebral of the median bundle and the central complex specifically in the fan shaped body (Ramaekers et al., 2001). DmGluRA expression has also been reported in the mushroom bodies (Sinakevitch et al., 2010), which is the structure important for learning and memory formation in insects, and in the neuromuscular junction (NMJ), where it is expressed presynaptically like mammalian Group II mGluRs (Bogdanik et al., 2004). At the NMJ, DmGluRA plays a role in transmitter release and modulating synaptic morphology (Bogdanik et al., 2004). Confocal imaging of *DmGluRA* mutant larvae which had their NMJ stained with presynaptic markers showed mild but significant in NMJ structure such as decreased in number of synaptic boutons and increased

bouton size. This indicates DmGluRA modulates the number of bouton. These phenotypes of these mutants are similar to hyperexcitable *Drosophila* mutants suggesting increased neuronal activity and a role for DmGluRA in regulating presynaptic excitability.

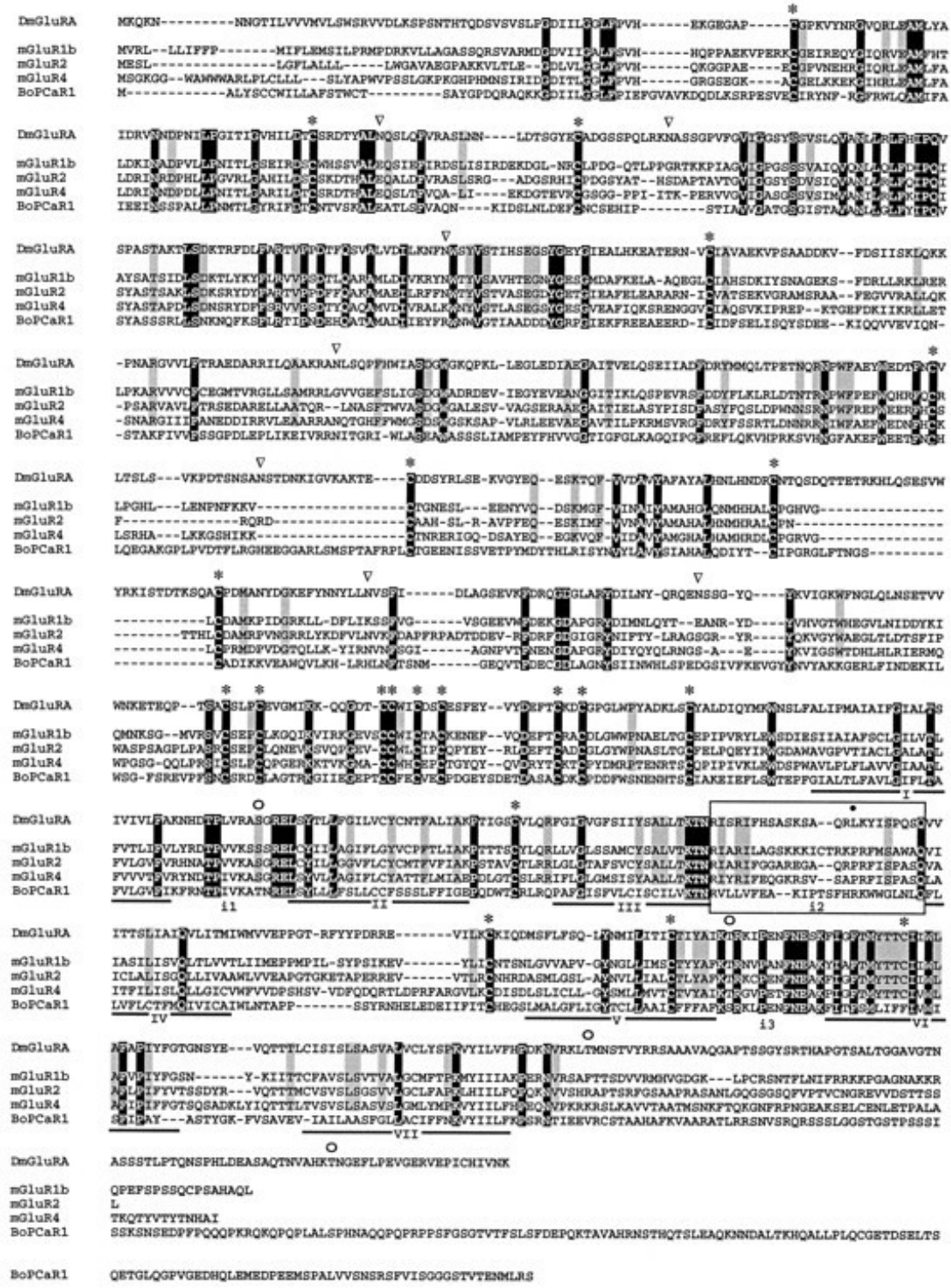


Figure 1.10 Sequence alignment of fly DmGluRA to its mammalian homologues
Drosophila DmGluRA aligned to mGluRs from each mammalian group. Regions of the proteins that are conserved in mammals and fly are shown in grey. Regions conserved in all mGluRs and in the calcium sensing receptor are in black. The 7-transmembrane domain is underlined in black and black box indicates the sequence of the second intracellular loop, which is involved in G protein specificity. The asterisks show conserved cysteine residues (Parmentier et al., 1996b).

A role for DmGluRA function in social behaviour and memory has been demonstrated by Schoenfeld et al. (2013). Male flies either heterozygous or homozygous for a null mutation in *DmGluRA* showed very little courtship behaviour, an indicator for social behaviour, in comparison to control flies. Courtship behaviour can also be used as a read-out for learning and memory in *Drosophila*. As homozygous *DmGluRA* mutants showed little courtship behaviour, only heterozygotes were used to measure learning and memory. The assay used is based on conditioned courtship, a learned behaviour in which males reduce their courtship behaviour after rejection from a mated female. Previously mated females usually reject a male's attempts of copulation. in response to this rejection, males would reduce courtship behaviour towards subsequent female target for a period of time.

Schoenfeld et al. (2013) found that short-term was impaired in *DmGluRA* heterozygote mutants compared to controls. When mutants were paired with a virgin female after a 1hr exposure with a pre-mated female, they failed to supress their courtship behaviour. This was compared to controls who exhibited supressed courtship towards the subsequent virgin female target. Heterozygous mutants also showed an impairment in long-term memory was impaired. The mutant males were paired with a pre-mated female for 7hr and subsequently 4 days after, paired with a virgin female. *DmGluRA* mutants were not able to suppress their courtship behaviour after the initial rejection compared to controls. both findings of long-term memory and short-term memory suggests DmGluRA is critical for the formation of memory.

The role of DmGluRA in social behaviour, cognition and memory has also been studied in a model for Fragile X in *Drosophila* (McBride et al., 2005). Fragile X is an inherited disease characterized by intellectual disability and caused by the lack of expression of Fragile X Mental Retardation Protein (FMRP) encoded by the *fragile X mental retardation 1* (*fmr1*) gene (Jin and Warren, 2000). Flies possess a homolog of *fmr1* (*dfmr1*) and a model for Fragile X in *Drosophila* is well-established (Wan et al., 2000). Flies mutant for *dfmr1* lack FMRP expression and show Fragile X-like symptoms including defects in social behaviour, immediate recall and short term memory (Bolduc et al., 2010). Inhibition of FMRP expression leads to upregulation of DmGluRA expression. McBride et al. (2005) found that inhibiting DmGluRA activity using the compound, 2-Methyl-6-(phenylethynyl)pyridine (MPEP), a selective antagonist for mGluR5, could improve the social behaviour and memory impairments observed in *dfmr1* mutant flies. Hence, these deficits in social behaviour and memory seen in *dfmr1* mutant flies may be caused by increased

expression of DmGluRA, highlighting the importance of DmGluRA in learning and memory and social behaviour in a similar manner to mammalian mGluRs.

Recent studies have also shown that DmGluRA plays a role in sleep (Ly and Naidoo, 2019, Ly et al., 2020). *DmGluRA* null mutant females displayed age-related loss of sleep and young flies carrying the null mutation showed reduced wakefulness during the day, i.e. increased amount of sleep during the day and reduced sleep during the night (Ly and Naidoo, 2019). The interaction between DmGluRA and Homer was found to be critical for this function of DmGluRA in sleep regulation. Homer and DmGluRA directly bind and disrupting the Homer binding site on DmGluRA, a proline-rich PPXXF motif, reduces the amount of sleep of the flies. Furthermore, promoting sleep deprivation or increasing wakefulness reduces the interaction between Homer and *DmGluRA* in wildtype flies (Ly et al., 2020).

1.7.2 Mammalian and *Drosophila* mGluRs interact with pro-longevity signalling pathways

Several studies have now shown that activation of mGluRs by glutamate binding stimulates intracellular signalling via both the PI3K/AKT and MAPK signalling pathways, both of which play important, evolutionary conserved roles in animal ageing, as discussed earlier. mGluR1 activation leads to PI3K-AKT-mTOR signalling via the protein Homer1 (Ronesi and Huber, 2008). In hippocampal neurons, disruption of mGluR-Homer interaction using a peptide containing mGluR5ct, the mGluR5 C-terminal tail containing the Homer ligand domain, prevented group I mGluR activation of PI3K-AKT-mTOR signalling (Ronesi and Huber, 2008).

Ligand activation of DmGluRA in D*rosophila* motor neurons stimulates PI3K/AKT via activation of calcium/calmodulin-dependent protein kinase II (CAMKII) and *Drosophila* focal adhesion kinase (DFak) (Chun-Jen Lin et al., 2011) . The study applied glutamate to activate DmGluRA in the motor neurons of larvae and PI3K/AKT activation was measured using an antibody against p-AKT, the phosphorylated form of AKT, which increases when PI3K is stimulated. P-AKT was increased by expression of $CaMKII^{T287D}$, a constitutively active form of CaMKII. Decreasing CAMKII activity, via expression of the CAMKII inhibitor peptide, *ala*, decreased p-AKT levels. DFAK was identified as another signalling intermediate, as the DFAK null mutation, $DFAK^{CG1}$, decreased levels of motor nerve terminal p-AKT, indicative of PI3K activation. Moreover, DFAK

null mutant larvae expressing CaMKII^{T287D} did not exhibit PI3K activation in motor neurons. Thus, DFak is required as in intermediate in PI3K activation by DmGluRA and CaMKII.

Inhibition of mGluR1 in human glioma U87 cells either using mGluR1 small interfering RNA (siRNA) to knockdown expression or the selective mGluR1 inverse agonists, Riluzole and BAY36-7620, also results in inhibition of PI3K, AKT and mTOR activation suggesting that P13K-AKT-mTOR signalling is diminished when mGluR1 is inhibited (Zhang et al., 2015). BAY36-7620 is the first described mGluR1 inverse agonist which acts in the transmembrane region of mGluR1, inhibiting constitutive activity (i.e. ligand independent activity) (Carroll et al., 2001). As BAY36-7620 acts on a site other than the glutamate binding site, it is a non-competitive mGluR1 antagonist. Riluzole is a drug used in amyotrophic lateral sclerosis treatment which blocks mGluR1 signalling by inhibiting glutamate release (Cheah et al., 2010).

Activation of the group I mGluR, mGluR5, using the selective agonist, DHPG, or a selective positive allosteric modulator (VU0092273) plays a PI3K-dependent neuroprotective role against oxygen-glucose deprivation in rat hippocampal slices (Cavallo et al., 2020). Inhibition of PI3K activation using the inhibitor, LY294002, blocked these neuroprotective effects of mGluR5 activation (Cavallo et al., 2020). DHPG is a highly potent agonist which has an efficacy similar to that of glutamate and be an agonist to both mGluR1 and mGluR5(Wisniewski and Car, 2002). Whereas VU0092273 has a high affinity for a site other than the glutamate binding site, it binds to an allosteric site in the seven transmembrane-spanning domain of mGluR which is targeted by compounds which inhibit coupling of the receptor to G-proteins(Rodriguez et al., 2010).

Inhibition of PI3K or mTOR can also block mGluR-dependent LTD during synaptic plasticity. Treatment with the PI3K inhibitors, LY294002 or Wortmannin, or the mTOR inhibitor, rapamycin, blocked mGluR-dependent LTD in the CA1 area of the hippocampus stimulated by DHPG (Hou and Klann, 2004).

There is also evidence that mitogen-activated protein kinase (MAPK) signalling is activated downstream of group I mGluR activation (Wang et al., 2007). DHPG-induced activation of group I mGluR increases ERK phosphorylation in the rat dorsal striatum *in vivo* as shown by semi-quantitative immunohistochemistry (Choe and Wang, 2001). P13K/AKT and RAS/MAPK pathways can also be stimulated by group II mGluR activation (Wang et al., 2007). For example for RAS/MAPK signalling, group II receptors have been showed to be coupled to ERK activation

in CHO cells, striatal neurons, and cerebellar granule neurons(Wang et al., 2004b). However Wang et al. (2007) suggests its is more likely to be through $G_{\beta\gamma}$ subunits which lead to activation of ERKvia RaS pathway.

Thus, mGluRs may offer a novel pharmacological target for the inhibition of known pro-longevity pathways. It is important to note here, existing studies have demonstrated the crucial role that mGluR plays in learning and memory (Schoenfeld et al., 2013, Conn and Pin, 1997) . From previous studies loss of mGluR in *Drosophila* has been seen to lead to impairments in memory and social behaviour(Schoenfeld et al., 2013), to consider its potential as a pharmacological target in longevity study, it's important to consider its potential effects it could have on the brain and its function particularly learning and memory.

1.7.3 Previous work on DmGluRA and ageing in *Drosophila*

The interaction between DmGluRA and known pro-longevity pathways such as PI3K/AKT and Ras-MAPK inspired previous studies in the laboratory to examine a role of DmGluRA in *Drosophila* ageing. Preliminary experiments found that null mutation for the single *Drosophila* mGluR orthologue, *DmGluRA*, resulted in longevity. Interestingly, these effects of loss of DmGluRA activity on lifespan were found to be sex-specific but were observed only in female flies and not in males suggesting that the effects of DmGluRA activity on lifespan are sex-specific (Cathy Slack, unpublished).

1.8 Thesis aims & objectives

The aim of this project was to determine the role of mGluR in biological ageing. To address this aim, *Drosophila* was used as an established ageing model to identify the mechanisms by which loss of mGluR activity in flies extends lifespan. The project also aimed to further characterise the sex-specific differences in the effects of DmGluRA expression on lifespan.

To address this aim, the following experimental approaches were taken:

Objective (1) To characterise in more detail the molecular mutant of the mutant line used. To confirm the longevity response across different backgrounds and to test whether other factors that are known to impact on longevity, such as the presence of the endosymbiotic bacteria,

Wolbachia pipientis, influenced the lifespan of the *DmGluRA*$^{[112b]}$ mutants. Also, to identify whether DmGluRA expression changes during ageing and compare gene expression across sexes.

Hypothesis & experimental approach

The hypothesis was that differences in expression of DmGluRA and the level of reduction in mutant flies may be different between males and females and so may underlie the observed sex-specific differences in the longevity response. To investigate this, qRT-PCR was used to measure gene expression in both sexes during ageing and in control and mutant flies (Chapter 3).

Objective (2) To determine whether the longevity response of mGluR mutants is accompanied by other phenotypic changes that may identify causal factors for the lifespan response.

Hypothesis & experimental approach

Other pro-longevity interventions are often accompanied by characteristic phenotypes, for example reduced fecundity, improved stress tolerance (such as starvation and oxidative stress). In these interventions, the lifespan enhancing effects have been attributed at least in part to these additional phenotypic responses. Therefore, the hypothesis was that the identification of these other mGluR-dependent phenotypes may highlight key physiological responses that underlie the longevity effects of mGluR inhibition. Based on the fact that mGluR signals via known pro-longevity mediators (e.g. PI3K and Ras/MAPK), experiments focused on phenotypes that are characteristic for down-regulation of their activity. Alongside these analyses of physiological responses to mGluR inhibition, *DmGluRA* mutants were also examined for indicators of age-related functional decline specifically locomotion and intestinal barrier function.

Objective (3) To describe the transcriptional response to loss of mGluR activity.

Hypothesis & experimental approach

mGluRs are GPCRs and therefore when activated these receptors modulate intracellular signal transduction cascades. This includes PI3K and MAP/ERK signalling pathways which play evolutionary conserved roles in animal ageing. Both signalling pathways lead to modulation of

downstream transcription factors such as FOXO and AOP which regulate different genes. Therefore, it is possible loss of mGluR in *Drosophila* could have an impact on gene expression and therefore may lead to phenotypic changes in mutants and affect longevity.

The hypothesis was therefore that differential gene expression analysis could highlight key transcriptional changes in *DmGluRA* mutants, leading to identification of key biological mechanisms that are associated with the longevity response. Therefore RNA-Seq analysis was used to identify gene expression changes in *DmGluRA* mutants and pathway analysis was used to assign the differentially expressed genes to functional groups.

Chapter 2 : Materials and Methods

2.1 Fly stocks and husbandry

Experiments were carried out using *white*^{Dahomey} (*w*^{Dah}), *white*^{1118} (*w*^{1118}) and *white*^{DahomeyT} (*w*^{DahT}) strains of *Drosophila*. *w*^{Dah} *was* created by backcrossing *w*^{1118} into a *Dahomey* background. *Dahomey* flies are an outbred wild-type strain, collected originally in Dahomey, West Africa, which is now known as Benin. Since their collection in 1970, they have been kept in large population cages at 25°C with overlapping generations on a 12h Light:12h Dark cycle (Bass et al., 2007). *w*^{Dah} flies naturally carry an endosymbiont, the bacteria *Wolbachia pipientis.* *w*^{DahT} lack this bacterium as this strain has been treated with the antibiotic, tetracycline. The *w*^{1118} strain does not contain *Wolbachia* (Grandison et al., 2009b). The *w*^{1118} stock was originally obtained from the Bloomington *Drosophila* Stock Centre (https://bdsc.indiana.edu).

The *DmGluRA* null mutation used in this study, *DmGluRA*$^{[112b]}$, was originally generated by P-element-induced imprecise excision, as previously described by (Bogdanik et al., 2004). The mutation was backcrossed for 6 generations into each of the wild-type strains before experiments were performed.

Drosophila stocks were maintained on standard sugar-yeast-agar food (SYA) containing 0.5 g/L sugar (Tate & Lyle, London, UK), 1 g/L brewer's yeast (MP Biomedicals, Solon, OH, USA) and 15 g/L agar (Sigma-Aldrich, Dorset, UK) (Bass et al., 2007) (See Table 2.1 for full fly food recipe). All experiments were carried out at 25°C with a 12 h:12 h light: dark cycle at a constant humidity (Bass et al., 2007).

Ingredient	1x SYA
Distilled water (ml)	700
Agar (g)	15
Sugar (g)	50
Yeast (g)	100
Water to add at the end (ml)	170
Nipagin- 10% in 100% ethanol (ml)	30
Propionic Acid (ml)	3

Table 2.1 Food recipe of fly media
All quantities listed are what is required in addition to distilled water to make 1L of food media. 30 ml of 10% (w/v) Nipagin (methylparaben, Genesee Scientific, San Diego, CA, USA) and 3 ml of propionic acid (Fisher Scientific, Surrey, UK) were added as preservatives.

2.2 Preparation of adult flies for experiments

Adult flies were raised from synchronised egg collections. Parental flies were transferred to collection cages and eggs were collected on grape juice agar plates overnight. Eggs were mobilized from the surface of the grape juice agar using a paintbrush and washed with phosphate buffered saline (PBS, Fisher Scientific, Surrey, UK). 18 µL of eggs were dispensed into bottles of fresh SYA food and incubated at 25°C for 10 days until the adult flies emerged. Adult flies were transferred to fresh SYA food and allowed to mate for 24 hours before being separated by sex using carbon dioxide anaesthesia. Flies were housed in single sexes in experimental vials (25cm x 95 cm polystyrene vials, from Regina industries Ltd.) at a density of 15 flies per vial.

2.3 Body size measurements

Flies were frozen in liquid nitrogen on day 10 post-eclosion. The body weights of individual male and female flies (10 flies per genotype) were measured using a precision balance (AR0640, Ohaus, Nänikon, Switzerland). The left wings of these same adult flies were removed, fixed in 50% (v/v) glycerol/ethanol (Fisher Scientific, Surrey, UK) overnight and then mounted onto a microscope slide. Images of wings were taken using a Leica MZ10F stereo microscope (Leica, Wetzlar, Germany) using a QIClick CCD monochrome microscope camera (model 74-0083-A0,

QI Imaging, Tucson, AZ, USA). Wing areas were measured using Image J software (Schneider et al., 2012).

2.4 Longevity Experiments

Flies for longevity experiments were transferred to fresh SYA food 3 times per week without anaesthesia, and during the transfer any deaths and/or censors were scored. Flies were censored from the experiment if they escaped or were still alive but stuck to the food.

2.5 Female Fecundity

To measure female fecundity, experimental flies were transferred to new vials and removed after 24 hours. The number of eggs per vial were counted manually using a Leica M80 dissecting light microscope (Leica, Wetzlar, Germany) and a hand counter. The mean number of eggs laid daily per fly was then calculated. Female fecundity was measured at 7 days and 14 days post-eclosion.

2.6 Stress resistance

For all stress assays, flies were kept and reared as for lifespan experiments till day 7. Flies were kept at density of 15 flies per vial with 10 vials per genotype.

2.6.1 Starvation

To test resistance to starvation, 7-day old flies were transferred to starvation media consisting of 1% (w/v) agar (Sigma-Aldrich, Dorset, UK) in distilled water. Deaths were scored over regular timed intervals (4 hours) 4 times per day and flies were tipped on to fresh agar vials every 3 days.

2.6.2 Oxidative stress

To measure resistance to oxidative stress, flies were transferred to vials containing either 5% (v/v) hydrogen peroxide (H_2O_2) diluted from a 30% (v/v) stock (Sigma-Aldrich, Dorset, UK) in

yeast-free media (0.5 g/L sugar (Tate & Lyle, London, UK) and 15 g/L agar (Sigma-Aldrich, Dorset, UK) or food containing 20 mM paraquat (N N'-dimethyl-4 4'-bipyridinium dichloride) diluted from a 1 M stock of methyl viologen dichloride hydrate (Sigma-Aldrich, Dorset, UK) prepared in distilled water. Deaths were scored over regular timed intervals 4 times per day.

2.7 Feeding behaviour

2.7.1 Capillary feeder (CAFE) assay

Food consumption of flies was measured using the Capillary feeder (CAFE) assay as previously described by Ja et al. (2007a). 10-day old flies were used for the CAFE assay. Individual flies (10 flies per condition) were placed in 7 ml bijou tubes containing 1% (w/v) agar (Sigma-Aldrich, Dorset, UK) which were then sealed with Parafilm (Bemis Company, Neenah, WI, USA) to prevent flies escaping. Each tube had a 5 µL graduated Hirschmann glass capillary (Hirschmann Laborgeräte GmbH, Eberstadt, Germany) held in place by a 200 µL plastic pipette tip. Liquid fly food consisting of 5% (w/v) sugar (Tate & Lyle, London, UK), 2% (w/v) brewer's yeast (MP Biomedicals, Solon, OH, USA) and 0.5 mg/mL Brilliant Blue FCF (FastColours, Huddersfield, UK) was fed through the capillary and the volume consumed was recorded after a 24-hour period. The CAFE assay was conducted over 3 days, replacing the glass capillary when necessary.

2.7.2 Proboscis extension (PE) assay

Feeding rates of flies were measured using the proboscis extension (PE) assay (Wong et al., 2009). Flies were reared as for lifespan studies and sorted as 5 flies per vial (30 vials per condition). On day 7 post-eclosion, the flies were transferred to blue food (SYA media with 2.5% (w/v) Brilliant Blue FCF (FastColours, Huddersfield, UK). Flies were observed every 3 minutes over a 60-minute period and the number of flies extending their proboscis onto the surface of the food was recorded. The observer was blinded to the conditions tested and vials were observed randomly to avoid any bias. The vials were decoded at the end of the experiment and feeding data was expressed as a proportion by experimental group (sum of scored feeding events divided by total number of feeding opportunities, where total number of feeding opportunities = number of flies in vial x number of vials in the group x number of observations).

2.8 Metabolic measurements

Flies were flash-frozen in liquid nitrogen and kept at -80°C until ready for metabolic measurements. For metabolic measurements after starvation, flies were raised and maintained as for lifespan experiments until day 7. They were then transferred to food vials containing 1% (w/v) agar (Sigma-Aldrich, Dorset, UK) in distilled water for 24, 48 or 72 hours before flash-freezing in liquid nitrogen.

2.8.1 Whole fly measurement of triacylglycerides

Triacelyglyceride (TAG) levels were quantified from whole fly extracts using Triacylglyceride Infinity Reagent (Thermo Fisher Diagnostics, Hertfordshire, UK). Groups of five flies were homogenized using glass beads in 150 µL of 0.05% (v/v) Tween20 (Fisher Scientific, Surrey, UK) in distilled water for 30 seconds using a Fastprep™ FP120 (MP Biomedicals, Solon, OH, USA) and then heated for 5 minutes at 70°C in a heat block. The samples were centrifuged for 1 minute at 5000 rpm. 150 µL of Triacylglyceride Infinity Reagent (Thermo Fisher Diagnostics, Hertfordshire, UK) were added to each well of a 96-well plate, 5 µL of each sample or standard was added to the appropriate well and the plate was incubated at 37°C for 5 minutes. The absorbance of each well was read at 574 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Surrey, UK). Triacelyglyceride (TAG) standards were prepared from a 25 mg/mL glycerol standard solution (Sigma-Aldrich, Dorset, UK). The standards (from 0 – 20 µg/µL) were prepared as serial dilutions in 0.05% (v/v) Tween20 (Fisher Scientific, Surrey, UK) in distilled water and processed alongside the fly samples.

2.8.2 Whole fly measurement of trehalose and glycogen

Groups of five flies were homogenised with glass beads in 50 µL of 0.2 M Na_2CO_3 (Fisher Scientific, Surrey, UK) and incubated for 2 hours at 95°C. The pH of the samples was adjusted to pH 5.2 by adding 30 µL of 1M acetic acid (Fisher Scientific, Surrey, UK) and 120 µL of 0.2M Na-acetate, pH 5.2 (Fisher Scientific, Surrey, UK). 50 µL of each fly homogenate was incubated with either 1 µL of trehalase enzyme suspension (Megazyme, Bray, Ireland) for trehalose measurements or 2 µL of amyloglucosidase (added from a 48 U/mL stock solution, Sigma-Aldrich, Dorset, UK) for glycogen measurements. Trehalose samples were incubated overnight at 37°C and glycogen samples were incubated at 57°C overnight. Trehalose standards (from 0 –

20 µg/µL) were prepared from serial dilutions of a 20 µg/µL stock solution of trehalose dihydrate (from *Saccharomyces cerevisiae*, Sigma-Aldrich, Dorset, UK) dissolved in 0.2 M Na_2CO_3. Glycogen standards (from 0 – 20 µg/µL) were prepared from a 20 µg/µL stock solution of glycogen (from oyster, Sigma-Aldrich, Dorset, UK) dissolved in 0.2 M Na_2CO_3. Standards were processed alongside the fly samples.

The amounts of glucose liberated during either the trehalase or amyloglucosidase digestion were measured using the Infinity Glucose Reagent (Thermo Fisher Diagnostics, Hertfordshire, UK). 150 µL of Infinity Glucose Reagent was added to each well of a 96-well plate. 5 µl of each standard or sample were added to the appropriate well and incubated at room temperature for 15 minutes. The absorbance of each well was read at 574 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Surrey, UK).

2.8.3 Whole fly measurement of protein

Protein content in flies was determined using the Pierce™ 660 nm Protein Assay Reagent (Thermo Scientific, Surrey, UK). Protein standards (from 0 – 2 µg/µL) were prepared from serial dilutions of a 2 mg/mL Pierce™ Bovine Serum Albumin Standard (Thermo Scientific, Surrey, UK). Serial dilutions were prepared in 0.2M Na_2CO_3 (for trehalose or glycogen assays) or 0.05% (v/v) Tween20 for TAG assays. 150 µL of the protein assay reagent was added to each well of a 96-well plate and 1 µL of each standard or sample was added to the appropriate well. The plate was incubated at room temperature for 5 minutes and then the absorbance of each well was read at 660 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Surrey, UK).

2.9 Dietary restriction

For dietary restriction experiments, flies were raised as for lifespan experiments and then females were sorted (15 flies per vial) onto different dietary regimes of SYA food with varying concentrations of dietary yeast (see Table 2.2). Flies were transferred to fresh food 3 times per week, without anaesthesia, and during the transfer any deaths and censors were scored.

Yeast Concentration	Agar (g/L)	Brewer's Yeast (g/L)	Sugar (g/L)
0.1x	15	10	50
0.5x	15	50	50
1.0x	15	100	50
1.5x	15	150	50
2.0x	15	200	50

Table 2.2 Food recipes used for dietary restriction experiments.
The concentration of dietary yeast was varied in the five different food concentrations. The amount of sugar and agar remained the same. All quantities listed are what is required in addition to distilled water to make 1L of food media. 30 ml of 10% (w/v) Nipagin (methylparaben, Genesee Scientific, San Diego, CA, USA) and 3 ml of propionic acid (Fisher Scientific, Surrey, UK) were added as preservatives.

2.10 Gut barrier analysis (Smurf assay)

In order to analyse intestinal barrier function, female flies were maintained on standard SYA food as for longevity experiments until they reached the desired age. On the day of the assay, flies were transferred to blue food (SYA media with 2.5% (w/v) Brilliant Blue FCF (FastColours, Huddersfield, UK). Smurf assays were performed essentially as previously described (Rera et al., 2012) except flies were maintained on blue food for 24 hours prior to scoring for Smurfs. After 24 hours, flies were scored for the presence of Smurfs where the blue dye coloration could be seen outside the digestive tract, having leaked into the hemolymph through the intestinal epithelia. After scoring, flies were returned to standard SYA. Smurf assays were carried out on Day 15, Day 30 and Day 45 post-eclosion.

2.11 Immunohistochemistry of adult guts

Flies were maintained as for longevity experiments until they had reached day 10 post-eclosion. The guts were dissected in ice cold phosphate buffer saline (PBS) and fixed in 4% (v/v) methanol-free formaldehyde in PBS (diluted from a 16% (w/v) stock solution, Fisher Scientific, Surrey, UK) for 15 minutes at room temperature. The fixed guts were then washed twice for 15 minutes in 0.2% (v/v) Triton X-100 (Fisher Scientific, Surrey, UK) in PBS (PBST), blocked in PBST containing 5% (v/v) normal goat serum (Stratech Scientific, Cambridge, UK) for 1 hour at room temperature then incubated at 4°C in primary antibody (rabbit anti-Fibrillarin (#ab5821) used at 1:200

dilution, Abcam, Cambridge, UK) diluted in PBST overnight. Guts were then washed 4x 15 minutes in PBST, incubated in secondary antibody (goat anti-rabbit AlexaFluor-488 used at 1:300 dilution, Thermo Fisher Scientific, Dorset, UK) diluted in PBST for 1.5 hours at room temperature and further washed in PBST for 4x 15 minutes.

Guts were then mounted in Vectashield containing DAPI (Vector Laboratories, Oxfordshire, UK) onto glass slides. Mounted slides were visualized and captured using a fluorescence Leica DMI400B microscope (Leica, Wetzlar, Germany) using the x63 oil-immersion objective and GFP filter cube. Nucleolar and nuclear areas were quantified using Image J software (Schneider et al., 2012).

2.12 Climbing assay for negative geotaxis

Climbing ability was assessed in adult flies at several timepoints throughout the lifespan. 15 flies were placed in a 25ml pipette (with the top cut off and bunged using cotton wool and the bottom sealed with parafilm and a 0.5 ml tube). Adult flies were gently tapped to the bottom of the pipette and observed for 45 seconds. The pipette was separated into 3 areas: top (above 25ml line), bottom (below 2ml line) and middle. After 45 seconds, flies that reached the top of the pipette (above 25ml) and flies remaining at the bottom (below 2ml) were counted and recorded. Three trials were performed for each experiment and three 25ml serological pipettes per genotype were used. The performance index (PI) was calculated which is defined as $0.5\times (n_{total} + n_{top}\text{-}n_{bottom})/n_{total}$ where n_{total} is the total number of flies, n_{top} is the total number of flies at the top and n_{bottom} is total number of flies at the bottom.

2.13 Genomic DNA preparation

2.13.1 Single fly gDNA preps

DNA was isolated from individual adult flies by homogenizing a single fly in 50 µL of 'Squishing Buffer' (10 mM Tris-Cl, pH 8.2, 1 mM EDTA, 25 mM NaCl containing 200 mg/mL Proteinase K (Fisher Scientific, Surrey, UK) added freshly diluted from a frozen stock) using a microtube pestle attached to an electric rotor. Fly homogenates were incubated at 37°C for 1 hour and the Proteinase K was inactivated by heating the sample to 95°C for 15 minutes.

2.13.2 Qiagen DNeasy method

DNA was isolated from 25 adult female flies using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) according to the manufacturers' instructions. Briefly, flies were homogenised in 180 µL of Buffer ATL using a microtube pestle attached to an electric rotor. 20 µL of Proteinase K was added, the sample was then mixed by vortexing and incubated at 56°C for 1 hour. Samples were then vortexed for 15 seconds before the addition of 200 µL of Buffer AL. Samples were again mixed by vortexing and 200 µL of 100% ethanol (Fisher Scientific, Surrey, UK) were added.

The homogenate was applied to a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded and the spin column was washed using 500 µl of Buffer AW1 and 500 µl of Buffer AW2. The columns were then centrifuged for 3 minutes at 14,000 rpm to dry the DNeasy membrane. The flow-through was discarded and the spin column was placed in a clean 1.5 ml microcentrifuge tube. Purified genomic DNA was eluted from the column by the addition of 200 µl Buffer AE and incubation at room temperature for 5 minutes. Eluted genomic DNA was then collected by centrifugation for 1 minute at 8000 rpm. The eluate was pipetted back onto the DNeasy membrane, incubated at room temperature for 5 minutes, and then centrifuged for 1 minute at 8000 rpm to maximise yields. Genomic DNA concentration and purity was then measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Surrey, UK). Genomic DNA quality was also assessed by agarose gel electrophoresis.

2.14 Polymerase Chain Reaction (PCR)

PCRs were performed using Invitrogen Platinum Hot Start PCR 2X Master Mix (Thermo Fisher, Surrey, UK). The Platinum Hot Start Master Mix contains Platinum Taq DNA polymerase in an optimized PCR buffer with Mg^{2+} and dNTPs. All primers were used at a final concentration of 200 µM. Primers used in this project are listed in Table 2.3.

Typical PCR cycling parameters were as follows: initial denaturation at 94°C for 2 minutes then 35 cycles of denaturation at 94°C for 30 seconds, annealing step at appropriate temperature (usually 5°C below the lowest melting temperature of the primer pair) for 30 seconds, extension step at 72°C for 1 minute followed by a final extension step at 72°C for 10 minutes. PCRs were

performed using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Warrington, UK). PCR products were analysed by agarose gel electrophoresis using Midori Green DNA stain (Geneflow, Staffordshire, UK) and visualised using a G:Box UV transilluminator (Syngene, Cambridge, UK).

Primer Name	Primer Sequence (5’ ® 3’)	Melting Temperature/Tm (°C)
AO1	GCCTGATAAAGATAAGGC	51.4
AO2	GGCTGATCGCTTTCCTATAC	57.3
AO3	GTAGTTGAACCACCAGGAAC	57.3
AO4	GTATAGGAAAGCGATCAGCC	57.3
AO5	GTTCCTGGTGGTTCAACTAC	62.0
AO6	GGCGATGGTTTGGCCAGATAC	66.0
AO7	GTATCTGGCCAAACCATCGCC	61.3
AO8	ATTCCTCTGATGCCCGCTGA	59.4
AO9	TCAGCGGGATCAGAGGAAT	59.4
AO10	TTCTGAGGAGCGAATGCGGCA	61.8
dmGlu5’F1	ATT TGA AAC ATC GAT AGC ACA	52
dmGlu5’R1	GAA CTA TGC TTG GAA CAG TCT	55.9
dmGlu5’F2	AGA CTG TTC CAA GCA TAG TTC	55.9
dmGlu5’R2	AGC TAA CCG TAC AAC CAA AG	55.2
dmGlu5’F3	CTT TGG TTG TAC GGT TAG CT	55.2
dmGlu5’R3	TTT AAT GGC CGT ACA AGT GA	53.2

dmGlu5'F4	TCA CTT GTA CGG CCA TTA AA	53.2
dmGlu5'R4	GCC AAT TTC GTA GCA ATA TGA	54
dmGlu5'F5	TCA TAT TGC TAC GAA ATT GGC	54
dmGlu5'R5	CTA ACT GTC CAT CTC TCA CG	57.3
DmGluR-DelF	ACA GAT GAT TAA ACG CCG CA	55.2
DmGluR-DelR	ACA ATT TTC TTA CAA ACA CCA TAC A	54.8
DmGluR-F1	TCCGGTCCATGAAAAAGGTGAAG	60.6
DmGluR- R1	GAGGGCTACGGGATTGGAAAGTGTC	64.4

Table 2.3 List of primers used for PCR and their 5'-3- sequences
All primers designed and used in PCR to map the *DmGluRA*$^{[112b]}$ mutation. Melting temperatures (Tm) are also listed for each primer, annealing temperatures for PCR were obtained by using temperatures 5°C below the lowest Tm of the pair of primers used.

2.14.1 DNA sequencing of PCR products

During mapping of the *DmGluRA*$^{[112b]}$ deletion, PCR products were purified using the Qiagen PCR purification kit according to the manufacturers' instructions and sequenced by Eurofins Genomics. DNA sequences were analysed using SerialCloner software.

2.15 RNA extraction

Total RNA was extracted from whole adult flies (10 pooled females or 20 pooled males) using Trizol extraction (Fisher Scientific, Surrey, UK) followed by RNeasy column purification (Qiagen, Manchester, UK). Flies were homogenised in 0.5 ml of Trizol reagent using a Fastprep™ FP120 (MP Biomedicals, Solon, OH, USA) and glass beads. Samples were then incubated at room temperature for 5 minutes. 100 µL of chloroform (Sigma-Aldrich, Dorset, UK) were added. Tubes

were then vortexed for 15 seconds and incubated at room temperature for 2 minutes. The upper colourless aqueous phase was separated by centrifuging at 14,000 rpm for 5 minutes at 4°C and then 250 µL were transferred to a new 1.5ml tube. 250 µL of 100% ethanol (Fisher Scientific, Surrey, UK) were added. Samples were mixed by pipetting and then 500 µL were transferred to a RNeasy column and centrifuged at 10,000 rpm for 30 seconds. The flow-through was discarded and the column was returned to the collection tube.

2.15.1 On-Column DNase digestion

The RNA was treated with DNase during the RNA extraction in the RNeasy column. 350 µL of Buffer RW1 were added to the column and centrifuged at 10,000 rpm for 15 seconds. 10 µL of DNase I stock solution was mixed with 70 µL Buffer RDD added to the directly to the RNeasy spin column membrane. The samples were then incubated at room temperature for 15 minutes after which 350 µL of Buffer RW1 were added and the column was centrifuged at 10,000 rpm for 15 seconds.

The column was then placed into a new collection tube, 500 µL of Buffer RPE (with added ethanol) were added and samples were centrifuged for 30 seconds at 10,000 rpm. 500 µL of Buffer RPE were added again and this time, samples were centrifuged for 2 minutes at 10,000 rpm. The column was then transferred into a new 1.5 ml collection tube and 30 µL of RNase-free water were directly added onto the column membrane. Following this, samples were incubated at room temperature for 5 minutes and then centrifuged at 10,000 rpm for 1 minute. 30 µL of the elute was returned to the column membrane, incubated for a further 5 minutes at room temperature, spun again, and the column was discarded. The quantity and quality of the RNA were measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Dorset, UK).

2.16 cDNA synthesis

Reverse transcription was performed using 2 µg of total RNA mixed with 1 µL Oligo(dT) (at 0.5 µg/µL, Invitrogen, Surrey, UK), 1 µL dNTP mix (from a 10 mM stock, Invitrogen, Surrey, UK) and RNase-free water (Ambion, Thermo Fisher, Surrey, UK) was added to make up a total volume of 12 µL. All incubation steps for cDNA synthesis were performed in a 5G Prime thermal cycler (Techne, Chelmsford, UK). Samples were incubated for 5 minutes at 65°C and then chilled for 2

minutes on ice. After brief centrifugation, 4 µL 5x First-Strand Buffer, 2 µL of 0.1 M DTT and 1 µL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Surrey, UK) were added. The reaction mixture was incubated at 42°C for 1 minute and 1 µl of SuperScript® II Reverse Transcriptase (RT) (Invitrogen, Surrey, UK) was added. The mixture was then incubated for a further 50 minutes at 42°C. The reaction was then inactivated by heating at 70°C for 15 minutes. The cDNA was stored at -20°C or immediately used for quantitative real-time PCR (qRT-PCR).

2.17 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) reactions were performed in duplicate for each standard or sample using a LightCycler® 480 (Roche, Basel, Switzerland). Each 20 µl reaction in a 96-well plate comprised of 2 µL of cDNA (diluted 1:5), 1 µL of each primer at a concentration of 6 nM (made from 100 µM stock, Eurofins Genomics, Ebersberg, Germany) and 10 µl 2x PrecisionPLUS qPCR master mix (containing 2x reaction buffer, 0.025 U/µl Taq Polymerase, 5 mM MgCl2, 100 µM dNTP Mix, PrimerDesign, Camberley, UK). Relative quantities of transcripts were determined using the relative standard curve made from a pool of cDNA generated from w^{Dah} females. Standards were also used to check for non-specific amplification and the presence of primer-dimers. As a negative control for each primer pair, distilled water (dH_2O) was used instead of cDNA template. The expression of the gene of interest was calculated relative to levels of *actin5C* (see Table 2.4 for primer sequences).

Gene name	Forward Sequence	Reverse sequence
DmGluRA	AGGAGGTGCGGTAGGAACA	TTGCTCACAAGACCAATGGAGA
actin5C	CACACCAAATCTTACAAAATGTGTGA	AATCCGGCCTTGCACATG
Hsp83	GCACGCCAGCCGCATCTACC	TCAACCAGCGAGGGGGCATCT
TotC	CTTGCCCTGCTCCTGATTAG	GA TTGATCTTCGATTCGGCG

Table 2.4 Primer sequences used for qRT-PCR

2.18 Western blots

Flies were snap frozen on day 10 in liquid nitrogen and stored at -80°C until ready for protein extraction. Proteins were extracted from whole flies as follows: five flies were homogenized on ice in 150 µL of lysis buffer (0.1 M DTT (1,4-Dithiothreitol, Sigma-Aldrich, Dorset, UK) in Laemmli sample buffer (Fisher Scientific, Surrey, UK). Samples were incubated at >90°C for 5 minutes and then centrifuged for 5 minutes at 14,500 rpm. Protein content was quantified using the Pierce™

660 nm Protein Assay Reagent (Thermo Scientific, Surrey, UK) as described above (see section 2.8.2).

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Fisher Scientific, Surrey, UK) by semi-dry transfer (Trans-Blot Turbo® Transfer™ system, Bio-Rad, Hertfordshire, UK) at 20 volts for 30 minutes. Membranes were blocked using 5% (w/v) skimmed milk in TBST (Tris-buffered saline containing 0.1% (v/v) Tween20). Membranes were incubated in primary antibody, diluted in 5% (w/v) skimmed milk in TBST overnight at 4°C under rotation. A list of the primary antibodies used in this study is provided in Table 2.5. Membranes were washed 5x in TBST for 5 minutes each and then incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibody (see Table 2.6) (diluted in 5% (w/v) skimmed milk in TBST) for 1 hour at room temperature. After further washing (5x for 5 minutes each), the protein bands were visualised by incubating blots in Lumina Forte chemiluminescent HRP substrate (Millipore, Burlington, MA, USA) for 5 minutes followed by signal detection using a G:BOX gel documentation system (SynGene, Cambridge, UK). Image analysis and densitometry of protein bands were performed using Image J (Schneider et al., 2012).

Blots were re-probed for beta actin as a loading control. Membranes were washed in TBST for 3x 5 minutes followed incubation with primary and secondary antibodies before visualisation as previously described.

Primary antibody	Species	Dilution	Source
anti-fibrillarin	Rabbit	1:1000	Abcam (ab5821)
anti-beta actin (D6A8)	Rabbit	1:1000	Cell Signalling Technology (8457S)

Table 2.5 Primary antibodies used for Western blots.

Secondary antibody	Species	Dilution	Source
anti-rabbit-HRP	Goat	1: 1000	Abcam (ab6721)

Table 2.6 Secondary antibodies used for Western blots.

2.19 RNA Sequencing

Total RNA was isolated from 10-day old w^{Dah} and $DmGluRA^{[112b]}$ females as described above. Library preparations and paired-end RNA sequencing were performed by Novogene Co. Ltd (Cambridge, UK) using an Illumina-based platform.

Data quality, alignment, read counts and differential gene expression analysis were all performed by Dr Irene Papatheodorou, EMBL-EBI, Cambridge UK.

2.20 Gene Ontology (GO) term enrichment analysis

Gene Ontology (GO) term enrichment analysis was carried out using the gene ontology enrichment and visualisation tool, GOrilla (Eden et al., 2009, Eden et al., 2007). The reference genome was set to *Drosophila melanogaster* and enrichment tests were performed using two unranked lists of genes where target represented the list of genes that showed differential expression (both upregulated and downregulated; adjusted $p<0.05$) and background was all the genes identified in the RNA-seq dataset.

2.21 Venn diagrams and statistical analysis of overlaps

Venn diagrams were created using Biovenn (Hulsen et al., 2008) and statistical analysis for the significance of overlaps was performed using Fisher's exact test and hypergeometric probability test (http://nemates.org/MA/progs/overlap_stats.html).

2.22 Statistical analysis

All statistical analyses were performed using JMP 14.0 statistical software (SAS Institute, Marlow, UK) apart from survival data. Excel (Microsoft) was used to perform statistical analyses of survival data using a custom Excel macro. Survival and stress data were analysed by Log-rank test. Fisher's exact test was used to analyse data from Smurf assays. The Chi-squared test was used to test the proboscis-extension feeding data. A Mixed Effects Linear Model was used to analyse qPCR data comparing *DmGluRA* levels between different ages. Climbing Data were analysed using an ordinal logistics regression model. The rest of the data was tested by Student's t-test for comparing means between two groups and One-way analyses of variance (ANOVA) or Two-way ANOVA for comparing means between more than two groups followed by the post hoc test Tukey's Honest Significant Difference (HSD) test.

Chapter 3 : Genetic analysis of *DmGluRA* and ageing

3.1 Introduction

Metabotropic glutamate receptors (mGluRs) are well known for their roles within the central nervous system including memory, synaptic plasticity and neuronal excitability (Niswender and Conn, 2010). However, their expression in tissues outside the CNS, such as the pancreas and lymphocytes, suggests that these highly conserved receptors have additional functions which are yet to be explored. Here*, Drosophila melanogaster* was used as a simple model to investigate additional functions of these highly conserved receptors and to define the underlying mechanisms. *Drosophila* are a well-suited system to study the functions of mGluRs due to their close homology (Parmentier et al., 1996a, Ly et al., 2020). The structural features of the mammalian mGluRs have all been conserved, suggesting that the functions have also been conserved in fly (Parmentier et al., 1996a).

Preliminary data from the laboratory using a null mutation of *DmGluRA*, which encodes the single fly orthologue of mGluR, had found that loss of mGluR signalling in *Drosophila* causes longevity via currently unknown mechanisms. The precise nature of the genetic deletion within this mutant line, $DmGluRA^{[112b]}$, was not well characterised although homozygous mutants were described as viable and fertile with an absence of DmGluRA protein expression (Bogdanik et al., 2004). A primary aim of this chapter was to therefore define the molecular end-points of the $DmGluRA^{[112b]}$ deletion to facilitate molecular detection of mutant animals.

Preliminary experiments had suggested that the effects of *DmGluRA* mutation on *Drosophila* lifespan were sex-specific: homozygous mutant females were long-lived but homozygous mutant males showed lifespans comparable to wild-type controls. Further investigations into the effects of *DmGluRA* loss of function on lifespan were therefore carried out to determine the influences of both genetic background and the presence of the naturally occurring endosymbiont, *Wolbachia pipientis*, on *DmGluRA*-dependent longevity especially in each sex.

Sex-specific effects of *DmGluRA* activity on lifespan could be induced by differences in *DmGluRA* expression between males and females. As such, *DmGluRA* expression was analysed in both wild-type and mutant flies and compared between sexes.

Studies have shown that gene expression from genes located in heterochromatic regions becomes dysregulated during ageing (Wood et al., 2016, Brown et al., 2020). For example, loss of heterochromatin with age has been observed as heterochromatin markers associated with heterochromatin formation such as H3K9me3 (trimethylation of the ninth lysine on H3 histones) and HP1 (heterochromatin protein 1) show reduction with age and accordingly genes that lose these marks with age shown increased expression (Wood et al., 2010). Such heterochromatic regions include the 4th chromosome. Expression of genes located within these regions has been found in tissues where they are not normally expressed as flies age (Wood et al., 2010). For example, Wood et al. (2016) reported increased transcript levels of many of these genes in fly heads and fat bodies with age. Together, this inappropriate gene expression may contribute to ageing pathologies (Larson et al., 2012, Wood et al., 2016).

As *DmGluRA* is located on the 4th chromosome within one of these heterochromatic regions, there is a possibility that its expression is misregulated during ageing - either in the wrong tissues or at the wrong level - which could be detrimental to health. *DmGluRA* is known to be overexpressed in *dfmr1* null animals, the *Drosophila* model of Fragile X Syndrome (FXS) (Pan et al., 2008). This increased *DmGluRA* level is associated with learning and memory deficits characteristic of FXS (Kanellopoulos et al., 2012). Furthermore, treating *dfmr1* mutants with mGluR antagonists rescues social behaviour defects and morphological defects seen in the mushroom bodies, brain structures linked to learning and memory (McBride et al., 2005). Therefore, it may be possible that mutation of *DmGluRA* may be rescuing deleterious effects of its misregulated expression which occurs with age. Therefore, *DmGluRA* expression was analysed during normal ageing to investigate whether it changes over the life-course.

3.2 Results

3.2.1 Molecular characterisation of the *DmGluRA$^{[112b]}$* deletion

The loss-of-function mutant utilised in this thesis, was previously described by Bogdanik et al. (2004). The mutants were generated by imprecise excision of the P-element, P39C42. P-elements are transposons, sequences of DNA that have the ability to move within the genome (Tettweiler and Lasko, 2007). They can be used to generate deletion mutants by inducing their mobilisation which is typically imprecise and so removes some flanking sequence. The precise

end-points of the *DmGluRA*$^{[112b]}$ deletion have not yet been defined but the deletion is known to extend within the coding sequence of the *DmGluRA* gene and homozygous mutants do not produce any DmGluRA protein but are viable and fertile. (Bogdanik et al., 2004).

For these studies, it was important that the *DmGluRA*$^{[112b]}$ mutants could be easily distinguished from wild-type flies but the *DmGluRA*$^{[112b]}$ mutation produces no obvious phenotypic differences in adult flies. Detection of the *DmGluRA*$^{[112b]}$ deletion was therefore achieved by PCR from genomic DNA prepared from adult flies using primers that anneal within the deleted region. Thus, the absence of a PCR product indicates the presence of the deletion. However, this method has a number of limitations. It does not allow detection of the *DmGluRA*$^{[112b]}$ deletion in heterozygous flies as the corresponding genomic region of the wild-type copy of the chromosome will be amplified. Furthermore, the absence of a PCR product could be attributed to a number of technical reasons other than the presence of the genetic deletion within this genomic region.

A primer-walking approach was therefore employed to determine the precise endpoints of this *DmGluRA*$^{[112b]}$ deletion. Starting at the 3' end of the *DmGluRA* locus, primers with overlapping sequences were designed to amplify consecutive regions of genomic sequence of ~ 1 kb in length from *DmGluRA*$^{[112b]}$ homozygotes (Figure 3.1). The primers used to identify the deletion endpoints are shown in Table 3.1.

The first four consecutive primer pairs that were tested, all resulted in amplification products, indicated by (+) in Table 3.1. This demonstrated that these genomic sequences were all still present within the mutant strain. However, primers AO9 and AO10 failed to produce any PCR product indicated by (-), revealing that at least one of the primer sequences sit within the deletion. As shown in Table 2.3 (See Chapter 2, section 2.14: Polymerase Chain Reaction (PCR)) primer AO9 is the reverse complement of primer AO7, which successfully amplified a PCR product, so this sequence must be present in the deletion strain. Thus, the 3' end-point must lie between the genomic locations of the primers AO9 and AO10.

Primers were then designed to primer-walk starting from the 5' end of the *DmGluRA* locus. Primers dmGlu5'F5 and dmGlu5'R5 failed to amplify any product in the mutant strain. Primer dmGlu5'F5 is the reverse complement of dmGLu5'R4, which successfully amplified a PCR product in combination with dmGlu5'F4. As such, this sequence must be present within the deletion strain and so the 5' end-point of the deletion sits between the genomic locations of the primers dmGlu5'F5 and dmGlu5'R5.

To identify the precise sequence that is missing in the deletion strain, the sequence between dmGlu5'F5 and AO9 was amplified by PCR and sequenced. This revealed that the deletion within *DmGluRA*$^{[112b]}$ removes 4.8 kb of the *DmGluRA* locus, including part of the promotor region and the translational start site, extending into the coding sequence (as shown in Figure 3.1).

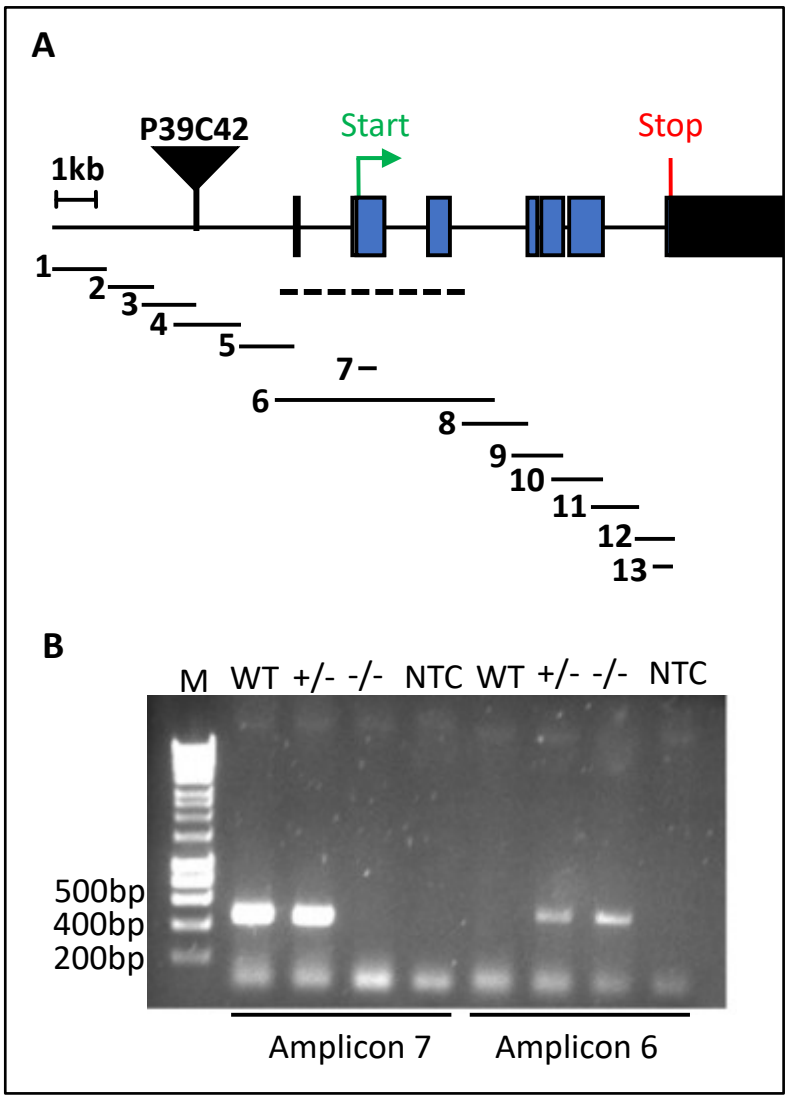


Figure 3.1 Mapping the end-points of the *DmGluRA*$^{[112b]}$ deletion.

(A) Schematic diagram of the *DmGluRA* gene locus. Exons are depicted as boxes and introns are represented as lines. The blue boxes indicate the coding sequence. The P-element insertion, P39C42, used to generate the *DmGluRA*$^{[112b]}$ deletion by imprecise excision is located within the promoter region. The amplicons used to map the extent of the deleted sequence are indicated. The black dashed line represents the sequence that has been deleted in the *DmGluRA*$^{[112b]}$ allele.

(B) Agarose gel electrophoresis of PCR amplification products using the primer combinations for amplicon 6 (primers located either side of the deleted region) and amplicon 7 (primers located within the deleted region) with wild-type genomic DNA (WT), heterozygous *DmGluRA*$^{[112b]}$ (+/-) or *DmGluRA*$^{[112b]}$ homozygous (-/-) genomic DNA as template. NTC represents no template control reactions. Predicted sizes of PCR products based on sequence are 472 bp for amplicon 7 and 5306 bp for amplicon 6.

Amplicon	Forward Primer	Reverse Primer	PCR product amplified in:	
			Wild-Type	*DmGluRA*$^{[112b]}$
1	dmGlu5'-F1	dmGlu5' -R1	+	+
2	dmGlu5'F2	dmGlu5'R2	+	+
3	dmGlu5'F3	dmGlu5'R3	+	+
4	dmGlu5'F4	dmGlu5'R4	+	+
5	dmGlu5'F5	dmGlu5'R5	+	-
6	DmGluR-DelF	DmGluR-DelR	-	+
7	DmGluR-F1	DmGluR-R1	+	-
8	AO10	AO9	+	-
9	AO8	AO7	+	+
10	AO6	AO5	+	+
11	AO3	AO4	+	+
12	AO2	R1	+	+
13	AO1	R1	+	+

Table 3.1 Summary of PCR results from primer-walking to detect *DmGluRA*$^{[112b]}$ deletion endpoints.

Amplicons as numbered in Figure 3.1 and their respective primer pairs produced from PCR using genomic DNA isolated from wild-type or *DmGluRA*$^{[112b]}$ homozygous mutant flies.

3.2.2 *DmGluRA*$^{[112b]}$ loss-of-function mutation extends lifespan

To confirm previous observations that the *DmGluRA*$^{[112b]}$ mutation extends lifespan in *Drosophila* in a sex-specific manner, the *DmGluRA*$^{[112b]}$ mutation was backcrossed for six generations into the w^{Dah} wild-type strain and the survival of both male and female *DmGluRA*$^{[112b]}$ homozygous mutants were compared to wild-type w^{Dah} controls (Figure 3.2).

Similar to previous preliminary studies, males homozygous mutant for the *DmGluRA*$^{[112b]}$ deletion were not long-lived compared to their wild-type control males (Figure 3.2, p=0.36) while female *DmGluRA*$^{[112b]}$ homozygous mutants were significantly longer lived than their wild-type control females (Figure 3.2, median lifespan +4%, $p<0.05$).

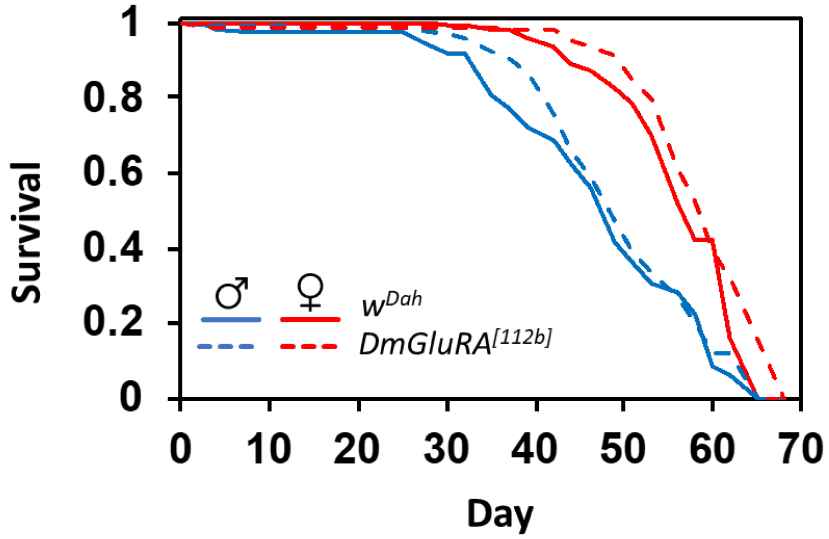


Figure 3.2 Survival of *DmGluRA*$^{[112b]}$ deletion mutants in the *w*^{Dah} genetic background.
Lifespan analysis of *DmGluRA*$^{[112b]}$ homozygous mutants compared to wild-type controls after backcrossing into *w*^{Dah}. Flies were maintained on standard sugar-yeast agar media. Median lifespans were: 48 days for *w*^{Dah} control males (n=130) and 48 days for *DmGluRA*$^{[112b]}$ homozygous mutant males (n=137), p=0.36 (Log-rank test); 57 days for *w*^{Dah} control, females (n=139) and 59 days for *DmGluRA*$^{[112b]}$ homozygous mutant males females (n=144), p=0.003 (Log-rank test).

The wild-type strain used for these studies, *w*^{Dah}, carries *Wolbachia pipientis*, a maternally-inherited, endosymbiotic bacteria. More than 60% of all insects species, including *Drosophila* (Hilgenboecker et al., 2008), and approximately 30% of the stocks at the Bloomington *Drosophila* Stock Centre carry *Wolbachia* (Clark et al., 2005). Several studies have shown that the presence of *Wolbachia* can affect longevity in *Drosophila*. For example, Min and Benzer (1997) found that wild-type flies treated with the antibiotic, tetracycline, which removes *Wolbachia*, live longer than *Wolbachia*-infected flies. *Wolbachia* can also have a negative effect on ageing by upregulating signalling via the IIS pathway (Ikeya et al., 2009) .

To test whether the lifespan effects of the *DmGluRA*$^{[112b]}$ mutants were influenced by the presence of *Wolbachia*, the *DmGluRA*$^{[112b]}$ mutation was backcrossed into a *Wolbachi*a deficient strain, *w*^{DahT}, which was previously generated in the laboratory by treating *w*^{Dah} flies with tetracycline. The survival of both male and female *w*^{DahT} *DmGluRA*$^{[112b]}$ homozygous mutants were then compared to *w*^{DahT} wild-type controls (Figure 3.3). Again, male flies homozygous mutant for the *DmGluRA*$^{[112b]}$ deletion were not long-lived compared to wild-type controls (Figure 3.3, p=0.8) while female flies homozygous mutant for the *DmGluRA*$^{[112b]}$ deletion did

show a significant increase in lifespan compared to their wild-type controls (Figure 3.3, median lifespan +15%, p<0.05).

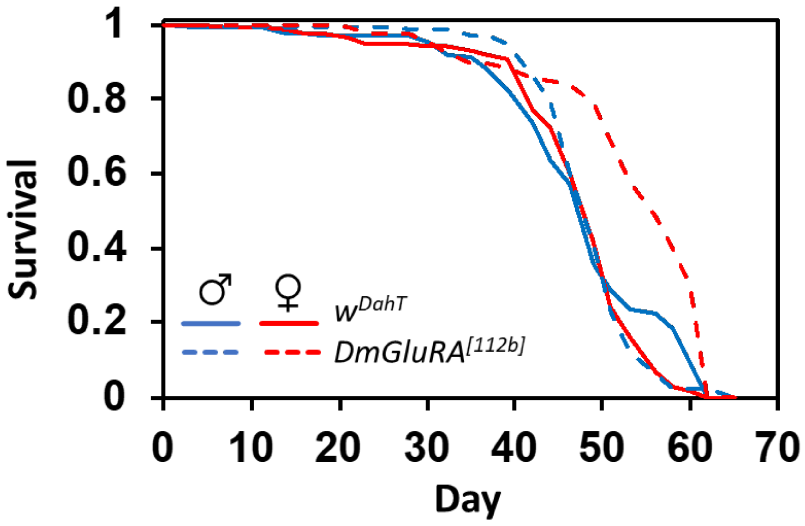


Figure 3.3 Survival of *DmGluRA*$^{[112b]}$ deletion mutants in the *Wolbachia* deficient *w*^{DahT} genetic background.
Lifespan analysis of *DmGluRA*$^{[112b]}$ homozygous mutants compared to wild-type controls after backcrossing into the *Wolbachia* deficient *w*^{DahT} strain. Flies were maintained on standard sugar-yeast agar media. Median lifespans were: 48 days for *w*^{DahT} control males (n=139) and 48 days for *DmGluRA*$^{[112b]}$ homozygous mutant males (n=141), p=0.8 (Log-rank test); 48 days for *w*^{DahT} control females (n=143) and 55 days for *DmGluRA*$^{[112b]}$ homozygous mutant females (n=141), p=5.3x10^{-16} (Log-rank test).

Genetic background can have a major influence on longevity (Spencer et al., 2003, Grandison et al., 2009b). To determine whether genetic background modulates the longevity phenotype of *DmGluRA*$^{[112b]}$ mutants, the *DmGluRA*$^{[112b]}$ mutation was also backcrossed into the inbred wild-type strain, *w*^{1118}. Again, the survival of both male and female *DmGluRA*$^{[112b]}$ homozygous mutants were compared to *w*^{1118} wild-type controls (Figure 3.4). Interestingly, in the *w*^{1118} genetic background, both males and females homozygous mutant for the *DmGluRA*$^{[112b]}$ deletion were significantly longer-lived compared to their wild-type controls (Figure 3.4, males: median lifespan +12%, p<0.05, females: median lifespan +6%, p<0.05).

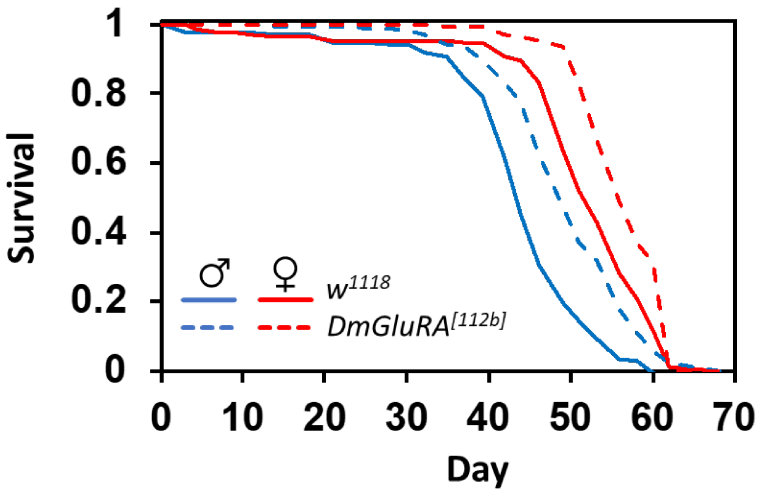


Figure 3.4 Survival of *DmGluRA*$^{[112b]}$ deletion mutants in *the* w^{1118} genetic background.
Lifespan analysis of *DmGluRA*$^{[112b]}$ homozygous mutants compared to wild-type controls across backcrossing into the w^{1118} genetic background. Flies were maintained on standard sugar-yeast agar media. Median lifespans were: 43 days for w^{1118} control males (n=136) and 48 days for *DmGluRA*$^{[112b]}$ homozygous mutant males (n=150), p=9.2x10^{-8} (Log-rank test); 52 days for w^{1118} control females (n=135) and 55 days for *DmGluRA*$^{[112b]}$ homozygous mutant females (n=140), p=1.6x10^{-6} (Log-rank test).

3.2.3 Analysis of *DmGluRA* gene expression

The transcript levels of several important longevity genes are often strongly correlated with their effects on lifespan (Rogina and Helfand, 2013, Huang et al., 2015, Symonenko et al., 2018). A good example of this is the long-lived *Indy* (*I'm Not Dead Yet*) mutant. Lifespan extension occurs in *Indy* mutants which have a reduction of *Indy* transcript levels between 25-75% of wild-type expression levels while flies in which *Indy* transcript levels are outside this range are not long lived (Wang et al., 2009a).

It was therefore possible that the sex-specific differences in lifespan observed in *DmGluRA*$^{[112b]}$ homozygous mutants in the w^{Dah} genetic background may reflect differential expression of *DmGluRA* between the two sexes. The lifespan extension may be associated with *DmGluRA* mRNA levels between a specific range of normal, and a reduction of *DmGluRA* transcription outside this range may abolish the lifespan effect. Hence, the *DmGluRA* mRNA levels in mutant males may be reduced either above or below the range needed to extend lifespan whereas levels in females may be reduced within this range.

Quantitative real-time PCR (qRT-PCR) was used to examine *DmGluRA* mRNA expression in both control and homozygous mutant age-matched males and females. Surprisingly, control

males had significantly higher relative expression of *DmGluRA* transcript compared to control females (Figure 3.5; p=0.0004). The *DmGluRA*$^{[112b]}$ mutation resulted in decreased expression of *DmGluRA* transcript by 43.2% and 61.2% in males and females, respectively (p=0.0313 males, p<0.0001 females). Interestingly, *DmGluRA* transcript levels remained significantly higher in *DmGluRA*$^{[112b]}$ homozygous mutant males compared to mutant females. *DmGluRA* transcript levels in mutant males were also higher than wildtype levels in females.

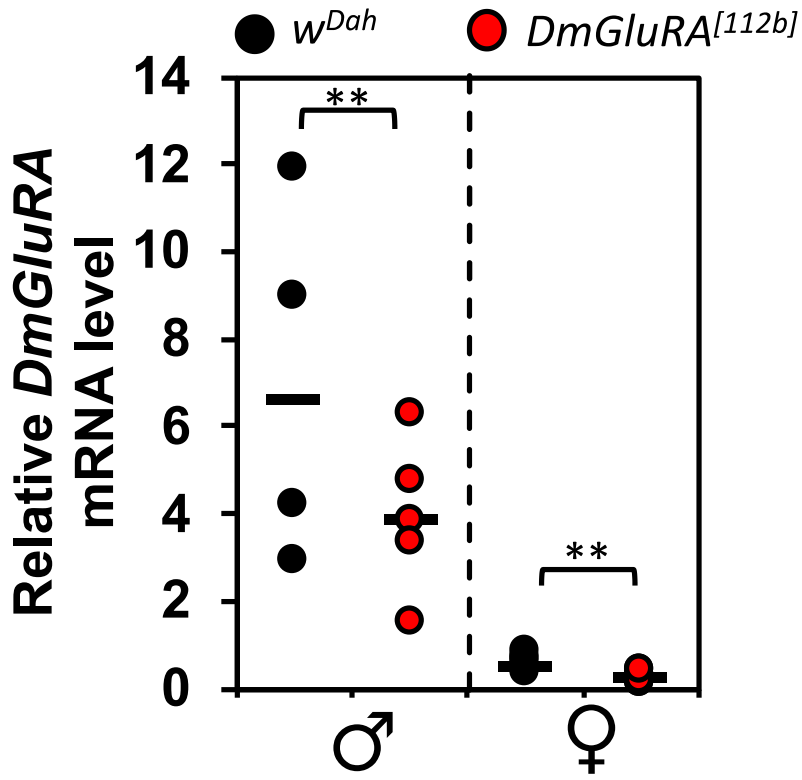


Figure 3.5 Analysis of *DmGluRA* mRNA expression by qRT-PCR.
DmGluRA mRNA expression was analysed by qRT-PCR and normalised to expression of *actin5C* mRNA in 10-day old adult flies. Data represent relative *DmGluRA* mRNA levels of 5 biological replicates for males and 10 for females. Bars represent means. ** indicates p<0.05, Student T-test.

The analysis of *DmGluRA* mRNA expression was performed on young, 10-day old flies. However, there is a possibility that *DmGluRA* expression changes during ageing. The gene *DmGluRA,* is located on the 4th chromosome, a highly heterochromatic region and smallest of *Drosophila* chromosomes (Sun et al., 2000). There is mounting evidence, that gene expression on the 4th chromosome becomes misregulated during ageing, and this has deleterious effects (Wood et al., 2016). This raises the possibility that removing DmGluRA activity in these mutants has

beneficial effects on ageing by removing a deleterious effect of misregulated *DmGluRA* expression.

DmGluRA mRNA expression was therefore examined in w^{Dah} females at three different time-points during their lifespan. However, no differences in *DmGluRA* transcript levels were observed across the different ages (Figure 3.6).

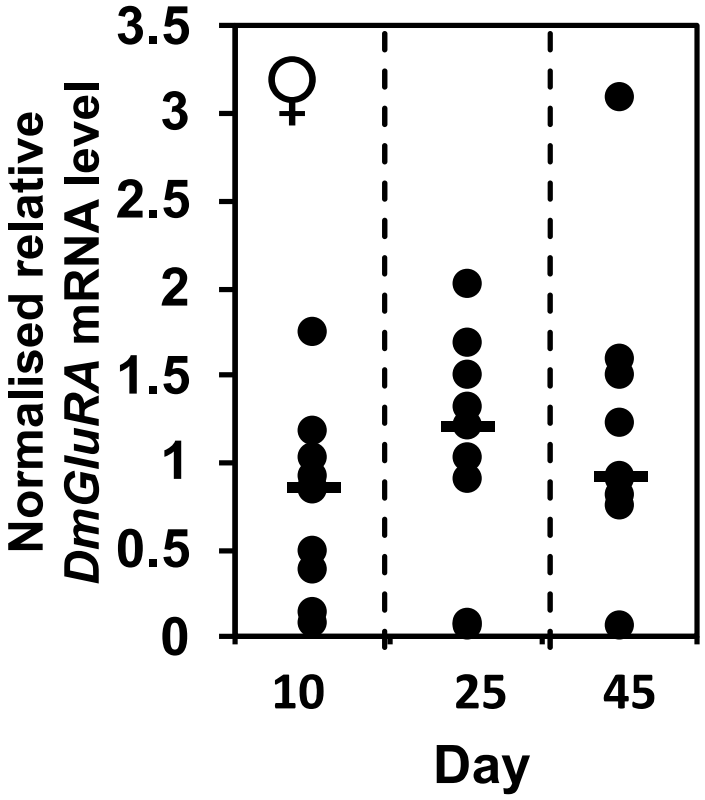


Figure 3.6 Analysis of *DmGluRA* mRNA expression during ageing.
DmGluRA mRNA expression was analysed by qRT-PCR and normalised to expression of *actin5C* mRNA in female w^{Dah} flies collected at 10, 25 and 45 days of age. Data represent normalised relative *DmGluRA* mRNA levels of 10 biological replicates across two independent experiments. Bars represent means. No significant differences in *DmGluRA* mRNA levels were observed across the different time-points. Data analysed using a Mixed Effects Linear Model with batch as a random effect.

3.3 Discussion

A primary focus of this chapter was to establish the nature of the $DmGluRA^{[112b]}$ null mutation, and its effects on *Drosophila* lifespan. Despite the use of this allele in several previous studies,

the precise nature of the mutant line was not well described (Bogdanik et al., 2004, Schoenfeld et al., 2013, Chun-Jen Lin et al., 2011). The *DmGluRA*$^{[112b]}$ mutation, was first described by Bogdanik et al. (2004), as a genetic deletion that removes part of the *DmGluRA* coding sequence and prevents expression of DmGluRA protein. However, the precise endpoints of this deletion remained unknown. In this chapter, the extent of the deleted genomic region within the *DmGluRA*$^{[112b]}$ allele has been fully characterised using a primer-walking method. The findings demonstrate that the deleted region spans 4.8kb of the *DmGluRA* locus, removing part of the promoter sequence and the translational start site and confirming the deletion of part of the coding sequence. Importantly, this finding enabled the development of a simple PCR-based assay that could successfully detect the presence of the mutant chromosome in adult flies. This was particularly useful as the *DmGluRA* mutation produced no obvious phenotypic differences in adult flies.

Preliminary data from the laboratory has suggested that *DmGluRA*$^{[112b]}$ mutants are long-lived but that this effect is specific to females. The data presented here confirms these previous observations, showing that loss of *DmGluRA* activity increases lifespan in *Drosophila* females irrespective of genetic background. For males, however, *DmGluRA*$^{[112b]}$ homozygotes showed increased lifespan in the inbred w^{1118} strain but no effects on lifespan were observed in the outbred w^{Dah} strain. This suggested that the effects of this mutation on male lifespan appear to be background specific.

Such sex differences in longevity are not uncommon. Often lifespan-extending interventions, particularly those that target nutrient-sensing pathways, show increased lifespan in females but with little or no effect on male survival (Austad and Fischer, 2016). For example, mutants of the *Drosophila* insulin-like receptor (dInR) or of the insulin receptor substrate, *chico,* and flies overexpressing the insulin-responsive transcription factor, dFOXO, in the adult fat body all show gender-specific effects on survival (Tatar et al., 2001, Clancy et al., 2002, Giannakou et al., 2004). For these genetic manipulations of the insulin/insulin-like growth factor signalling pathway, the underlying causes of these sex differences in longevity remain unknown.

The longevity of *DmGluRA*$^{[112b]}$ mutants described here is in contradiction to previous studies that have reported that loss of *DmGluRA* activity shortens lifespan in *Drosophila* (Ly and Naidoo, 2019). This discrepancy between the presented findings and previous published studies, is likely to be explained by differences in diet and/or genetic background used for the experimental work. In their study, Ly and Naidoo (2019) only examined lifespan in females and used a

different genetic background, *white Canton-Special* (w^{CS10}), maintained on a standard dextrose media. Previous work on long-lived *Drosophila* mutants has shown that genetic background greatly influences lifespan. For example, in the originally reported long-lived $Indy^{206}$ (*I'm Not Dead Yet*) mutants, males failed to show lifespan extension after the mutation was backcrossed into other genetic backgrounds (Toivonen et al., 2007). Moreover, the percentage increase of median survival times varies across different genetic backgrounds for individual *Indy* mutations (Rogina and Helfand, 2013). Diet also influences *Drosophila* lifespan, specifically diets with reduced yeast or sugar lead to increase lifespan, although reducing yeast has a greater effect on lifespan (Mair et al., 2005). The diet used for lifespan studies throughout this thesis, has been optimised for lifespan (Bass et al., 2007). This means the yeast concentration of the diet used is optimal for both lifespan and fecundity, where increases in nutrient availability beyond this concentration reduces lifespan despite a continuous increase in fecundity (Chapman and Partridge, 1996). The diet used in the published data may have different compositions of components such as sugar and yeast. An example of a mutation which shows different lifespan effects on different diets is *rpd3*, a histone deacetylase. *Rpd3* heterozygous mutants are long lived but by varying sugar and yeast, mean lifespan decreases or increases, for example (Frankel et al., 2015). Studies have demonstrated the role protein or the balance between proteins and carbohydrates in a diet play on lifespan regulation. For example, mice fed diets low in protein and high in carbohydrates have extended lifespan and improvements in health including brain function (Wahl et al., 2018) and some metabolic parameters such as insulin and glucose (Solon-Biet et al., 2015). This highlights the importance of the yeast/protein content of the diet on lifespan, if the longevity effects of *DmGluRA* mutation is dependent on protein then any changes to this component may affect the lifespan effects. This can be tested by observing the lifespan of *DmGluRA* mutants on varying yeast concentrations. If this is not the case then the lifespan extension should be seen at varying yeast concentrations.

The findings in this chapter also revealed that the lifespan extension observed in $DmGluRA^{[112b]}$ mutant females was not dependent on the presence or absence of the naturally occurring endosymbiotic bacteria, *Wolbachia pipientis,* as lifespan extension was observed in both *Wolbachia* positive and negative strains of w^{Dah}. *Wolbachia* has been shown to cause both positive (Brummel et al., 2004, Aleksandrov et al., 2007) and negative effects (Min and Benzer, 1997) on *Drosophila* lifespan. It has also been shown to modulate the effects of reduced IIS signalling on lifespan (Ikeya et al., 2009). However, no such interactions with reduced *DmGluRA* activity were observed here.

Sex-specific effects of the *DmGluRA*$^{[112b]}$ mutation have not been reported before mainly due to the fact that the effects of this mutation on phenotypes such as lifespan, sleep, social behaviour and memory (Ly and Naidoo, 2019, Schoenfeld et al., 2013) have only been studied in one sex. Ly and Naidoo (2019) studied lifespan and sleep in females only whereas Schoenfeld et al. (2013) used male flies to examine the role of *DmGluRA* in social behaviour and memory.

Importantly, the effects loss of *DmGluRA* activity on male lifespan showed differences between the w^{1118} and w^{Dah} genetic strains. This suggests that perhaps males may age differently in these different strains and there is some evidence to support this. Studies have shown that w^{1118} and w^{Dah} show male-specific differences in intestinal pathology during ageing. As flies age, they show age-related deterioration in intestinal physiology that manifests as deterioration of intestinal barrier function associated with increased numbers of dividing intestinal stem cells (ISCs) within the intestinal epithelium. In w^{1118} flies, this age-related intestinal pathology is observed in both males and females but in w^{Dah} flies, only females show the same age-related changes in intestinal physiology. Thus, intestinal pathology may be life-limiting for w^{1118} males but not w^{Dah} males (Regan et al., 2016). Any effect of the *DmGluRA*$^{[112b]}$ mutation on this age-dependent breakdown of the intestinal barrier could therefore form the basis for the lifespan differences observed in males of these two genetic strains.

The differential effects of the *DmGluRA*$^{[112b]}$ mutation on male and female lifespan in the w^{Dah} genetic background could reflect sex-specific effects of the mutation on *DmGluRA* expression. Strikingly, wild-type males exhibited significantly higher levels of *DmGluRA* transcript than females. Such sex differences in *DmGluRA* expression have not been previously reported. Flies homozygous for the *DmGluRA*$^{[112b]}$ show reduced but not absence of *DmGluRA* transcript expression despite previous studies reporting that no functional protein is produced in these mutants (Bogdanik et al., 2004). However, its important point to note here that the nature of the *DmGluRA* transcripts expressed in the mutants is not known or whether they would produce any functional protein. Although the epitope recognised by the antibody used by Bogdanik et al. (2004) is encoded by sequences close to the amplicon region for qRT-PCR and as such, it can be assumed that any protein produced in the mutants would still be detected with this antibody. Interestingly, although *DmGluRA* expression is reduced in mutants of both sexes, *DmGluRA* transcript levels in mutant males are still higher than those of wild-type females. It is possible that expression of *DmGluRA* below a threshold level is required for lifespan extension. The sex-

specific differences in lifespan may therefore reflect these higher levels of *DmGluRA* expression in mutant males compared to females.

The *DmGluRA* gene is located on the fourth chromosome, which is the smallest autosome and contains relatively few genes (Sun et al., 2000). The fourth chromosome is also highly heterochromatic and studies have shown that expression of genes located in these heterochromatic regions of the genome becomes dysregulated during ageing. Thus, as flies age these genes are expressed in tissues in which they are not normally expressed or their levels of expression change. Together, this inappropriate gene expression may contribute to ageing pathologies (Wood et al., 2010) and interventions that promote longevity have been shown to reduce the detrimental effects of this misregulated gene expression (Wood et al., 2016). The location of *DmGluRA* within one of these heterochromatic regions suggests that it may also show misregulated expression during ageing. As such, it is possible that in *DmGluRA*$^{[112b]}$ mutants, the longevity effect are a consequence, at least in part, of preventing dysregulation of *DmGluRA* expression. There is existing evidence that overexpression of *DmGluRA* is associated with FXS symptoms in the *Drosophila* FXS model(Kanellopoulos et al., 2012). However, in wild-type females, the levels of *DmGluRA* transcript expression did not change over time suggesting that the loss of *DmGluRA* activity itself exerts a direct effect on ageing.
From the qPCR analysis in both mutants and wildtype flies, further experiments are needed to fully characterise the mutant. For instance, using western blotting to measure protein levels in *DmGluRA* mutants to ensure the D*mGluRA* transcripts do not produced any protein. Due to time restraint of the project, such experiments were not performed but it will be useful also to study expression level of *DmGluRA* in males and mutants at different ages. It would be interesting to know if the levels of *DmGluRA* remain higher in males during ageing and also if the transcripts still produced by the mutants changes with age in either males or females.

The next chapter will attempt to further understand the mechanisms by which loss of *DmGluRA* extends lifespan, by examining *DmGluRA*$^{[112b]}$ mutants for phenotypes that commonly associate with increased lifespan. Alongside, *DmGluRA*$^{[112b]}$ mutants were also examined for improvements in health parameters during ageing.

Chapter 4 : Effects of *DmGluRA* mutation on phenotypes associated with changes in lifespan

4.1 Introduction

The data so far supported a role for *DmGluRA* signalling in lifespan regulation (Chapter 3). However, the mechanisms by which loss of *DmGluRA* activity extends lifespan was yet to be elucidated. The aim of this chapter was to study phenotypes that are associated with changes in lifespan to identify potential mechanism/s by which this mutation extended lifespan. For example, differences in female fecundity, growth, stress resistance, metabolic function and feeding behaviour have all been observed in long-lived strains and so these phenotypes were analysed in *DmGluRA* mutants. In addition, as flies age, like in mammals, they exhibit physiological declines in various functions including climbing behaviour and intestinal barrier function (Grotewiel et al., 2005, Rera et al., 2012). Therefore, additionally this chapter set out to examine changes in physiological functions during aging in *DmGluRA* mutants to establish whether loss of *DmGluRA* activity improved any of these phenotypes. This will also test if loss of *DmGluRA* improves healthspan in *Drosophila,* in addition to the lifespan extension.

As previously seen in Chapter 3, $DmGluRA^{[112b]}$ mutation extended lifespan in a sex-specific manner, at least in the w^{Dah} background. Therefore, it was important, for at least some of the phenotypes assayed, to analyse both sexes. Similarly, as sex-specific differences were observed between different genetic backgrounds, it was important to characterise the mutant phenotypes in both w^{Dah} and w^{1118} genetic strains. Any phenotypic differences that correlated with the lifespan effects may therefore explain the differences observed in lifespan.

4.2 Results

4.2.1 Effects of $DmGluRA^{[112b]}$ on female fecundity

Long-lived strains tend to be associated with reduced early life fecundity in females. It has been suggested that a trade-off between reproduction and lifespan exists (Flat,2011). Processes such as reproduction, somatic repair and maintenance, and growth are costly processes which require resources such as nutrients and energy. Trade-offs are thought to occur because resources cannot be invested in all these processes at once (Partridge et al., 2005) so investing

resources in producing many offspring compromises and therefore shortens survival. Additionally, reproduction may be damaging to the female leading to mortality.

To test whether long-lived *DmGluRA*$^{[112b]}$ mutant females also showed reduced fecundity, egg production on day 7 and day 14 of adult lifespan was measured. In the w^{1118} genetic background, females homozygous for the *DmGluRA*$^{[112b]}$ mutant did not show a significant decrease in egg laying compared to their wild-type controls (Figure 4.1, day 7: p=0.4003, day 14: p= 0.8339). Similarly, in the w^{Dah} genetic background, no differences in fecundity between *DmGluRA*$^{[112b]}$ mutant mutants and their wildtype controls were observed (Figure 4.1, day 7: p= 0.4285; day 14: p=0.2786). In both genetic backgrounds, both the mutants and controls show a similar reduction in egg laying between 7 and 14 days of age.

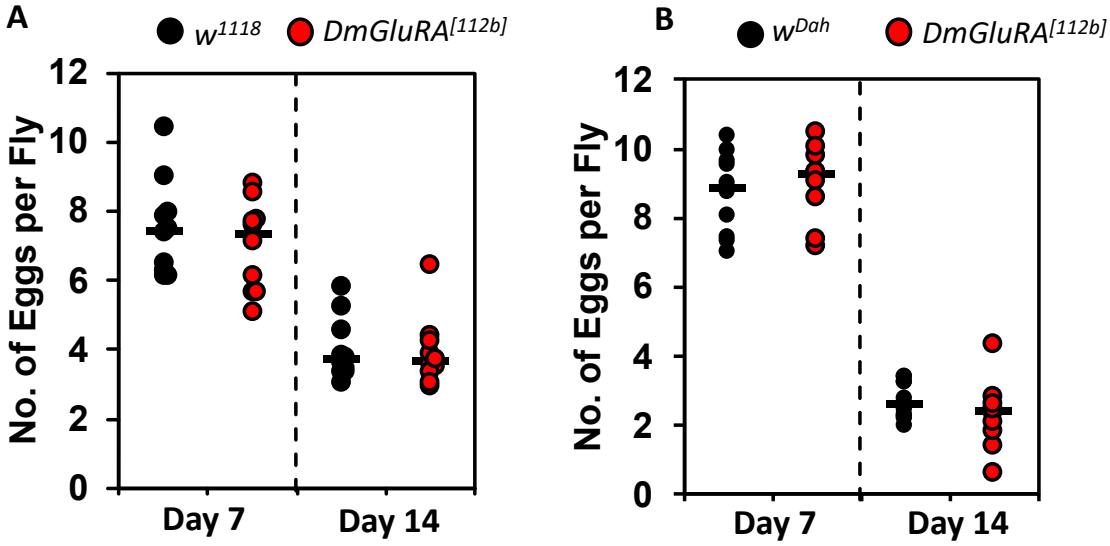


Figure 4.1 Number of eggs per 24hr period per fly.
Eggs were counted from 10 vials containing 15 females each after 24-hour collections taken on days 7 and 14 of adult lifespan. Data represents number of eggs laid per fly per vial. Bar represents the average number of eggs laid over the 10 vials.
(A) No significant differences were observed between $w^{111\ 8}$*DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls at either of the timepoints (Student's *t* test, p>0.05; n=10 vials of 15 flies per genotype).
(B) No significant differences were observed between w^{Dah} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls at any of the timepoints (Student's *t* test, p>0.05; n=10 vials of 15 flies per genotype).

4.2.2 Effects of *DmGluRA*$^{[112b]}$ on growth

Reduced adult body size is a phenotype observed in many long-lived mutant fly strains associated with reduced nutrient signalling during development (Slack et al., 2010, Post et al.,

2019). In mammals, the opposite effect is observed, as larger body size is generally associated with longer lifespan (Speakman, 2005). In *Drosophila*, the body size of the adult is determined during development and so differences in adult body size are indicative of effects on developmental growth. To determine if mutation of *DmGluRA* was associated with effects on growth during development, body weights and wing sizes were measured of individual adult *DmGluRA*$^{[112b]}$ mutants and their controls.

In the *w*^{1118} genetic background, *DmGluRA*$^{[112b]}$ mutant males and females showed a significant increase in body weight compared to their controls ($p<0.0001$; Figure 4.2A). In the *w*^{Dah} background, no differences were observed in the body weights of *DmGluRA*$^{[112b]}$ mutant males compared to controls (Figure 4.2B) but body weights were significantly increased in *DmGluRA*$^{[112b]}$ mutant females compared to controls ($p<0.001$, Figure 4.2B).

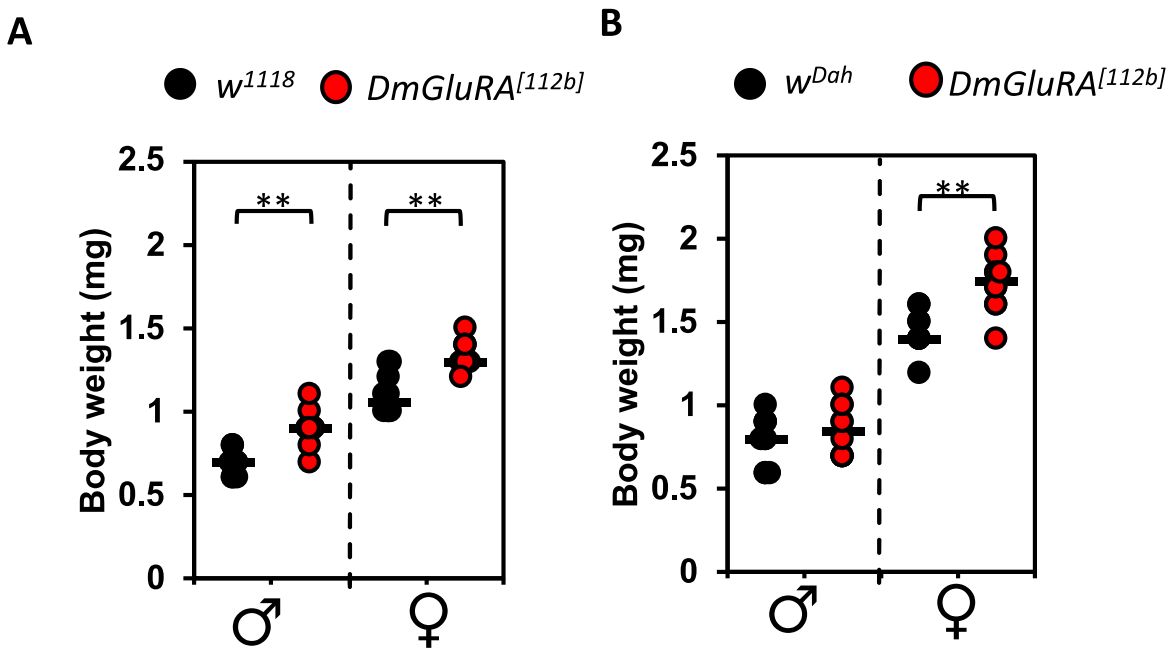


Figure 4.2 Body weights in *DmGluRA* mutants and wild-type controls.
Individual 10-day old flies of the indicated genotypes were weighed (n=10 flies per condition). Data represents individual body weights (mg). Bars represent the mean. ** represents $p<0.05$, (Student's *t* test).
(A) *w*^{1118} *DmGluRA*$^{[112b]}$ mutant males and females showed increased mean body weights compared to their wildtype controls.
(B) No differences in mean body weight were observed for *w*^{Dah} *DmGluRA*$^{[112b]}$ mutant males compared to controls. *w*^{Dah} *DmGluRA*$^{[112b]}$ mutant females showed increased mean body weight compared to controls.

No significant differences in wing area were observed for w^{1118} $DmGluRA^{[112b]}$ mutant males compared to controls while female w^{1118} $DmGluRA^{[112b]}$ showed a small but significant increase in wing area (Figure 4.3A). In the w^{Dah} background, no differences in wing size were observed for either male or female $DmGluRA^{[112b]}$ mutants compared to controls (Figure 4.3B).

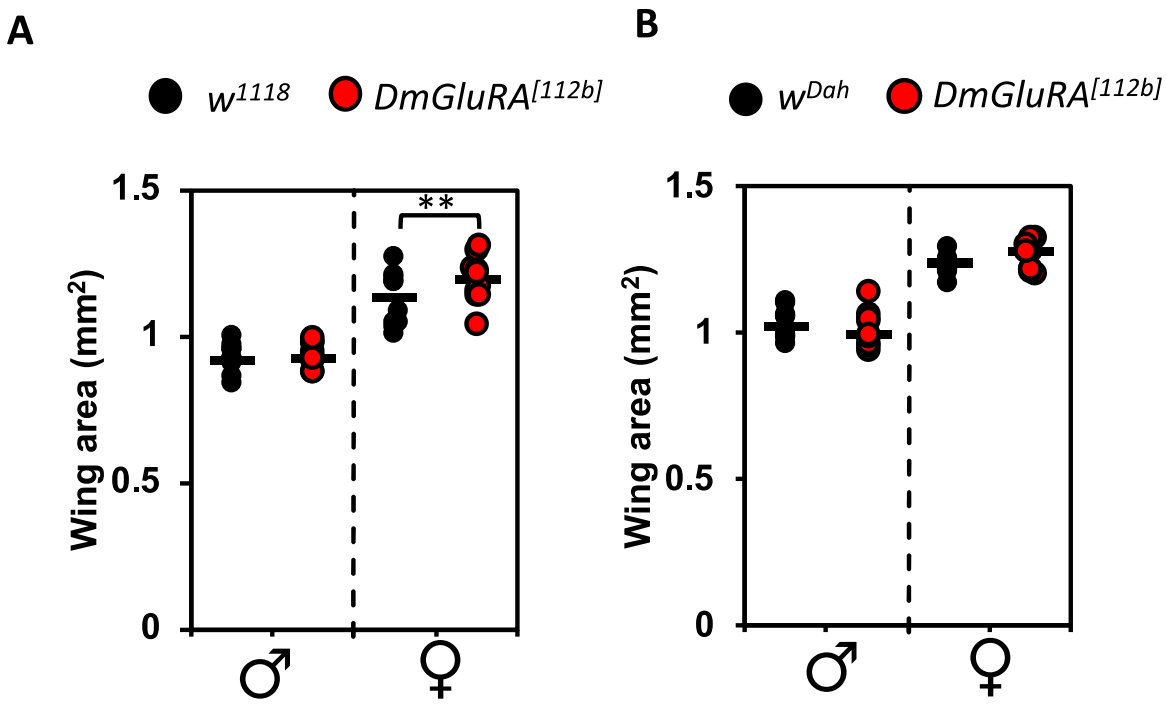


Figure 4.3 Wing sizes in *DmGluRA* mutants and wild-type controls.
The left wing of individual 10-day old flies of the indicated genotypes were removed and the wing size measured (n=10 flies per condition). Data represents individual wing areas (mm^2). Bars represent the mean. ** represents $p<0.05$, (Student's *t* test).
(A) w^{1118} $DmGluRA^{[112b}$ mutant females showed a small increase in wing area compared to wildtype controls. No differences were observed in wing area between w^{1118} $DmGluRA^{[112b]}$ mutant males and control males.
(B) No differences were observed in wing size between w^{Dah} $DmGluRA^{[112b]}$ mutants and controls for either males or females.

4.2.3 Effects of $DmGluRA^{[112b]}$ on stress resistance

Several long-lived animals have increased resistance to various stressors (Lin et al., 1998, Rogers and Rogina, 2014, Moskalev et al., 2019) including oxidative stress and starvation.

Therefore, these previous studies have suggested that increased resistance against stressors may be a potential mechanism for lifespan extension.

Firstly, the *DmGluRA*$^{[112b]}$ mutant flies were tested for survival under oxidative stress, induced by feeding flies with either paraquat or hydrogen peroxide. Paraquat, a widely used pesticide, works by disrupting complex I and III of the electron transport chain and inducing mitochondrial dysfunction (Derek & Patel, 2009). This catalyses the production of reactive oxygen species (ROS) particularly superoxide anions that cause the oxidative damage of DNA, proteins and lipids. Hydrogen peroxide (H_2O_2) is a reactive oxygen species itself and leads to the generation of hydroxyl radicals (Minois et al., 2012).

In the w^{1118} genetic background, long-lived *DmGluRA*$^{[112b]}$ mutant females upon paraquat treatment, showed an 18.8% increase in median survival time compared to their wildtype controls (Figure 4.4A, females: $p=2.8 \times 10^{-6}$). Similarly, long-lived *DmGluRA*$^{[112b]}$ mutant males showed increased survival upon paraquat treatment with an increase in median survival time of 56.5% compared to their controls (Figure 4.4A, males: $p= 2.8 \times 10^{-4}$).

In the w^{Dah} genetic background, again both males and females homozygous for the *DmGluRA*$^{[112b]}$ mutation showed significantly increased median survival times upon paraquat treatment compared to their wildtype controls (Figure 4.4B, males: median survival increased by 50%, $p= 5.1 \times 10^{-5}$; females: median survival increase by 40%, $p=4.2 \times 10^{-5}$).

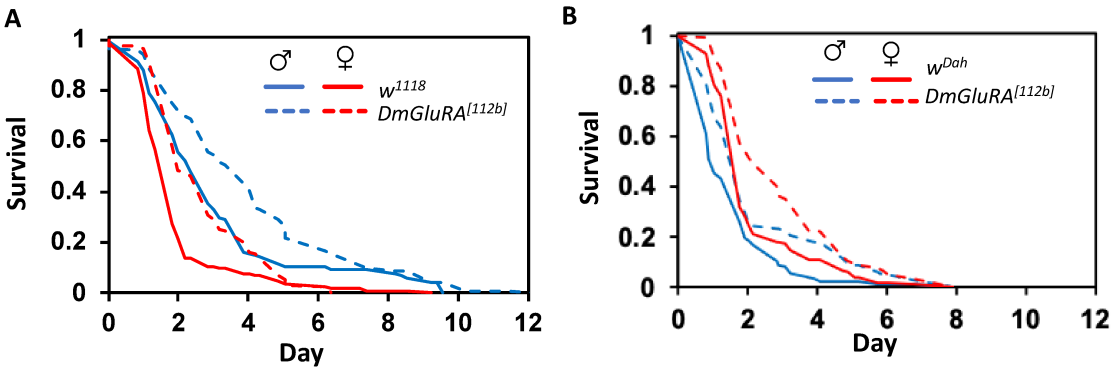


Figure 4.4 Survival of *DmGluRA*$^{[112b]}$ mutants during paraquat-induced oxidative stress.
Survival curves of flies maintained on media containing 20 mM paraquat from 7 days of age
(A) w^{1118} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls. Median survival times were: 2.3 days for w^{1118} males (n=147) and 3.6 days for *DmGluRA*$^{[112b]}$ males (n=150), $p= 2.8 \times 10^{-4}$ (Log rank test) and 1.6 days for w^{1118} females (n=130) 1.9 days for *DmGluRA*$^{[112b]}$ females (n=141), $p=2.8 \times 10^{-6}$ (Log rank test).
(B) w^{Dah} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls. Median survival times were: 1 day for w^{Dah} males (n= 147) and 1.5 days for *DmGluRA*$^{[112b]}$ males (n=147), $p= 5.1 \times 10^{-5}$ (Log rank test) and 1.5 days for w^{Dah} females (n=150) 2.1 days for *DmGluRA*$^{[112b]}$ females (n=148), $p=4.2 \times 10^{-5}$ (Log rank test).

In the w^{1118} genetic background, *DmGluRA*$^{[112b]}$ homozygous mutant males again were more resistant to hydrogen peroxide than their wildtype controls, exhibiting an increase in median survival time of 72.7% (Figure 4.5A, p= 1.1 x 10^{-46}). In females, though the median lifespan of mutants and controls were similar (Figure 4.5A; 2.8 days), there was an increase in maximum survival of *DmGluRA*$^{[112b]}$ mutants of 18.2% (p=2.1 x 10^{-2}).

In the w^{Dah} genetic background, *DmGluRA*$^{[112b]}$ homozygous mutant males again showed increased survival in the presence of hydrogen peroxide with a 13.9% increase in median survival (Figure 4.5B, p=1.6 x 10^{-10}). However, *DmGluRA*$^{[112b]}$ females exhibited reduced survival in the presence of hydrogen peroxide compared to their wildtype controls, with a 8.9% reduction in median survival (Figure 4.5B, p = 6.6 x 10^{-5}).

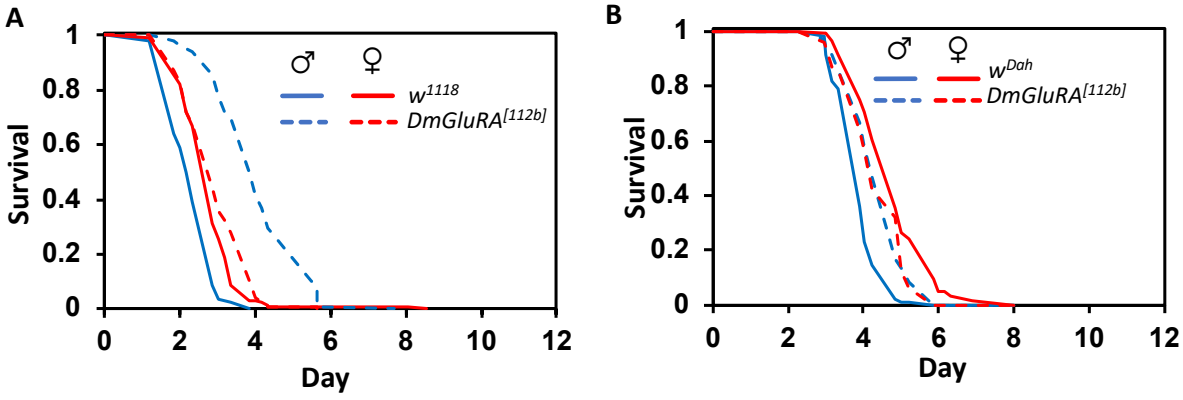


Figure 4.5 Survival of *DmGluRA*$^{[112b]}$ mutants during hydrogen peroxide-induced oxidative stress.

Survival curves of flies maintained on media containing 5% (v/v) hydrogen peroxide from 7 days of age. (A) w^{1118} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls. Median survival times were: 2.2 days for w^{1118} males (n=138) and 3.8 days for *DmGluRA*$^{[112b]}$ males (n=138), p= 1.1 x 10^{-46} (Log rank test) and 2.6 days for w^{1118} females (n=129) 2.6 days for *DmGluRA*$^{[112b]}$ females (n=126), p=2.1 x 10^{-2} (Log rank test). (B) w^{Dah} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls. Median survival times were: 3.6 days for w^{Dah} males (n= 149) and 4.1 days for *DmGluRA*$^{[112b]}$ males (n=142), p= 1.6 x 10^{-10} (Log rank test) and 4.5 days for w^{Dah} females (n=149) 4.1 days for *DmGluRA*$^{[112b]}$ females (n=146), p=6.6 x 10^{-5} (Log rank test).

The response of *DmGluRA*$^{[112b]}$ mutants to starvation stress was then assessed by removing the external intake of nutrients, which causes flies to use their internal metabolic stores and once this is exhausted, this leads to death (Sun et al., 2013).

In the w^{1118} genetic background, significant increases in survival during starvation were observed in both *DmGluRA*$^{[112b]}$ homozygous mutant males and females compared to their wildtype controls although the response in females was not as great as that observed in males (Figure 4.6A males: median survival increased by 28.6%, p= 1.0 x 10^{-18} and females: median lifespan increased by 7.8%, p=4.2 x 10^{-5}).

In the w^{Dah} genetic background, *DmGluRA*$^{[112b]}$ homozygous mutant males showed a significant increase in survival during starvation (median survival increased by 25%, p= 1.4 x 10^{-23}) but female *DmGluRA*$^{[112b]}$ homozygous mutants did not show any differences in starvation survival compared to their controls (Figure 4.6B, p=0.234).

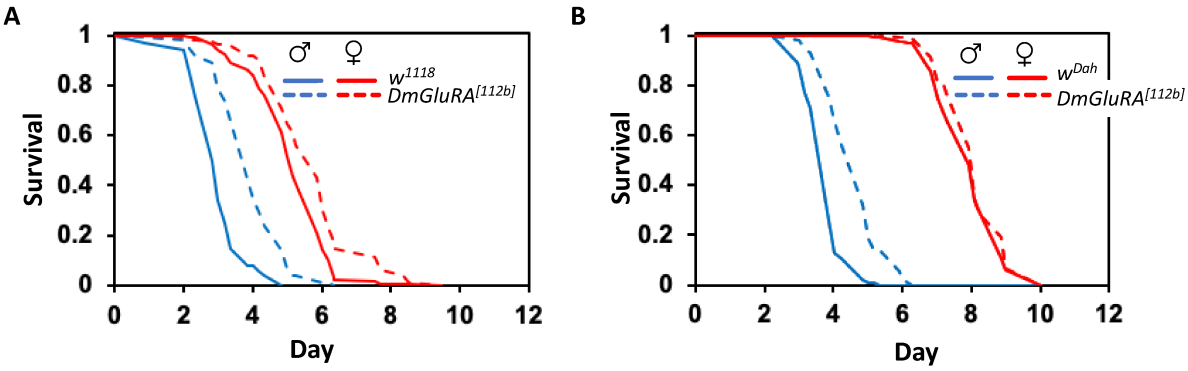


Figure 4.6 Survival of *DmGluRA*$^{[112b]}$ mutants during starvation.
Survival of adult flies of the indicated genotypes maintained on 1% (w/v) agar media from 7 days of age. (A) w^{1118} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls. Median survival times were: 2.8 days for w^{1118} males (n=140) and 3.6 days for *DmGluRA*$^{[112b]}$ males (n=130), p= 1.0 x 10^{-18}(Log rank test) and 5.1 days for *w1118* females (n=147) and 5.5 days for *DmGluRA*$^{[112b]}$ females (n=142), p=4.2 x 10^{-5} (Log rank test).
(B) w^{Dah} *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls. Median survival times were: 3.6 day for w^{Dah} males (n= 145) and 4.5 days for *DmGluRA*$^{[112b]}$ males (n=149), p= 1.4 x 10^{-23} (Log rank test) and 7.6 days for w^{Dah} females (n=144) 8.0 days for *DmGluRA*$^{[112b]}$ females (n=142), p=0.234 (Log rank test).

4.2.4 Effects of *DmGluRA*$^{[112b]}$ on metabolism

Increased resistance to starvation tends to be associated with increased levels of energy reserves (Bjedov et al., 2010, Rion and Kawecki, 2007). Flies survive longer during starvation because they have more energy reserves. Similar to mammals, *Drosophila* store lipids as triacylglycerides (TAGs) which are stored within the fat body. *Drosophila* have two main forms of sugars in their circulation: glucose, which they obtain from their diet and trehalose, which is glucose stored as a disaccharide. Excess glucose is also stored as glycogen.

To test if the enhanced resistance to starvation observed in *DmGluRA*$^{[112b]}$ mutants was due to increased energy reserves, levels of the three major storage macromolecules: TAGs, trehalose and glycogen, were measured in whole adult flies.

In both the w^{1118} and w^{Dah} genetic backgrounds, no significant differences were observed in the levels of TAGs in in either males or females homozygous mutant for *DmGluRA*$^{[112b]}$ compared to wildtype controls (Figure 4.7).

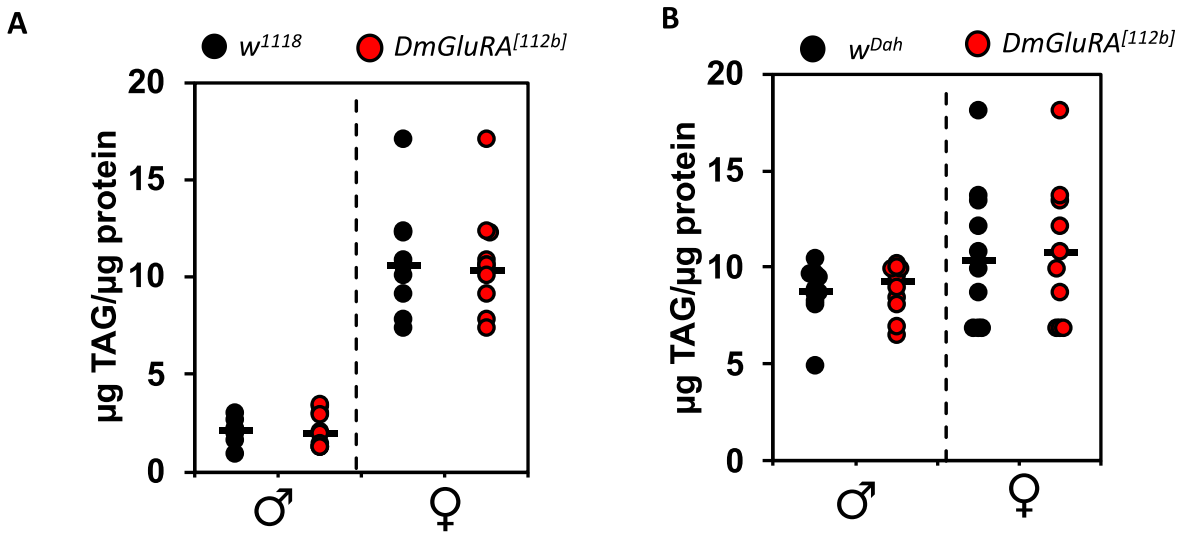


Figure 4.7 Measurements of triaceylglyceride (TAG) stores in *DmGluRA*$^{[112b]}$ mutants and wildtype controls.
Whole-fly extracts of the indicated genotypes were prepared from groups of 5 flies. Data represent µg of triacylglycerides (TAGs) per µg of protein. Bars represent the mean. No significant differences were observed in the levels of TAG between *DmGluRA*$^{[112b]}$ homozygous mutants and wildtype controls in either genetic background (One-way ANOVA with Turkey's post hoc test, n=10).

Similarly, no significant differences were observed in the levels of trehalose (Figure 4.8) or glycogen (figure 4.9) in either males or females homozygous mutant for *DmGluRA*$^{[112b]}$ compared to wildtype controls.

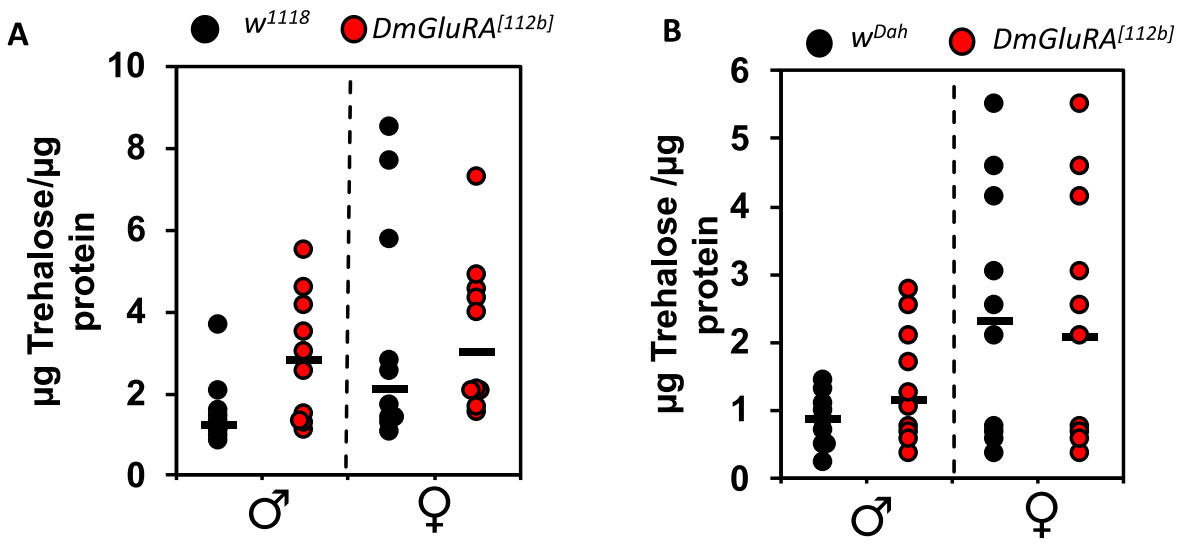


Figure 4.8 Measurements of whole-fly trehalose in *DmGluRA[112b]* mutants and wildtype controls.

Whole-fly extracts of the indicated genotypes were prepared from groups of 5 flies. Data represent µg µg of trehalose per µg of protein. Bars represent the mean. No significant differences were observed in the levels of the three metabolic storage molecules between *DmGluRA[112b]* homozygous mutants and wildtype controls in either genetic backgrounds (One-way ANOVA with Turkey's post hoc test, n=10).

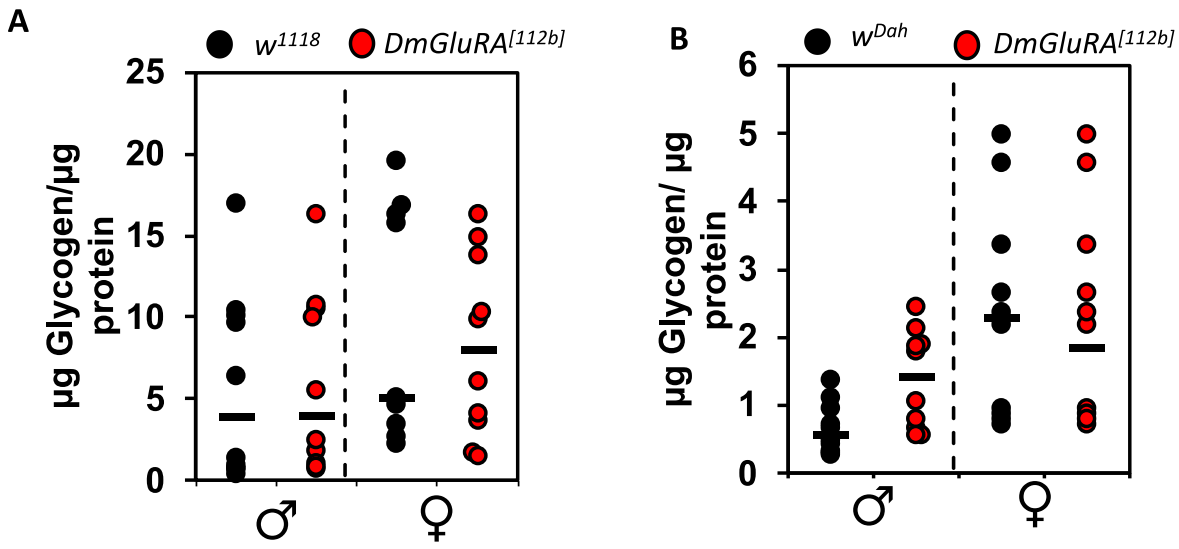


Figure 4.9 Measurements of glycogen in *DmGluRA[112b]* mutants and wildtype controls.

Whole-fly extracts of the indicated genotypes were prepared from groups of 5 flies. Data represent µg of glycogen per µg of protein. Bars represent the mean. No significant differences were observed in the levels of the three metabolic storage molecules between *DmGluRA[112b]* homozygous mutants and wildtype controls in either genetic backgrounds (One-way ANOVA with Turkey's post hoc test, n=10).

While there were no obvious differences in the amounts of metabolic storage molecules between the *DmGluRA* mutants and their controls under fully-fed conditions, enhanced resistance under starvation stress may reflect the more efficient mobilisation and/or usage of metabolic stores during starvation in the mutants (Rion and Kawecki, 2007). To examine this, levels of TAGs, trehalose and glycogen were measured in 7-day old flies over a 48-hour starvation time-course.

In wildtype male flies, the levels of TAGs decreased by ~50% after 24 hours of starvation but did not decrease further after 48 hours of starvation. *DmGluRA*$^{[112b]}$ homozygous mutant males showed significantly elevated levels of TAGs compared to the wildtype controls at all time-points (Figure 4.10A). Similarly, in wildtype females flies, the levels of TAGs also decreased after 24 hours of starvation and also did not decrease any further after 48 hours of starvation (Figure 4.10B). Unlike for males, in *DmGluRA*$^{[112b]}$ homozygous mutant females, levels of TAGs were similar to controls at all timepoints.

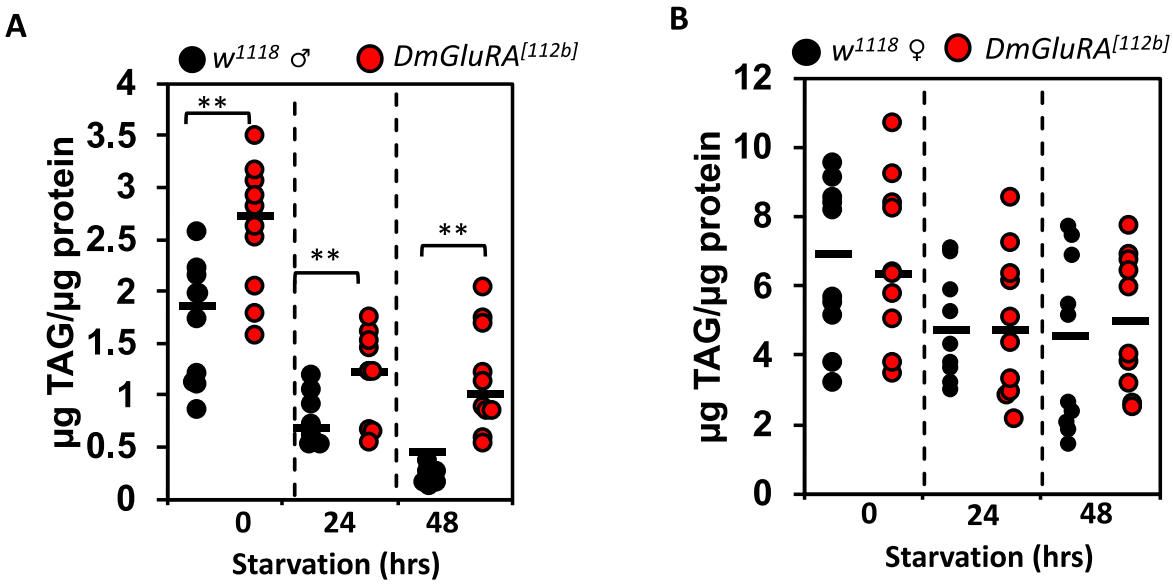


Figure 4.10 Measurements of triacelgylcerides (TAGs) during starvation.
Whole-fly extracts of the indicated genotypes were prepared from groups of 5 males (A) or females (B) under fully-fed (0 hr starvation) and starved conditions (24 hr and 48 hr starvation). Data represent µg of triacelyglycerides per µg of protein content. Bar represents the mean. ** denotes $p<0.001$ (Two-way ANOVA with Turkey's post hoc test, n=10).

In wildtype males, there was no significant change in the levels of trehalose over 0, 24 and 48 hrs of starvation (Figure 4.11A). There were also no differences in trehalose observed between mutants and controls at any of the timepoints. In wildtype females, similar to what was observed in males, trehalose levels did not decrease during starvation and there were no differences between mutants and controls at any of the starvation timepoints (Figure 4.11B).

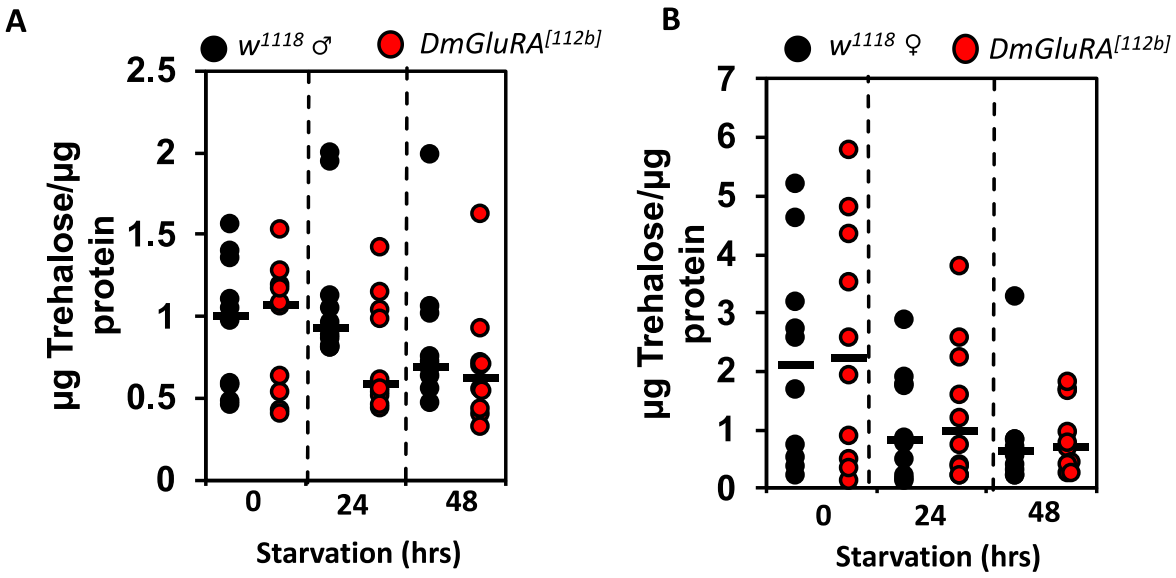


Figure 4.11 Measurements of trehalose during starvation.
Whole-fly extracts of the indicated genotypes were prepared from groups of 5 male (A) or female (B) flies under fully-fed (0 hr starvation) and starved conditions (24 hr and 48 hr starvation). Data represent µg of trehalose per µg of protein Bars represent the means. Data were analysed using Two-way ANOVA with Turkey's post hoc test (n=10).

Glycogen levels in wildtype males again did not decrease after 24 hrs or 48hrs of starvation (Figure 4.12A). There were also no differences in glycogen levels between control and mutant at any timepoints. In wildtype females, the levels of glycogen decreased by 50% after 24 hrs of starvation (Figure 4.12B, p=0.0076) but did not decrease further. Levels of glycogen were also similar in mutants and controls at all timepoints.

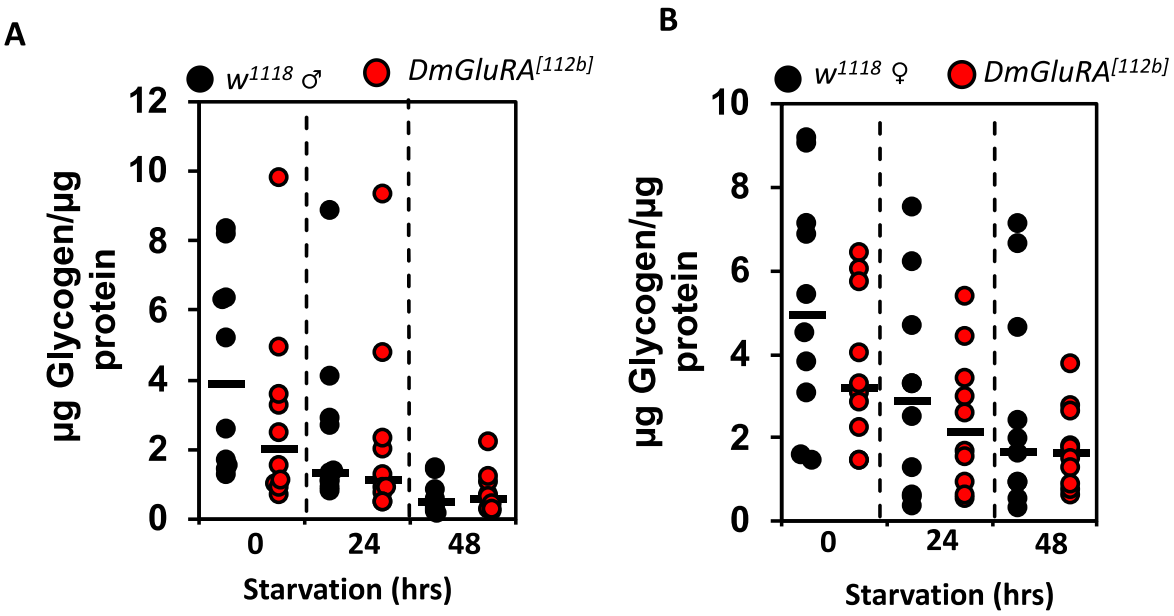


Figure 4.12 Measurements of glycogen during starvation.
Whole-fly extracts of the indicated genotypes were prepared from groups of 5 male (A) or female (B) flies under fully-fed (0 hr starvation) and starved conditions (24 hr and 48 hr starvation). Data represent µg of glycogen per µg of protein. Bars represent the means. Data were analysed using Two-way ANOVA with Turkey's post hoc test (n=10).

4.2.5 The effects of *DmGluRA[112b]* on food intake

One important factor to control when studying the effects of genetic interventions on ageing is dietary intake (Sun et al., 2013) . Reducing food intake without starvation, termed as dietary restriction (DR), extends animal lifespan and improves health during old age (Piper and Partridge, 2007). It is was therefore possible that *DmGluRA[112b]* mutants eat less and so induce DR resulting in the observed lifespan extension. *DmGluRA[112b]* mutants also showed differences in body weights, this may also reflect differences in food intake i.e. the mutants may eat more.

To determine whether there were any differences in food consumption between *w1118 DmGluRA[112b]* mutants and their wildtype controls, the CApillary FEeder assay (CAFÉ) was performed over three consecutive days on 7-day old flies to directly measure food intake (Ja et al., 2007a). However, no differences were observed in the volume of food consumed between *DmGluRA[112b]* mutants and their wildtype controls at any of the three time points for either males or females (Figure 4.13).

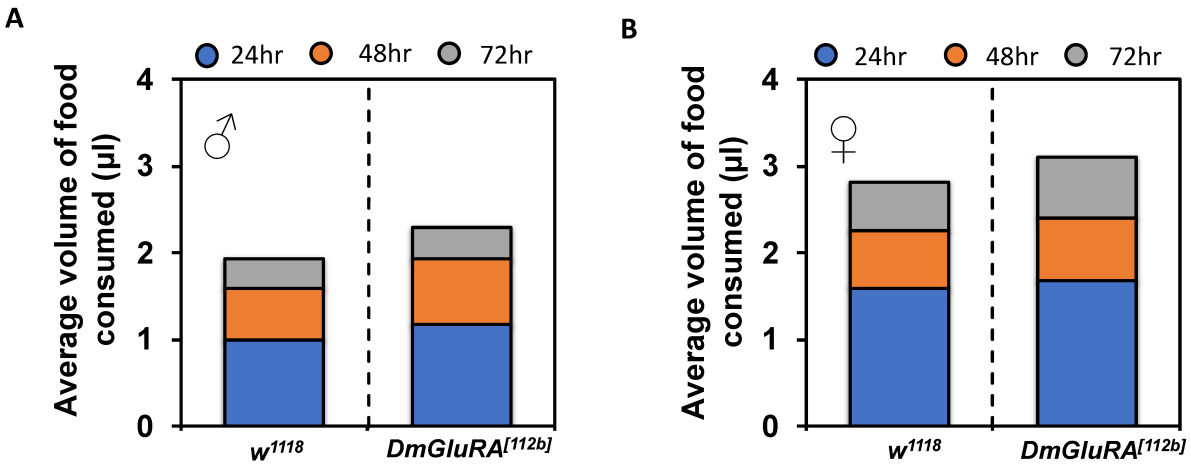


Figure 4.13 Measurement of food consumption in *DmGluRA*$^{[112b]}$ mutants and controls using the CAFÉ assay.

The volumes of food consumed by 15 individual 7-day old flies per genotype were measured over three consecutive days using the CAFÉ assay. Data represent the average volume of food consumed in µl after 24, 48 and 72 hours in males (A) and females (B). No significant differences were observed between *DmGluRA*$^{[112b]}$ mutants and their wildtype controls for either sex at any of the three time-points (Student's *t* test).

Though the CAFE assay allows direct measurement of food intake, it is does not reflect how flies feed during lifespan. This is because in this assay they eat on liquid food, rather than solid food like the other experimental work in this thesis. To overcome this limitation, feeding rate was measured using another feeding assay, the proboscis extension (PE) assay which better reflects the situation the flies were housed in for the other experiments.

DmGluRA$^{[112b]}$ mutants fed at similar rate as their wildtype controls across the 60-min period tested (Figure 4.14). Overall, the mutants showed no differences in the average feeding rate compared to their wildtype controls for both males and females (Figure 4.14).

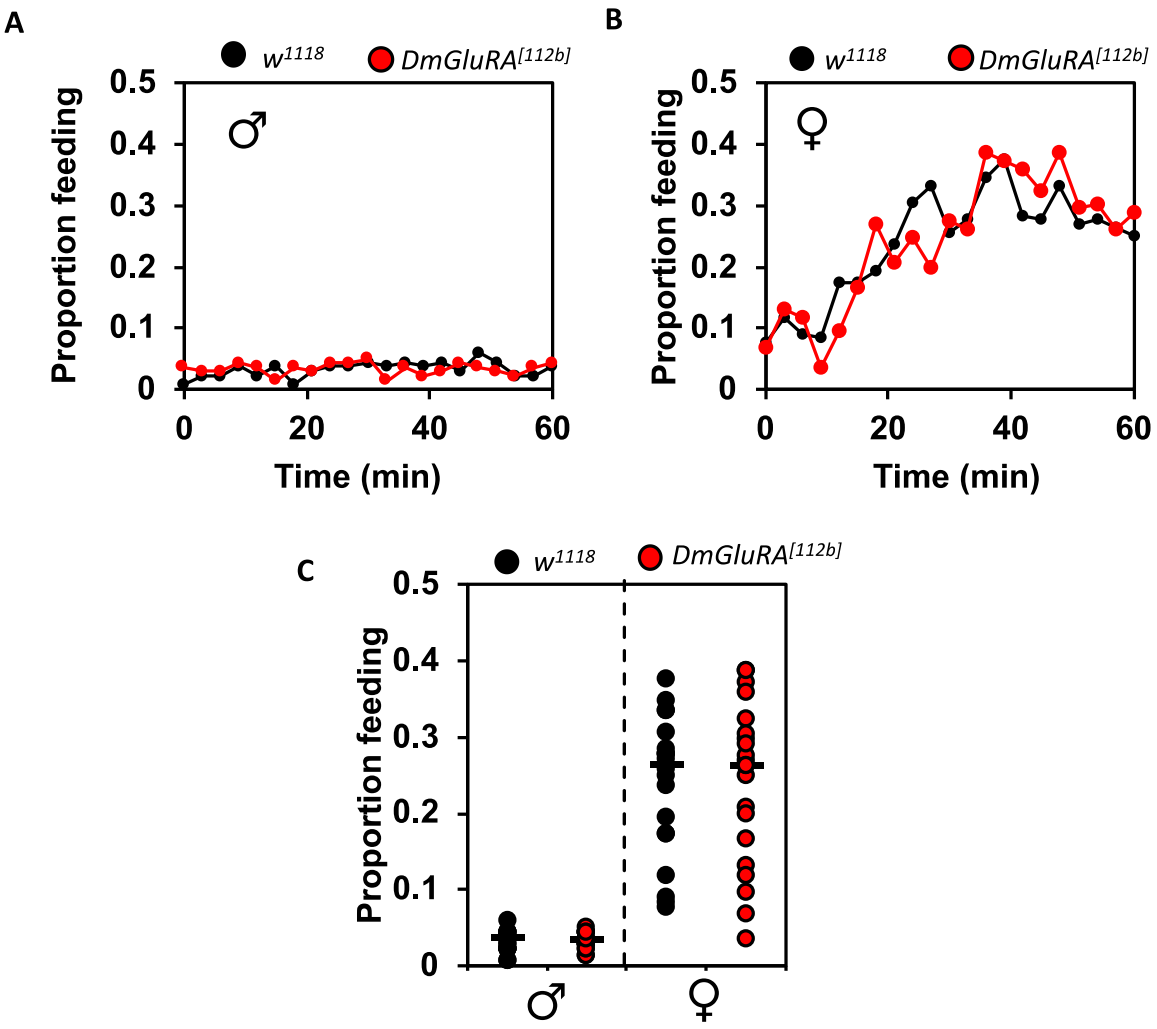


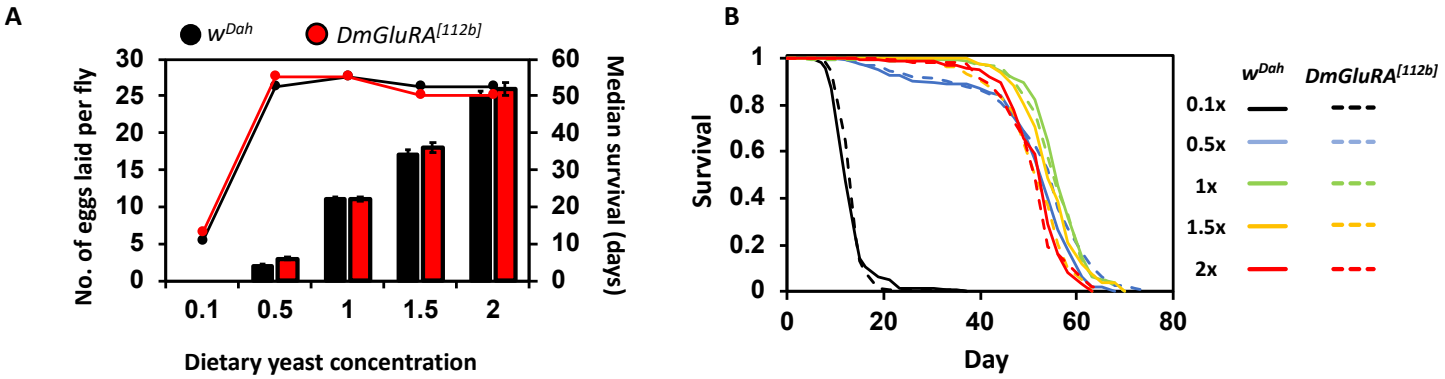
Figure 4.14 Measurement of feeding rate in *DmGluRA[112b]* mutants and controls using the PE assay.

Flies were maintained on normal sugar-yeast-agar media for 7 days at a density of 5 flies per vial and then transferred to SYA containing 2.5% blue dye. Feeding behaviour was observed over a 60 min period. The proportion of feeding events over the 60 min period in (A) males and (B) females.

(C) Data are presented as the proportion of feeding events/possible feeding events ± SEM. No significant difference was observed in feeding behaviour between mutants and their controls (Males: p=0.9999, females: p=0.08003, Chi-square test)

Dietary restriction (DR) is characterized by the reduction of dietary intake without inducing malnutrition. It remains a robust environmental intervention which extends life span in various organisms including flies (Emran et al., 2014). In *Drosophila*, DR is induced by diluting the yeast component of the food, which provides the source of protein but keeping the sugar component constant (Bass et al., 2007).

To test if the lifespan extension associated with loss of *DmGluRA* activity overlapped with DR, survival and fecundity was examined across a food dilution series on SYA food, varying from 0.1x to 2x yeast concentration. Egg laying increased with yeast concentration in both wildtype controls and mutants (Figure 4.15A). The controls exhibited the typical tent-shaped response to DR with peak median lifespan at 1x yeast concentration. The DR tent changed in the mutants, they exhibited a left-shifted response to DR and median lifespan peaked at 0.5x yeast concentration (Figure 4.15A). Lifespan extension was not observed at 1x yeast concentration as previously observed in controls (Figure 4.15B). Median lifespan was also higher than controls at 0.5x. At high concentrations, 1.5x and 2x, mutants were shorter lived than controls.



Concentration	Genotype	Median lifespan	n	P value
0.1x	w^{Dah}	10.6	141	0.394
	DmGluRA$^{[112b]}$	13.0	148	
0.5x	w^{Dah}	52.6	128	0.031*
	DmGluRA$^{[112b]}$	55	143	
1x	w^{Dah}	55	139	
	DmGluRA$^{[112b]}$	55	137	0.560
1.5x	w^{Dah}	52.6	129	
	DmGluRA$^{[112b]}$	50.1	137	0.0004*
2x	w^{Dah}	52.6	138	
	DmGluRA$^{[112b]}$	50.1	125	0.424

Figure 4.15 The response of *DmGluRA*$^{[112b]}$ mutants to Dietary Restriction (DR).
The effects of increasing concentrations of dietary yeast on lifespan and egg laying were measured in w^{Dah} wildtype and *DmGluRA*$^{[112b]}$ mutant females.
(A) Eggs were counted from 10 vials containing 15 females each after 24-hour collection taken 7 days after flies were transferred onto their respective food concentrations. Bars represent average number of eggs laid over the 10 vials. Error bars represent S.E.M. Connected points represent the median lifespan of the indicated genotypes with increasing dietary yeast.
(B) Survival of w^{Dah} wildtype and *DmGluRA*$^{[112b]}$ mutant females in response to changes in dietary yeast. Median survival times for each genotype on the different food concentrations are shown, red asterisk shows where there is a significant difference (Log rank test, $p<0.05$).

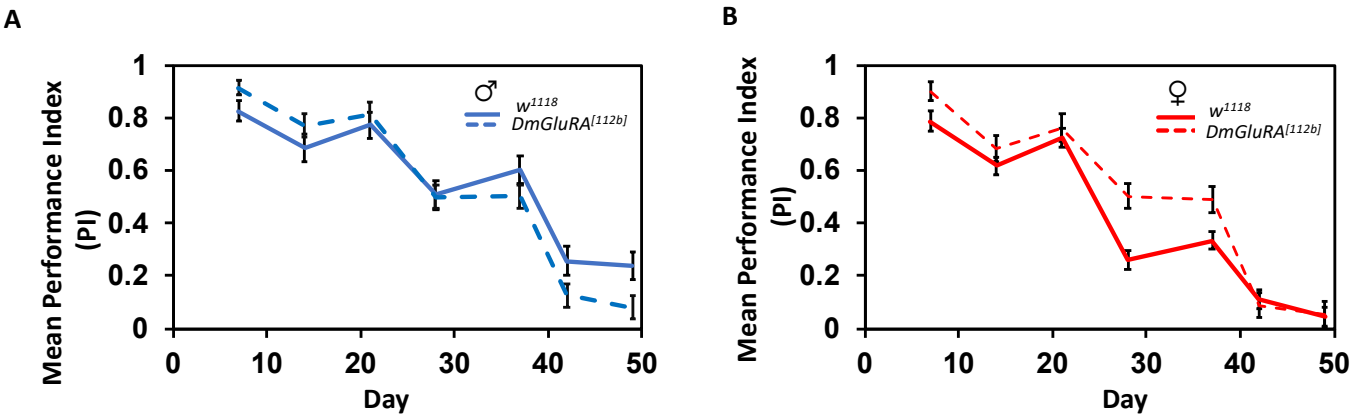
4.2.6 The effects of *DmGluRA*$^{[112b]}$ mutation on age-related functional decline

As flies age they show physiological declines in various tissues including the neuromuscular system and intestinal epithelium system. The aim of this section was to see if the extended lifespan of the mutants was associated with any improvements in age-related physiological parameters.

As the neuromuscular system declines with age, it causes impairments in locomotor activity. Locomotor activity can be measured in flies using the negative geotaxis assay that exploits an innate response displayed by *Drosophila.* When flies are tapped to the bottom of a vertical column they will move against gravity and climb towards the top of the vial in response (Ali et al., 2011). This ability to climb a vertical surface is lost as flies age.

To test whether *DmGluRA*$^{[112b]}$ mutants show improved neuromuscular function as they age, negative geotaxis was examined at multiple time-points over the course of their lifespan and compared to age-matched w^{1118} controls (Figure 4.16). The mean performance index (PI) for both mutants and controls decreased over time in males and females (Figure 4.16)

An age-related decrease in mean PI was observed for both control and *DmGluRA*$^{[112b]}$ mutant flies for both sexes confirming that as these flies aged there was an age-dependent loss of neuromuscular function which impaired their ability to climb upwards. In males, a significant interaction between age and genotype was detected indicative of a difference in the rate of this age-related decline between controls and mutants. However, rather than improving their neuromuscular function, loss of *DmGluRA* expression in male flies accelerated the rate of decline for this behaviour as the flies aged. For female flies, the rate of this age-dependent decline in neuromuscular function was comparable between controls and *DmGluRA*$^{[112b]}$ mutants but mean PI was generally higher for *DmGluRA*$^{[112b]}$ females compared to controls across the time-course of the assay.



Ordinal Regression Model Fit – Effect Tests			
Males		Females	
Age:	<0.0001	Age:	<0.0001
Genotype:	0.4990	Genotype:	0.0277
Age*Genotype:	0.0143	Age*Genotype:	0.4848

Genotype	P-value for each time-point (in days)						
	7	14	21	28	35	42	49
Males: *w*^{1118} *to DmGluRA*$^{[112b]}$	0.06	0.22	0.53	0.93	0.26	0.12	0.12
Females: *w*^{1118} *to DmGluRA*$^{[112b]}$	0.11	0.24	0.46	0.08	0.18	0.53	0.88

Figure 4.16 Age-dependent effects of *DmGluRA*$^{[112b]}$ mutation on locomotion using the climbing/negative geotaxis assay

Age-dependent changes in locomotor function were assessed in *w*^{1118} *DmGluRA*$^{[112b]}$ homozygous mutants and their wildtype controls. Performance Index (PI) for each of the indicated genotypes at each time point was calculated as PI= 0.5´ (n_{total} + n_{top}-n_{bottom})/n_{total} where n_{total} was the total number of flies. Data represent the mean performance index.

(A) *DmGluRA*$^{[112b]}$ female flies compared to *w*^{1118} control (B) *DmGluRA*$^{[112b]}$ male flies compared to *w*^{1118} controls.

Error bars are +/- SEM. Data were analysed using an ordinal logistics regression model looking for age and genotype effects and age*genotype interaction. Post hoc pairwise comparisons were carried out using Student's t-test. Red colour text highlights where there is a significant difference (p<0.05).

.4.2.7 The effect of *DmGluRA* on intestinal barrier function

The intestinal epithelium acts as barrier that permits the entry of nutrients while restricting the diffusion of harmful toxins and pathogens. As flies age, the integrity of the intestinal epithelium breaks down and the intestine becomes 'leaky'. This can be visualised using the Smurf assay, which involves feeding flies with food containing a non-absorbable blue dye (Rera et al., 2012). Flies are referred to as 'Smurfs' when upon ingestion of the blue food, the dye does not remain in the digestive tract but is seen within the hemolymph causing the fly to appear blue. This indicates that the barrier function of the gut epithelium has been compromised allowing the dye to leak through into the haemocoel (Rera et al., 2012).

To examine if *DmGluRA*$^{[112b]}$ mutants showed improved intestinal barrier function as they age, males and females were scored for the presence of Smurfs at three time-points over the course of their lifespan compared to age-matched controls (Figure 4.17).

In the w^{1118} genetic background, the percentage of Smurfs in the population did not increase between day 15 and day 45 of age for either males (Figure 4.17A) or females in both the controls and mutants (Figure 4.17B). Furthermore, no significant differences were observed between *DmGluRA*$^{[112b]}$ mutants when compared to controls for either sex at any of the time points.

In the w^{Dah} genetic background, intestinal barrier function was only examined in females as previous studies have shown that w^{Dah} males do not display the Smurf phenotype (Regan et al. (2016). Unlike in the w^{1118} background, both wildtype and *DmGluRA* mutants showed an increase in the percentage of Smurfs with age (Figure 4.17C, controls: 1.1% at day 15 to 7% on day 45, p=0.0003; *DmGluRA*$^{[112b]}$ mutants: 1.6% at day 15 to 10% at day 45, p=0.0019). However, *DmGluRA*$^{[112b]}$ mutants showed a similar increase as their wildtype controls suggesting that intestinal barrier function is not preserved in these flies.

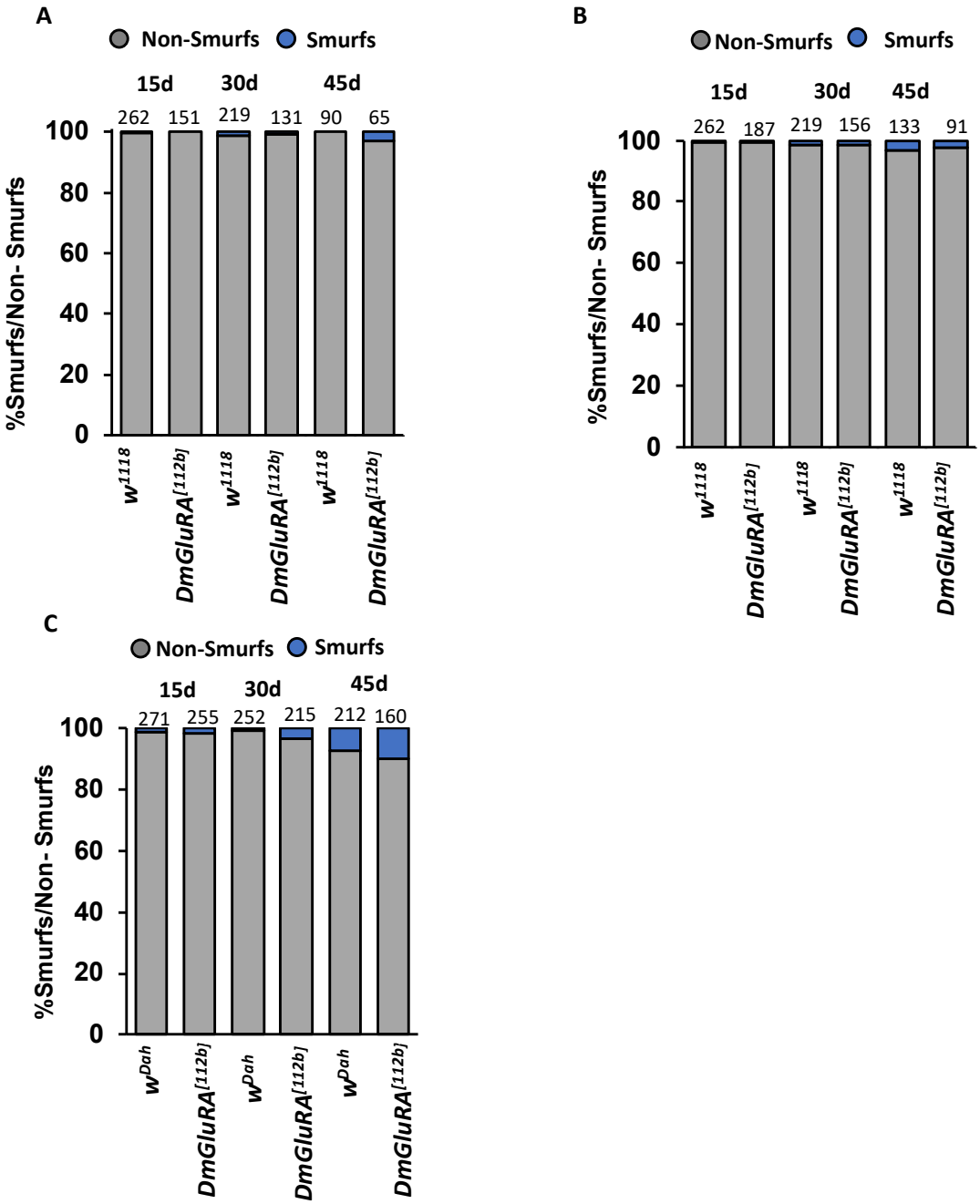


Figure 4.17 Age-dependent effects on increased intestinal barrier permeability measured using the Smurf assay.

Flies were aged under standard conditions (15 flies per vial) and exposed to standard SYA media containing 2.5% blue dye (w/v) for 24 hours prior to scoring for intestinal barrier breakdown by the presence of blue dye within the haemolymph. Data represents the proportion of Smurfs (blue bar) and non-Smurfs (grey column) for (A) w^{1118} males, (B) w^{1118} females or (C) w^{Dah} females. Numbers above the bars indicate the total number of flies observed at that timepoint. No significant differences were observed in the proportion of Smurfs between wildtype controls and *DmGluRA*$^{[112b]}$ mutants at any of the time points (Fisher's exact test).

4.2.8 Summary

Together, the data presented in this chapter shows the phenotypic characterization of *DmGluRA*$^{[112b]}$ mutants in both sexes across different genetic backgrounds. The different phenotypes and the effects that were observed in males and females in the two different genetic backgrounds, w^{Dah} and w^{1118}, have been summarised in Table 4.1.

Phenotype			w^{Dah}		w^{1118}	
			Male	Female	Male	Female
Lifespan	**Standard Food conditions**		Normal	Long-Lived	Long-Lived	Long-Lived
	DR	**0.1x** **0.5x** **1x** **1.5x** **2x**	ND	Normal Long-lived Normal Short-lived Short-lived	ND	ND
Fecundity			ND	Normal	ND	Normal
Growth			Normal body weight Normal wing size	Increased body weight Normal wing size	Increased body weight Normal wing size	Increased body weight Normal wing size
Paraquat Resistant			Resistant	Resistant	Resistant	Resistant
Hydrogen Peroxide Resistant			Resistant	Resistant	Resistant	Normal - increase in only maximum lifespan not median
Starvation Resistant			Resistant	Normal	Resistant	Resistant
Feeding Behaviour			Normal	Normal	Normal	Normal
Climbing Behaviour			ND	ND	Normal	Normal
Intestinal Barrier Function			ND	Normal	Normal	Normal

Table 4.1 Phenotypes of *DmGluRA*$^{[112b]}$ mutants associated with changes in lifespan
Summary of the different phenotypes observed in *DmGluRA*$^{[112b]}$ mutant flies and the effects observed in males and females in two different genetic backgrounds.

4.3 Discussion

This chapter set out to understand the mechanism/s by which loss of mGluR activity in flies leads to longevity. Other genetic manipulations that increase lifespan in *Drosophila* also show phenotypic changes in several characteristic physiological parameters. For example, mutants in the nutrient sensing signalling pathways, such as those that reduce insulin/insulin-like signalling (IIS), exhibit a wide array of phenotypes including reduced developmental growth, reduced fecundity, altered lipid and carbohydrate metabolism and enhanced resistance to stress (Broughton et al., 2005, Clancy et al., 2001, Brogiolo et al., 2001, Giannakou et al., 2004).

Thus, by examining *DmGluRA*$^{[112b]}$ mutant phenotypes and comparing to other long-lived strains, the underlying biological processes that are altered in *DmGluRA* that may contribute to their longevity could be identified. Another major question that this chapter aimed to address, was whether the *DmGluRA*$^{[112b]}$ mutants showed any improvements in age-related physiological decline. This could indicate that loss of DmGluRA function delayed the onset of age-related pathologies thereby promoting longevity. Together, these data would identify the mechanisms by which DmGluRA activity influences biological ageing.

Longevity is often associated with reduced lifetime female fecundity. For example, genetic manipulation of the insulin/insulin-like signalling pathway reduces egg laying in females including *chico* mutants (Clancy et al., 2001), *insulin receptor* (*dInR*) mutants (Tatar et al., 2001) and ablation of the DILP-producing median neurosecretory cells (mNSCs) (Broughton et al., 2005). Overexpression of dFOXO in adult fat body increased female lifespan by 20 to 50% and reduced fecundity of females by 50% whereas no effect was seen on male lifespan (Giannakou et al., 2004). Loss of the GPCR, *methuselah* (*mth*) in flies is an example of other long-lived mutants which show normal egg laying at early stages of the lifespan but reduced egg laying as the flies age (Baldal et al., 2006). The trade-off theory proposes a trade-off relationship between reproduction and lifespan. Where an organism invests its resources into reproductive processes compromising survival, as resources cannot be allocated to all these costly processes at once (Partridge et al., 2005). Alternatively, it suggests that reproduction is in fact itself damaging to females leading to mortality. Increased lifespan could therefore occur by one of these two mechanisms, costing reproduction.

However, reduced egg laying was not observed in the *DmGluRA* mutants at two different time-points suggesting the longevity effects of *DmGluRA* mutation are not attributable to reduced

fecundity. Furthermore, these data suggest that extended lifespan and fecundity can be uncoupled in these mutants. This has been observed previously in other long-lived strains such as flies overexpressing the DNA repair gene, D-GADD45 (*Growth Arrest and DNA Damage-inducible 45*) in the nervous system (Plyusnina et al., 2011), *rpd3*-downregulated flies, a histone deacetylase (Woods et al., 2017), and more recently in long-lived *E(z)* histone methyltransferase heterozygous mutants (Moskalev et al., 2019). Overexpression of *dFOXO* in the adult head fat body is sufficient to extend lifespan without decreasing female fecundity (Hwangbo et al., 2004).

Administration of plant extract, curcumin at doses of 50µm and 100µm extends lifespan in flies but reduced fecundity was not observed at these doses (Lee et al., 2010).
Examples of such an uncoupling between increased lifespan and reduced fecundity also exist in worms – long lived *age-1* mutants are not associated with loss of fecundity (Johnson et al., 1993) and RNAi knockdown of the worm insulin receptor, daf-2 increases lifespan without reducing fecundity(Dillin et al., 2002).

Taken together, these findings demonstrate that the lifespan extension observed in *DmGluRA*$^{[112b]}$ mutants does not represent a trade off with fecundity but that other mechanisms are involved.

The fact that normal egg laying is observed in the *DmGluRA* mutants, suggests that the DmGluRA receptor does not form part of the nutrient sensing network. However, to confirm this, developmental growth was also studied in the mutants. Perturbations in nutrient signalling usually lead to smaller body size (Nijhout, 2003) but in *DmGluRA* mutants, wing size was normal. Wing size is indictive of developmental growth as the final wing size is influenced by nutrient availability during development (Vijendravarma et al., 2011, Gronke et al., 2010).

Interestingly, body weight was increased in those flies that also show longer lifespan - long lived w^{1118} males and females and females in the w^{Dah} background had increased body mass than controls. This increase in body weight could be indicative of behavioural changes in the adult such as increased feeding or decreased locomotion, or could reflect metabolic dysfunction. Loss of function of components of the network sensing network usually produce developmental phenotypes such as reduced body size as well as phenotypes like reduced fecundity (Tatar et al., 2001, Clancy et al., 2001, Slack et al., 2010) and the DmGluRA mutants do not show the

classic canonical phenotypes therefore suggesting that it does not form part of the nutrient sensing network.

Improved tolerance to several different forms of stress is often associated with longevity (Broughton et al., 2005, Lin et al., 1998, Slack et al., 2010, Alic et al., 2011b). For example, flies carrying the *E(z)* histone methyltransferase heterozygous mutation are long lived and exhibit resistance to oxidative stress and starvation (Siebold et al., 2010) and hyperthermia and endoplasmic stress (Moskalev et al., 2019). Also flies artificially selected for increased resistance to stress are long lived (Rose et al., 1992). Furthermore, stress resistance is thought to increase lifespan as manipulation of stress responsive genes can be sufficient to extend lifespan. For instance, overexpression of the heat shock protein genes, *hsp26* and *hsp27,* which are induced in response to multiple forms of stress have been shown to increase lifespan (Wang et al., 2004a). Lifespan extension is not always linked to improved stress resistance, for instance long-lived mutants also show sensitivities to some stresses such as heat and cold shock(Broughton et al., 2005, Emran et al., 2014). As an example, Broughton et al. (2005) found that long lived flies with ablated median neurosecretory cells have increased sensitivity to both heat and cold shock. Also adult flies fed with the petal extract *Rosa damascena* were long lived but these flies showed increased sensitivity to heat stress at 37°C (Schriner et al., 2012).

After backcrossing into the w^{1118} background, long-lived $DmGluRA^{[112b]}$ males and females showed resistance to oxidative stress induced by both paraquat and hydrogen peroxide. However, in the w^{Dah} background, both $DmGluRA^{[112b]}$ mutant males and females showed enhanced resistance to paraquat despite the differential effects of this mutation on male and female lifespan. Furthermore, while $DmGluRA^{[112b]}$ mutant males were also resistant to hydrogen peroxide, long-lived $DmGluRA^{[112b]}$ females were actually sensitive to hydrogen peroxide. These data suggest that resistance to oxidative stress and lifespan can also be uncoupled. Other examples that also show this uncoupling of oxidative stress resistance and longevity have been described. For instance, overexpression of the circadian clock regulated gene, *takeout*, extends *Drosophila* lifespan but these flies do not survive longer upon hydrogen peroxide treatment (Chamseddin et al., 2012). The differences in response to oxidative stress induced by either paraquat or hydrogen peroxide may be due to the fact that these two stressors work by different mechanisms. Interestingly, Girardot et al. (2004) found more genes are differentially expressed in flies upon exposure to paraquat compared to hydrogen peroxide. In addition, some of these gene expression changes were specific to each compound. A further

examination of gene expression changes in *DmGluRA*$^{[112b]}$ mutants may be beneficial to see if any of these genes are differentially expressed in the mutants compared to explain their differential responses to these two compounds.

Differential responses to starvation were also observed depending on genetic background. After backcrossing into w^{1118}, both males and females were starvation resistant. However, in the w^{Dah} background, males but not females showed starvation resistance despite their longevity. Thus, starvation resistance in *DmGluRA*$^{[112b]}$ mutants does not correlate with lifespan extension. Examples of such uncoupling between starvation resistance and lifespan have been previously described. For example, *takeout* overexpressing flies are long-lived but do not display increased resistance to starvation (Chamseddin et al., 2012). Rapamycin-treated mNSC-ablated flies show increased survival under starvation, yet they are not long lived (Bjedov et al., 2010). Similarly, rapamycin improves the stress resistance of flies overexpressing a constitutively active form of the ribosomal protein S6 kinase (S6K), *4E-BP* null mutants and flies with downregulation of the essential autophagy gene, *Atg5*, but lifespan extension was not associated with any of these conditions (Bjedov et al., 2010).

Taken together, the findings presented here using *DmGluRA*$^{[112b]}$ mutants imply that the longevity effects of *DmGluRA* loss-of-function does not represent a general stress resistance response. Similar observations have been reported in other long-lived flies. Overexpression of the human uncoupling protein 2 (hUCP2), a cytoprotective protein, in *Drosophila* neurons, has pro-longevity effects. Yet while these flies are resistant to paraquat stress, they are sensitive to starvation stress (Fridell et al., 2005). Similarly, the administration of Korean red ginseng (KRG) to adult flies was associated with increased longevity and resistance to paraquat-induced oxidative stress but these flies showed decreased survival under starvation conditions (Lee et al., 2019).

Enhanced starvation resistance, as observed in the *DmGluRA* mutants, is often associated with an increase in metabolic stores. Alternatively, enhanced survival under conditions of starvation could reflect differences in the ability of mutant flies to mobilise and/or use their metabolic stores during periods of high demand such as starvation. Therefore, levels of stored fat in the form of TAG and carbohydrates (glycogen and trehalose) were measured in the mutants during a fully fed state and during starvation. Differences in the levels of metabolic stores may also explain the observed differences in body weight.

In the *DmGluRA*$^{[112b]}$ mutants, no differences were observed in the levels of lipid or carbohydrate stores in day 10 fully-fed flies. This suggests that the increased survival during starvation is not consistent with elevated levels of metabolic stores. This also suggests that the differences observed in body weight cannot be attributed to differences in metabolic stores. However, it is worth noting, for the metabolic studies during starvation, 7-day old flies were used. Interestingly in males, a difference was observed in the fully fed state. It is possible this is due to the age of the flies used i.e. 10 days vs 7 days. Wat et al. (2020) show that TAG levels reduce in males over these early days of adulthood and this is dependent on a gene called *brummer* (*bmm*). *bmm* is a adipose triacylglyceride lipase which is the homolog of the human adipocyte triglyceride lipase (ATGL) (Gronke et al., 2005). (Gronke et al., 2005) demonstrated flies that lacking functional *bbm* are obese and also show increased sensitivity under starvation conditions, displaying the critical role in lipid metabolism/TAG mobilisation of *bbm.* This increase in TAG stores in *bmm* mutants is also associated with reduced lifespan but during starvation *bmm* mutants use this increase stored fat and actually are long lived compared to controls due to this (Gronke et al., 2005).

Wat et al. (2020) found that though females start off with more levels of TAG than males, males even one day after eclosion show decreased TAG levels compared to newly eclosed males and 5-day post eclosion males show even lower TAG levels than 1-day post eclosion males. In contrast, females do not show any reductions to TAG levels during these early adulthood days, therefore leading to a sexual dimorphism in TAG storage. The increase in TAG levels observed in fully-fed *DmGluRA*$^{[112b]}$ mutant males at day 7 but not day 10 could be due to these differences in the dynamics of TAG breakdown during early adulthood in males.

In *DmGluRA*$^{[112b]}$ mutants, male flies showed improved survival during starvation in both w^{Dah} and w^{1118} genetic backgrounds while females showed no increase in survival under starvation in the w^{Dah} background and a reduced response to starvation in w^{1118}. In w^{1118} flies, these sex differences in the starvation response were associated with differential effects of starvation on TAG breakdown in the two sexes with *DmGluRA*$^{[112b]}$ mutant males showing higher levels of TAG than controls during 48 hours of starvation while *DmGluRA*$^{[112b]}$ mutant females did not show any differences in TAG levels compared to controls over the same time-course of starvation.

It appears, upon starvation male mutant flies seem to retain their TAG stores for longer but female flies do not show the same response. Based on Wat et al. (2020) it may be that DmGluRA activity may be involved in the sex-specific regulation of *bmm* – so when DmGluRA is removed as in the mutants, the flies are no longer able to breakdown TAG as efficiently in males.

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During starvation, *DmGluRA*$^{[112b]}$ mutant males showed higher levels of TAGs but not glycogen or trehalose during starvation compared to control males. However, in *DmGluRA*$^{[112b]}$ mutant females, the levels of both TAGs and carbohydrates decreased at similar rates to controls during starvation yet they were also starvation resistant. These data suggest that starvation resistance in *DmGluRA*$^{[112b]}$ mutants may be mediated by different mechanisms between males and females. *DmGluRA*$^{[112b]}$ mutant males may retain their lipid-based energy stores for longer allowing them to survive starvation for longer but *DmGluRA*$^{[112b]}$ mutant females may use another mechanism. For instance, reduced activity - *DmGluRA*$^{[112b]}$ mutant females could exhibit reduced locomotor activity during starvation and therefore decrease energy expenditure. During starvation, flies tend to be hyperactive, increasing their locomotor activity, which is thought to be a food-seeking behaviour (Yang et al., 2015). In some genotypes, increased starvation resistance has been attributed to reduced starvation-induced hyperactivity, such as flies with reduced AMP-activated protein kinase (AMPK) function, an important metabolic regulator and nutrient sensor (Braco et al., 2012).

Despite their bigger body weight, *DmGluRA* mutants do not contain elevated levels of fat or carbohydrate stores. However, body size was measured in flies that do not show TAG differences (day 10) therefore this might be reason this does not reflect this. An increase in food intake, rather the mutants may eat more, may account for the increase in body weight. Food intake was therefore measured directly using the CAFÉ assay. No differences in the volume of food consumed during the assay were observed in either male or female *DmGluRA*$^{[112b]}$ mutants compared to controls. Feeding behaviour was also assessed using the proboscis extension (PE) assay. The proportion of time spent feeding has been directly correlated with food intake (Wong et al., 2009). Again, no differences were observed using this behavioural parameter of feeding in either male or female *DmGluRA*$^{[112b]}$ mutants compared to controls. This suggests that increased body weight in *DmGluRA*$^{[112b]}$ mutant flies does not occur as a result of increased food intake.

Dietary restriction (DR) refers to the reduction of food intake without malnutrition, implemented in *Drosophila*, through the dilution of the protein component of diet whilst

maintaining a constant sugar concentration (Bass et al., 2007). The normal response of lifespan to DR is tent-shaped, with lifespan peaking at an intermediate food concentration and decreasing due to starvation at lower food concentrations and also decreasing at higher concentrations but with a concomitant increase in egg laying (Piper and Partridge, 2007).

Dietary restriction was previously thought to be caused by reduction in calorie intake but this has since been disputed (Mair et al., 2005). Some studies have suggested that DR is mediated by IIS (Clancy et al., 2002), but there is now evidence that DR can occur in the absence of dFOXO, a key downstream transcriptional effector of IIS (Giannakou et al., 2004) although their findings still suggest a role for dFOXO in the normal response to DR. Despite the volume of work on DR, the mechanisms by which DR is thought to occur and affect lifespan still remain poorly understood. To see if there was an interaction between *DmGluRA*-induced longevity and DR, the survival of *DmGluRA*$^{[112b]}$ mutants was tested on various yeast concentrations.

Both *DmGluRA* mutants and controls responded normally to DR with respect to egg laying. Hence, both mutants and controls proportionally increased their egg laying as yeast concentration increased. However, differential responses on lifespan were observed across different yeast concentrations. For control flies, lifespan peaked at 1x SYA concentration whereas for *DmGluRA*$^{[112b]}$ mutants, the DR tent was left-shifted with median lifespan peaking at 0.5x SYA. From these findings, it is unlikely that DmGluRA is involved in the normal DR response as the *DmGluRA*$^{[112b]}$ mutants still respond to DR, with respect to both egg laying and survival. These findings do show, however, that the lifespan response of *DmGluRA*$^{[112b]}$ mutants is influenced by the nutritional environment. It should be noted that in the DR experiment presented here, no lifespan extension was observed at 1x SYA as has been previously presented in Chapter 3. This difference may be attributed to these nutritional effects on DmGluRA-mediated lifespan extension and may reflect differential nutritional compositions of the yeast batch used in these two different sets of experiments. This variable effect on lifespan under standard and DR conditions could be tested using a holidic diet rather than a standard diet. A holidic diet is a chemically defined diet where the exact composition and concentration of every ingredient is known(Piper et al., 2014). Therefore it is possible to control the nutrient content of the diet i.e. the protein concentration and assess the lifespan effects. The holidic diet yields more consistent lifespan and egg-laying between different experiments compared to standard SY diet (Piper et al., 2014). A holidic diet could also be used to confirm the left-shift of DR, by

diluting the amino acids in the holidic medium. Piper et al. (2014) shown dilution of amino acids increases lifespan similar to dietary restriction on standard media.

This shift of the DR lifespan response with median lifespan peaking at lower yeast concentrations is unusual and there are no obvious examples in the literature. Genetic and environmental manipulations that are thought to function as DR mimetics, that is extend lifespan via similar mechanism to DR, usually show a right-shifted survival response in the DR tent, as observed in *chico*^{1} mutants (Clancy et al., 2002). *Chico*^{1} mutants were longer lived than controls on normal yeast concentration and under DR they show a similar peak as controls but their maximum is different (i.e. right shifted controls peak at 0.65x whereas *Chico*^{1} mutants peak at 0.8x). *Chico*^{1} mutants were short lived on low yeast concentrations compared to controls and were long-lived on normal and high yeast concentrations. Thus, suggesting there is an overlap between the two pathways that lead to lifespan extension in *chico* mutants and DR flies as chico^{1} flies act as if they were already partially dietary-restricted. Nevertheless, from the findings presented in this chapter, it would appear that DmGluRA does not contribute to the DR response.

Drosophila exhibit complex behaviours, many of which undergo age-related decline. Healthspan is the ability of longevity-inducing interventions to improve age-related physiological function. Loss of locomotor function and loss of intestinal epithelium integrity, two outputs indicative of age-related pathology, were tested.

Negative geotaxis is an innate behaviour in which flies climb a vertical surface when startled. This is tested by this calculating the performance index or PI. Performance index (PI) = 0.5´ $(n_{total} + n_{top}-n_{bottom})/n_{total}$ where n_{total} was the total number of flies, n_{top}, the total number of flies at top and n_{bottom} was the total number of flies at bottom.

For male flies, loss of *DmGluRA* expression did not delay or reduce this decline in climbing ability. In fact, despite their increased lifespan, *DmGluRA* mutant males showed a faster decline than controls. In contrast, climbing ability in long-lived *DmGluRA* mutant females was generally better than controls but the rate at which this behaviour declines with age was comparable to control flies suggesting that loss of DmGluRA activity in females does not prevent the age-related loss of neuromuscular function but these flies generally perform better than controls in this assay.

In the w^{1118} background- both males and females – mutants and controls show reduction in climbing behaviour with age. For males, there is a difference in the rate of this age-related decline- unexpectedly, the rate of decline is actually slower in controls than in mutants. In females, no difference in the rate of this age-related decline but the mutant females actually have a higher PI across agespan compared to the controls.

Long-lived models tend to be associated with improvements in negative geotaxis, for instance, long-lived flies with ubiquitous IIS reduction demonstrated improvements in negative geotaxis behaviour in both males and females (Ismail et al., 2015) . Also, ubiquitous and tissue specific (intestines and brain) downregulation of S-adenosyl-homocysteine (AHCY) -like protein 1 (*dAchyL1)* or AHCY-like protein 2 in male and female adult by RNAi extends *Drosophila* lifespan (Parkhitko et al., 2016). The two genes are components of the Methionine pathway. When negative geotaxis were tested in 40-day old flies, it was found that these RNAi lines were significantly better at climbing, indicating this lifespan extension supresses the age-related decrease in negative geotaxis (Parkhitko et al., 2016).

The Smurf assay was used to determine intestinal integrity during ageing in *DmGluRA* mutants. This assay detects intestinal permeability *in vivo*, using a non-absorbable blue dye.
The intestinal epithelial barrier breaks down with age and the intestine becomes 'leaky' allowing the ingested blue dye to leak into the hemolymph producing 'Smurfs'. This age-related disruption to intestinal structure and function has been linked to over-proliferation of the intestinal stem cells (ISCs) (Regan et al., 2016, Biteau et al., 2010). Most of the somatic cells within the adult fly are post mitotic, but a small population of mitotically active ISCs reside within the intestinal epithelium where they play an important role in epithelial regeneration (Lucchetta and Ohlstein, 2012). The proliferation of ISCs must be strictly controlled to ensure proliferative homeostasis and prevent hyper-proliferative diseases including cancer (Biteau et al., 2008). Previous studies have shown that the number of mitotically active ISCs within the intestinal epithelium increases with age in *Drosophila* and that this is associated with morphological changes in the structure of the epithelial layer (Biteau et al., 2010, Regan et al., 2016). Several interventions that increase lifespan have been shown to reduce ISC proliferation such as flies overexpressing *Drosophila* PGC-1 (Peroxisome proliferator-activated receptor gamma coactivator 1) homolog, dPGC-1/spargel (Rera et al., 2011) and Rapamycin-treated flies

(Schinaman et al., 2019) . Also, long-lived *Indy* mutant flies show an increase in dPGC-1 activity leading to an improvement in intestinal integrity in old age (Rogers and Rogina, 2014).

Age related intestinal integrity in both control and *DmGluRA* mutant flies was therefore examined, to see whether the lifespan extension of the $DmGluRA^{[112b]}$ mutants was associated with improvements in intestinal barrier function. Unlike previously reported, experiments conducted in the w^{1118} background did not show an increase in the proportion of Smurfs with age and no differences were observed between $DmGluRA^{[112b]}$ mutants and controls for either sex. Age-related decline in intestinal barrier function was observed in w^{Dah} animals but again, mutation of did not ameliorate this effect, suggesting that $DmGluRA^{[112b]}$ mutation extends lifespan through an alternative mechanism than improvements in intestinal physiology.

Not all lifespan-extending interventions ameliorate the age-related decline in intestinal barrier function. For example, flies treated with trametinib, a highly specific inhibitor of the MEK kinase, are long-lived but show no obvious improvements in gut health with age (Slack et al., 2015). However, the lack of effects on intestinal physiology in $DmGluRA^{[112b]}$ mutants was somewhat surprising as a role for *DmGluRA* in ISC proliferation and gut growth has previously been described (Deng et al., 2015). Dietary glutamate, the ligand of *DmGluRA*, has a positive effect on both ISC proliferation and intestinal epithelial growth. Deng et al. (2015) reported from 4 hours post-feeding, with food supplemented with L-Glutamate (L-Glu), that the gut increased in size, specifically both the anterior and posterior parts. Furthermore, this positive effect of dietary L-Glu is dependent on *DmGluRA* as this response was not seen when flies with ISC-specific knock down of *DmGluRA* and homozygous $DmGluRA^{[112b]}$ mutants were fed L-Glu. However, despite this previous link to ISC proliferation, loss of DmGluRA did not appear to improve intestinal barrier function with age.

That said, intestinal barrier function was not assessed in males in the w^{Dah} strain. Although previous studies reported no Smurfs were produced by w^{Dah} males even at oldest age examined (i.e. 80 days old) (Regan et al., 2016), it would be interesting to know whether this is also the true for the *DmGluRA* mutants and if intestinal barrier function is still intact during old age.

In this chapter, in an attempt to understand the mechanisms by which *DmGluRA* mutation extends lifespan, the physiological and metabolic phenotypes of these mutants have been described. Several different phenotypes have been characterised in the *DmGluRA* mutants – the

physiological effects of this mutation are obviously complex and depend in some respect on genetic background, sex and diet. The only phenotype that fully correlated with the lifespan extension was increased body weight which was increased in long-lived flies.

So as yet the mechanisms for the lifespan extension remain unknown – in the next chapter (chapter 5) the potential mechanisms will be explored using a different approach – by examining the transcriptional responses to DmGluRA loss-of function.

Chapter 5 : Transcriptional responses to *DmGluRA* loss-of-function

5.1 Introduction

The previous chapter has given further insight into the physiological functions of DmGluRA – as described earlier, it was found that increased body weight (but not wing area) and increased resistance to oxidative stress and starvation (background-dependent) were characteristics of long-lived *DmGluRA*$^{[112b]}$ flies. However, although these phenotypes were found to be associated with loss of DmGluRA activity, many of the examined phenotypes did not correlate with the lifespan effects. Therefore, the underlying mechanisms by which the *DmGluRA*$^{[112b]}$ mutation induces longevity were still not entirely obvious. In this chapter, another approach was taken to try and identify these mechanisms, turning to RNA-sequencing (RNA-seq) to see if the transcriptional response to loss of DmGluRA activity can shed some light as to why *DmGluRA* mutants are long-lived.

As a GPCR, DmGluRA can signal through several downstream signalling pathways due to its ability to couple to different G proteins (Parmentier et al., 1996a). For example, DmGluRA signals via PI3K/AKT by activating calcium/calmodulin-dependent protein kinase II (CaMKII) and *Drosophila* focal adhesion kinase (dFak) (Chun-Jen Lin et al., 2011). It was subsequently found that this DmGluRA-dependent PI3K activation is dependent on the small GTPase, Ras (Johnson et al., 2012). Downstream of Ras activation, the RAS/MAPK signalling pathway is activated as Ras activates Raf. DmGluRA has also been linked to RAS/MAPK signalling through its activation of G proteins and it is possible that the G protein beta and gamma subunits can lead to Ras activation thereby activating RAS/MAPK signalling (Ito et al., 1995). Both PI3K/AKT and RAS/MAPK pathways lead to the modulation of transcription factors such as dFOXO and AOP, and so can in turn affect the transcription of the genes they regulate. Therefore, it may be possible that loss of mGluR signalling has an impact on gene expression which may underlie the phenotypes observed in the *DmGluRA*$^{[112b]}$ mutants and ultimately affect lifespan.

The aim of this chapter was to therefore examine the transcriptional response to *DmGluRA* loss-of-function in order to elucidate the molecular mechanisms by which loss of DmGluRA activity extends lifespan. To identify changes in expression levels, RNA-seq was performed to compare the transcriptome of *DmGluRA* mutants with the wildtype control.

RNA-seq is a high throughput next-generation sequencing (NGS) technology in which isolated RNA is converted into cDNA by reverse transcription. The cDNA is then fragmented and used to produce a cDNA library. Adaptors are added to both ends of the cDNA fragments which are then sequenced to produce either single reads or paired-end reads. Through NGS, millions of short reads are produced and the reads are then aligned to a reference genome (Wang et al., 2009b). By alignment to the genome, the genomic origins of the reads can be determined and gene expression can then be inferred by determining the relative amount of reads associated with a particular gene locus.

Previous studies have already successfully taken this approach of looking at transcriptional responses in long-lived mutants in order to elucidate the mechanisms for the lifespan extension. Moskalev et al. (2019) explored the mechanisms of lifespan extension of *E(z)* mutants using genome-wide transcriptome analysis. Heterozygous mutation of *E(z)*, a histone methyltransferase, leads to longevity in both males and females and is associated with other phenotypes such as reduced fecundity and increased tolerance to oxidative stress and starvation. Moskalev et al. (2019) found that the genes which showed expression changes were genes involved in metabolism including carbohydrate, lipid, drug and nucleotide metabolism. They also found that though this mutation does not cause sex-specific effects on lifespan, they observed sex-specific effects on gene expression.

Soule et al. (2020) measured the changes in the transcriptome within the intestines of *jouvence* (*jou*)-deleted flies. *Jou* is a small nucleolar RNA, and its mutation shortens lifespan. They found many genes changed in expression, particularly showing down-regulation of their expression. Interestingly, through their analysis, they were able to identify two genes *ninD* (an upregulated gene) and *CG6296* (a downregulated gene) of which when the mRNA level is decreased or increased respectively, restores normal lifespan to these mutants.

Most recently, RNA-seq was used to determine the mechanism by which glucose-enriched food (GEF) extends lifespan in *Drosophila* (Galenza and Foley, 2021). By comparing gene expression between flies fed GEF and flies maintained on a chemically-defined holidic media, they uncovered differential expression across many genes, amongst which they found an upregulation in genes involved in cell adhesion. This led Galenza and Foley (2021) to study intestinal epithelial barrier function in GEF flies and they discovered that is likely GEF extends lifespan by improving intestinal epithelial barrier integrity.

In this chapter, the differential gene expression in *DmGluRA* mutants was examined for specific gene expression changes that could be associated with the phenotypes described in Chapter 4. Increased resistance to oxidative stress was one of the robust phenotypes observed in *DmGluRA* mutants. Therefore, genes associated with increased tolerance to oxidative stress were specifically looked at. Previous studies have identified gene expression changes in stress responsive genes such as *catalase* and *superoxide dismutase (SOD)* in several long-lived mutants which display enhanced resistance to oxidative stress (Clancy et al., 2002, Kabil et al., 2007, Chattopadhyay and Thirumurugan, 2020). Soule et al. (2020) found that short-lived *jou*-deleted flies showed a decrease in stress resistance. They found that several genes that were downregulated by loss of *jou* were involved in stress resistance, which could explain the sensitivity of these mutants to stress . Moskalev et al. (2019) also reported for long-lived *E(z)* mutants changes in gene expression for genes encoding antimicrobial peptides and genes belonging to the *Turandot* family, that have been linked to stress resistance and ageing. These gene expression changes correlated with the enhanced stress resistance seen in these mutants. Therefore, a particular focus of the transcriptomic analysis described in this chapter, was to look for changes in the expression of stress responsive genes which could explain this phenotype in *DmGluRA* mutants.

Such high-throughput transcriptional profiling often produces very large amounts of data which can be difficult to interpret or assign biological relevance to at the gene-by-gene level. Here, Gene Ontology (GO) enrichment analysis of the differentially expressed genes identified from the RNA-seq analysis was applied. This analysis uses GO terms to assign functional significance within a list of differentially expressed genes compared to all genes detected in the RNA-seq data set. It essentially groups the differentially expressed genes (DEGs) based on their function – so the biological processes and cellular components with which they are associated, and their molecular function, as assigned by GO terms. This approach therefore revealed a list of GO terms that are associated with the genes that show altered expression in the *DmGluRA* mutants.

Similar GO analysis was successfully employed in the study cited previously by Galenza and Foley (2021) who found a large number of genes (1043) were differentially expressed upon feeding flies with glucose-enriched food. GO analysis of the upregulated genes revealed GO terms associated with cell-cell junctions including cell-cell junction assembly. As cell-cell junctions are important in the maintenance of the intestinal epithelium (Rera et al., 2012), this prompted the authors to test whether the intestinal epithelium may play a role in the lifespan extension of flies fed with a glucose-enriched diet.

Similarly, GO analysis of the differentially expressed gene in *E(z)* mutant flies revealed a reduction in the expression of the majority of the genes related to the immune response. Normal ageing is normally associated with upregulation of immune response genes and so this finding was an important contribution to the authors understanding of how *E(z)* mutation results in longevity (Moskalev et al. (2019)).

One of the most significantly enriched GO terms identified within the DEGs associated with *DmGluRA* mutation was ribosomal RNA (rRNA) processing. This was of particular interest because studies have shown that the nucleolus, the organelle within the nucleus which plays an essential role in rRNA transcription and processing and ribosome biogenesis, has been linked to ageing (Tiku et al., 2017, Lam et al., 2005). For example, Larson et al. (2012) reported loss of heterochromatin in *Drosophila* results in an increase in rRNA transcription. Conversely, they found that conditions that increase heterochromatin such as heterochromatin protein 1 (HP1) overexpression - a component of heterochromatin that controls heterochromatin levels - leads to a reduction in rRNA transcription. Therefore, suggesting rRNA synthesis is controlled by heterochromatin. They also described a role for heterochromatin in nucleolar stability, since loss of heterochromatin is associated with nucleolar instability and increased heterochromatin preserves the structure of the nucleolus. Moreover, they reported that there is a decline of heterochromatin during normal ageing, and that increasing heterochromatin levels results in increased lifespan .

Various long-lived interventions such as DR or rapamycin-treatment are associated with small nucleoli and decreased rRNA synthesis in *Drosophila, C. elegans* and mice (Tiku et al., 2017). Furthermore, inhibition of RNA polymerase I (Pol I), which transcribes ribosomal RNAs in the nucleolus thereby initiating ribosome biogenesis, extends lifespan (Martinez Corrales et al., 2020). Also, reduction of Myc expression, which regulates ribosome biogenesis, has been shown to increase lifespan (Greer et al., 2013). Mounting evidence suggests that the role of the nucleolus in ageing is also linked to the key lifespan regulator, mTOR. For example, mTOR activates transcription initiation factor-1A (TIF-IA) which promotes rRNA synthesis (Grewal et al., 2007).

nucleolar function seems to play an important role in ageing, any differences in these markers of nucleolar function in *DmGluRA*$^{[112b]}$ mutants could explain the lifespan effects of this mutation.

5.2 Results

5.2.1 Analysis of differential gene expression by RNA sequencing (RNA-seq)

To identify gene expression changes in *DmGluRA* loss of function mutants, RNA-seq was performed on RNA isolated from whole 10-day old adult *DmGluRA*$^{[112b]}$ mutant females and w^{Dah} control flies. Results were considered statistically significant at an adjusted p-value < 0.05 which identified 2194 genes with expression level changes that were associated with the *DmGluRA* mutation (Figure 5.1). Of these, 944 genes showed a significant increase in expression while 1250 showed a significant decrease in expression.

The top 10 upregulated genes in *DmGluRA* mutants were *CG3841, CG31233, dpr6, IA-2, CNT2, CG13868, CG17597, Mal-A1, Cyp6d4* and *Uxs*. The top 10 downregulated genes were *Adh*, *thetaTry*, CG5550, CG5724, CG1213, CG2064, CG43680, *Mal-A4, Npc2f* and *Damm* (≥1.5 Fold change, FDR <0.05), Figure 5.1). Notably, *DmGluRA* itself was amongst the significantly downregulated genes (Fold change -1, adjusted p-value<0.05).

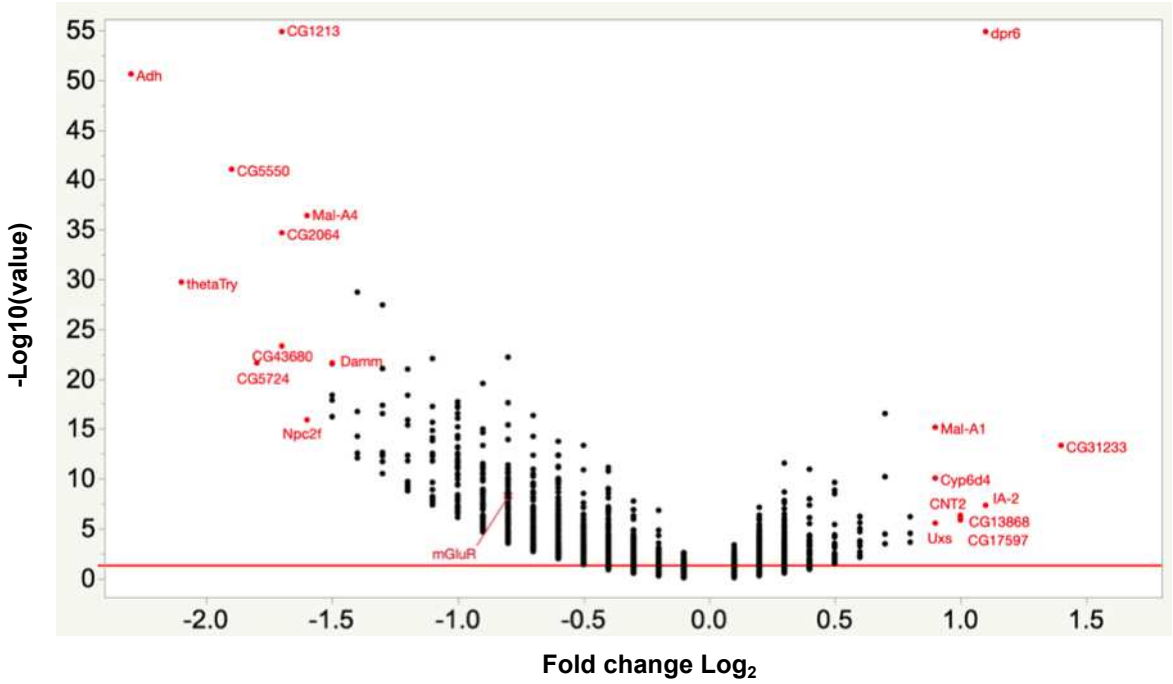

Figure 5.1 Volcano plot representation of differential expression analysis in *DmGluRA*$^{[112b]}$ mutants compared to controls.

Each dot represents an individual gene plotted as -Log_{10}(p-value) as a function of Log_2 fold-change. Positive fold-change values represent genes that were upregulated (n=944) and negative values represent genes that were downregulated (n=1250). The top 10 significantly downregulated (left) and upregulated genes (right) are highlighted in red with their gene names indicated. *DmGluRA* is also highlighted in red and its position indicated. Horizontal red line denotes significance level of the adjusted p-value threshold (-log (0.05). Genes below this line were classified as non-significant.

To validate the gene expression changes identified in the RNA-seq data, qRT-PCR was performed using RNA isolated from an independent cohort of flies. Three genes were selected for qRT-PCR expression analysis: two upregulated genes, *TotC* (fold change: 0.4) and *hsp83* (fold change: 0.1) and one downregulated gene, *mGluR* (fold change: -1). qRT-qPCR analysis confirmed upregulation of *TotC* (p=0.0264) and downregulation of *mGluR* (p=0.0031) in *DmGluRA*$^{[112b]}$ mutants but *hsp83* did not show altered expression (p=0.8659) (Figure 5.2). Thus, two of the three genes analysed by qRT-PCR showed altered gene expression, consistent with the expression changes detected by RNA-seq.

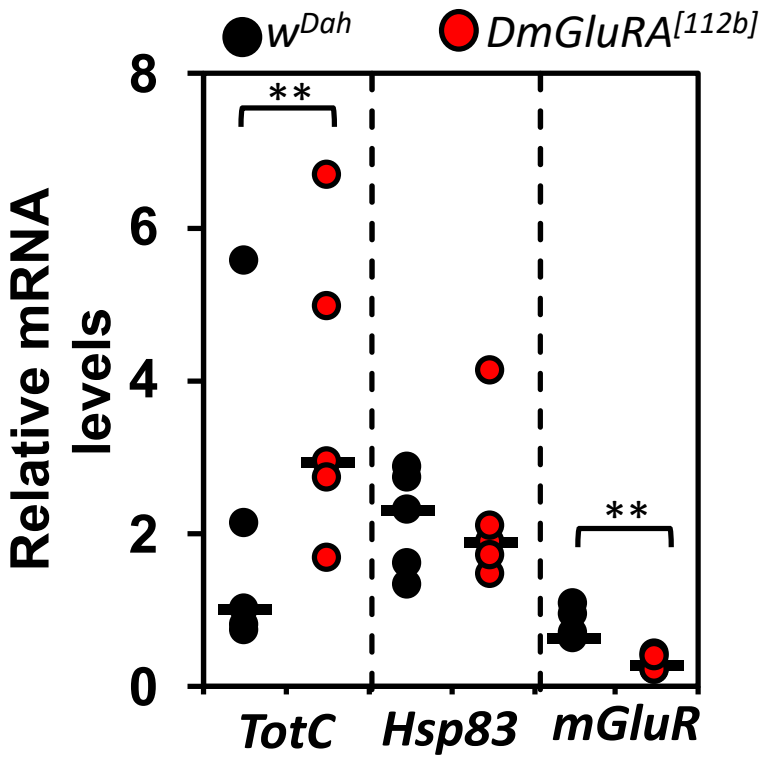

Figure 5.2 qRT-PCR analysis of *TotC*, *Hsp83* and *mGluR* expression in *DmGluRA*$^{[112b]}$ mutants. mRNA expression of *TotC*, *Hsp83* and *mGluR* were analysed by qRT-PCR and normalised to expression of *actin5C* in 10-day old female flies of the indicated genotypes. Data represent 5 biological replicates. Bar represent means ** indicates p<0.05, Student T-test.

The expression and/or activity of several proteins have been previously described to change in response to DmGluRA activity. For example, the expression of dFMRP (*Drosophila* fragile X mental retardation protein) which is encoded by *FMR1* increases in *DmGluRA* mutants (Pan et al., 2008). Also, PI3K is known to be activated by glutamate-dependent activation of DmGluRA via dFak and CaMKII activity although no changes in dFak/CaMKII protein expression were observed (Chun-Jen Lin et al., 2011). DmGluRA in neurons inhibits the activity of the adenyl cyclase *rutabaga* (*rut*) at the cell membrane leading to decreased cyclic AMP (cAMP) levels (Kanellopoulos et al., 2012).

As such, the expression of the genes encoding these key proteins that are influenced by DmGluRA activity were examined within the RNA-seq data. However, *PI3K, CAMKII* and *Fak* did not show any changes in expression, and while *rut* was significantly differentially expressed, *rut* expression was decreased compared to controls (-0.4 fold, adjusted p- value<0.05) which was in the opposite direction to what might be expected based on the reported changes in adenyl cyclase activity.

5.2.2 Analysis of oxidative stress responsive gene expression in *DmGluRA*$^{[112b]}$ mutants

Earlier in this study, novel phenotypic responses to *DmGluRA* loss-of-function including enhanced resistance to oxidative stress were identified. Protection against oxidative stress has been shown to be achieved by increased expression of genes encoding proteins such as SOD and catalase (Clancy et al., 2002, Mockett et al., 2003, Kabil et al., 2007). For example, increased *SOD* (specifically *SOD1/Manganese Superoxide Dismutase* (*Mn-SOD*)) or *catalase* expression was associated with increased resistance to oxidative stress and increased lifespan in flies treated with the flavonol glycoside, Rutin (Chattopadhyay and Thirumurugan, 2020). Therefore, the DEGs identified by RNA-seq analysis in the *DmGluRA* mutants were examined for gene expression changes that could be associated with increased resistance to oxidative stress.

Although *SOD1/Mn-SOD* and *catalase* did not show any changes in expression in *DmGluRA* mutants, other oxidative stress responsive genes were found to show altered expression (Table 5.1). The following stress-related genes were upregulated: DJ-1β, an antioxidant gene (+0.2 fold), heat shock protein-encoding genes *hsp83* and *hsp26* (+0.1 fold) and the MAP kinase kinase kinase (MAPKKK), *MEKK1* (-0.2 fold). Among the stress-related genes significantly downregulated were *CG6006* and *CG16727* (-0.4 fold).

Oxidative stress response genes	Log_2 fold change	P_{adj}
DJ-*1β*	0.2	0.006070532
HSP83	0.1	0.008304746
HSP26	0.1	0.013578192
MEKK1	-0.2	0.037027052
CG6006	-0.4	0.010649173
CG16727	-0.4	0.0005093

Table 5.1 Oxidative stress responsive genes with altered expression in *DmGluRA*$^{[112b]}$ mutants.
Table of oxidative stress responsive genes which are significantly increased or decreased in the *DmGluRA*$^{[112b]}$ mutants. Red shading represents genes that are upregulated and blue represents downregulated gene. The Log_2 fold change and adjusted p-value have been included.

5.2.3 Transcriptional changes in components of PI3K/AKT and RAS/MAPK signalling in *DmGluRA*$^{[112b]}$ mutants

DmGluRA has been shown to interact with both PI3K/AKT and RAS/MAPK signalling (Chun-Jen Lin et al., 2011, Johnson et al., 2012). These pathways were of particular interest as they are both known to influence ageing downstream of IIS (Slack et al., 2015). The RNA-seq data was therefore examined for any transcriptional changes in key signalling molecules of IIS pathway. However, many of the key IIS molecules did not show changes in gene expression (Figure 5.3) although significant changes in expression were seen for *dInR*, *Ras* and *Raf* (Table 5.2). Lastly, there was also increased expression of the mTOR pathway gene, *dTsc1*, encoding part of tuberculosis complex (Tsc1/Tsc2) which acts to inhibit TOR (Table 5.2).

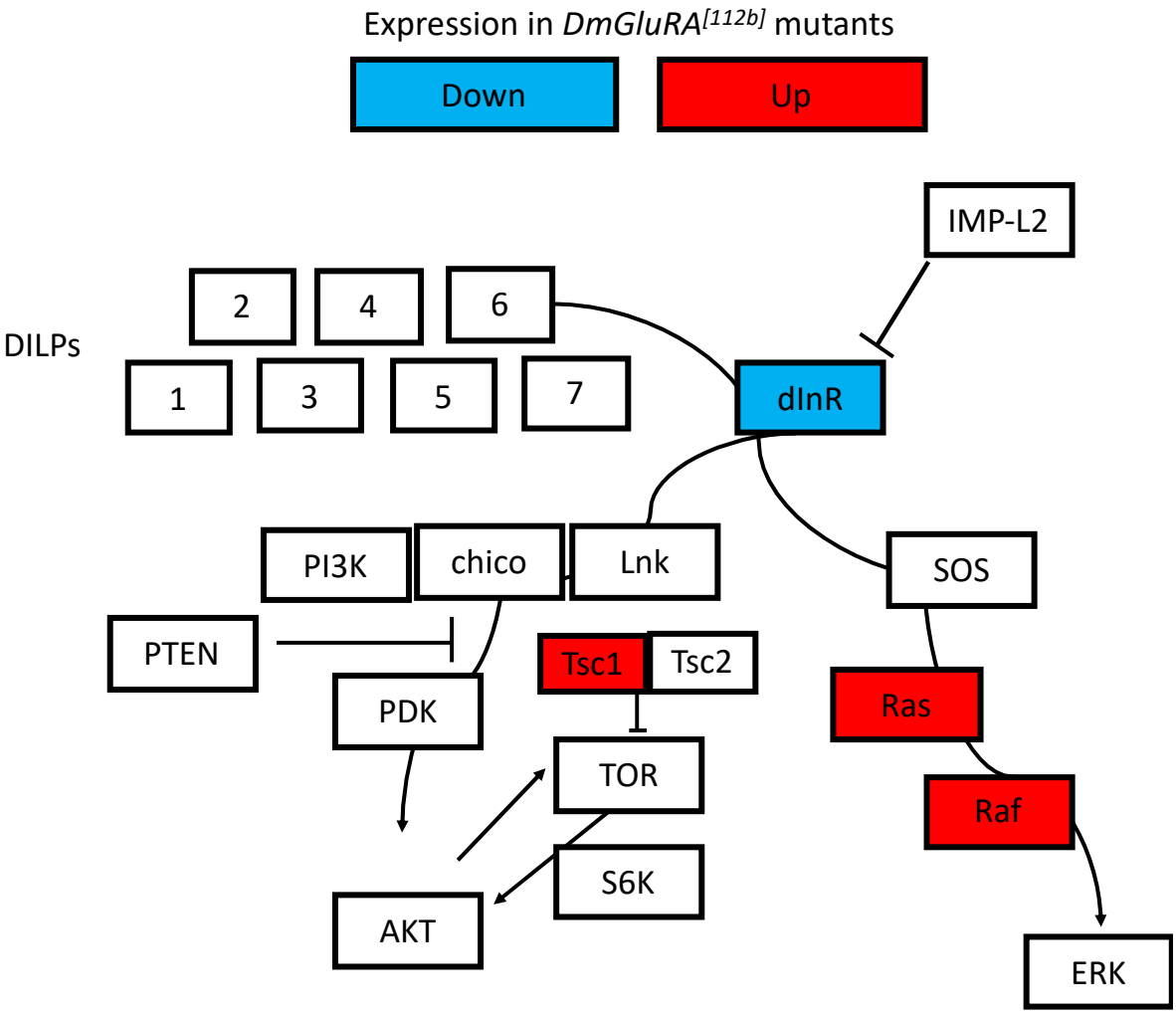

Figure 5.3 Schematic of IIS signalling and gene expression changes in *DmGluRA*[112b] mutants. Altered transcript levels in *DmGluRA* mutant flies were mapped onto a schematic of IIS. Genes showing downregulated expression in the *DmGluRA* mutants are blue in colour and red colour represents genes which show upregulation in these mutants. White filled boxes show genes unchanged in *DmGluRA* mutants.

Key IIS molecules	Log_2 fold change	P_{adj}
Raf	0.1	0.031042282
Ras	0.1	0.018115351
dTsc1	0.1	0.02831966
dInR	-0.2	0.013578192

Table 5.2 Key IIS components that show altered expression in *DmGluRA*$^{[112b]}$ mutants.
Table of key molecules of IIS signalling pathway which are significantly increased or decreased in the *DmGluRA*$^{[112b]}$ mutants. Red shading represents genes that are upregulated and blue represents downregulated gene. The Log_2 fold change and adjusted p-value have been included.

Two key transcription factors, FOXO and AOP, act downstream of reduced IIS to mediate lifespan responses (Slack et al., 2015, Alic et al., 2014). As these transcription factors have been implicated in lifespan regulation, the DEGs were examined for changes in these transcription factors but neither showed any changes in expression in *DmGluRA* mutants. However, it was important to also examine whether key target genes that are directly bound and regulated by FOXO or AOP show changes in expression. The DEGs from *DmGluRA* mutants was therefore compared to a previously published dataset in which 358 direct targets of FOXO were identified (Alic et al., 2014) . Of these 358 direct FOXO target genes, 57 genes were also differentially expressed in *DmGluRA* mutants representing a statistically significant overlap (p=0.0376; Figure 5.4A). Genes were then grouped according to the direction of change in their expression in response to FOXO binding or *DmGluRA* mutation. Thus, of the 84 FOXO target genes that that increase in expression in response to FOXO activation, 13 genes were also upregulated in the *DmGluRA* mutants representing a significant overlap ($p=8.06x10^{-3}$, Figure 5.4B).

The representation factor or R factor is based on the hypogeometric distribution and represents the number of overlapping genes divided by the expected number of overlapping genes if drawn from two independent groups. A representation factor > 1 indicates that there is more overlap than would be expected between two independent groups of genes while a representation factor < 1 indicates that there is less overlap than would be expected. A representation factor equal to 1 indicates that overlap between two groups of genes is what would be expected if the two groups are independently regulated. The representation factor of the overlap for the

upregulated genes was calculated to be 2.26 suggesting that the two groups of upregulated genes may be regulated by similar mechanisms.

Similar comparisons were performed for downregulated genes. But of the 256 FOXO target genes that decrease in expression in response to FOXO activation, only 3 genes were also downregulated in the *DmGluRA* mutants. The calculated representation factor of 0.145 suggests that there is no significant overlap between the two groups.

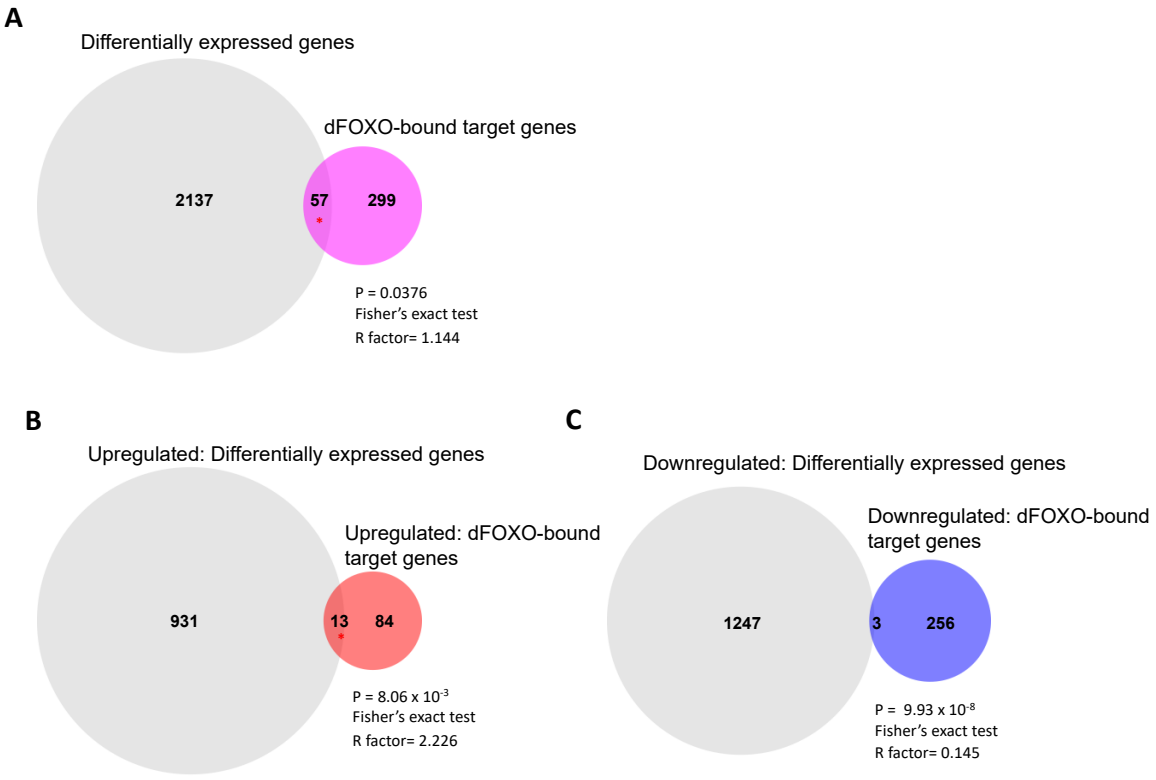

Figure 5.4 Overlap between FOXO target genes and DEGs associated with *DmGluRA* mutation.

Proportional Venn diagrams displaying the overlap of genes differentially expressed in *DmGluRA*$^{[112b]}$ mutants with direct FOXO target genes using (A) all *DmGluRA* DEGs with all 358 direct FOXO target genes, (B) genes upregulated in *DmGluRA* mutants with FOXO target genes that are upregulated in FOXO mutants and (C) genes downregulated in *DmGluRA* mutants with FOXO target genes that are downregulated in FOXO mutants. Red asterisks (*) denotes a statistically significant overlap with the calculated p-values (All DEGs: p=0.0376, upregulated: 8.06x10^{-3}, Fisher's exact test) and representation (R) factors. Venn diagrams were generated using BioVenn (Hulsen et al., 2008).

The DEGs from *DmGluRA* mutants was were also compared to a published dataset of 2828 genes identified as direct target genes of AOP by chromatin immunoprecipitation (ChIP) (Alic et al., 2014) Of these 2858 direct AOP target genes, 551 genes were also differentially expressed in *DmGluRA* mutants representing a statistically significant overlap (p=1.11x10^{-10}; Figure 5.5).

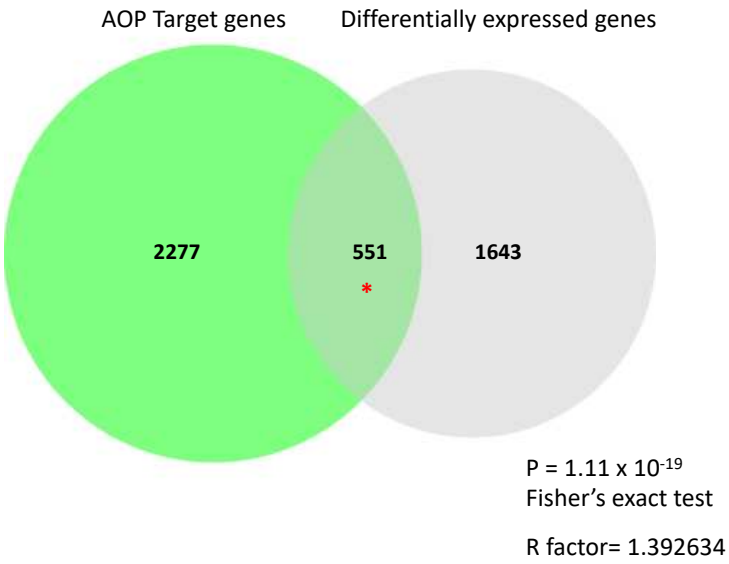

Figure 5.5 Overlap between AOP target genes and DEGs in *DmGluRA* mutants.
Proportional Venn diagrams displaying the overlap of genes differentially expressed in *DmGluRA*$^{[112b]}$ mutants with direct AOP target genes using all *DmGluRA* DEGs with all 2828 direct AOP target genes. Red asterisks (*) denotes a statistically significant overlap with the calculated p-values (p=1.1 x 10^{-}19; Fisher's exact test) and representation (R) factor >1 indicating more overlap than expected between the two independent groups. Venn diagrams were generated using BioVenn (Hulsen et al., 2008).

5.2.4 Gene ontology (GO) analysis of RNA-seq data

Due to the large number of differentially expressed genes between control and *DmGluRA*$^{[112b]}$ mutants, it was difficult to study genes on a gene-by-gene basis. Therefore, Gene Ontology (GO) enrichment analysis was performed using GOrilla (Eden et al., 2009) to classify the differentially expressed genes (DEGs) into functional groups based on biological process, molecular function and cellular component (Figure 5.6).

For biological processes, an enrichment for GO categories associated with ribosomal function and ribosomal RNA processing including ribosomal large subunit biogenesis, rRNA processing, ncRNA processing was observed, as well as for metabolic processes such as UDP-glucose, ncRNA, RNA, pyrimidine-containing compound metabolic process and proteolysis. There was also an enrichment in salivary gland development including salivary gland boundary specification (Figure 5.6).

For molecular function, there was enrichment for GO categories associated with catalytic activity such as hydrolase, peptidase, transferase, serine hydrolase activity and peptidase such as serine-type endopeptidase activity. Also, the binding of compounds such as iron-ion, heme, tetrapyrrole, galactose binding and RNA binding including snoRNA binding (Figure 5.6).

For cellular component, several GO terms associated with the nucleolus and ribosome were enriched including the pre-ribosome, small-subunit processome and nucleolus, nucleolar part, preribosome large subunit and small nucleolar ribonucleoprotein complex. ATPase dependent transmembrane transport complex was also significantly enriched (Figure 5.6).

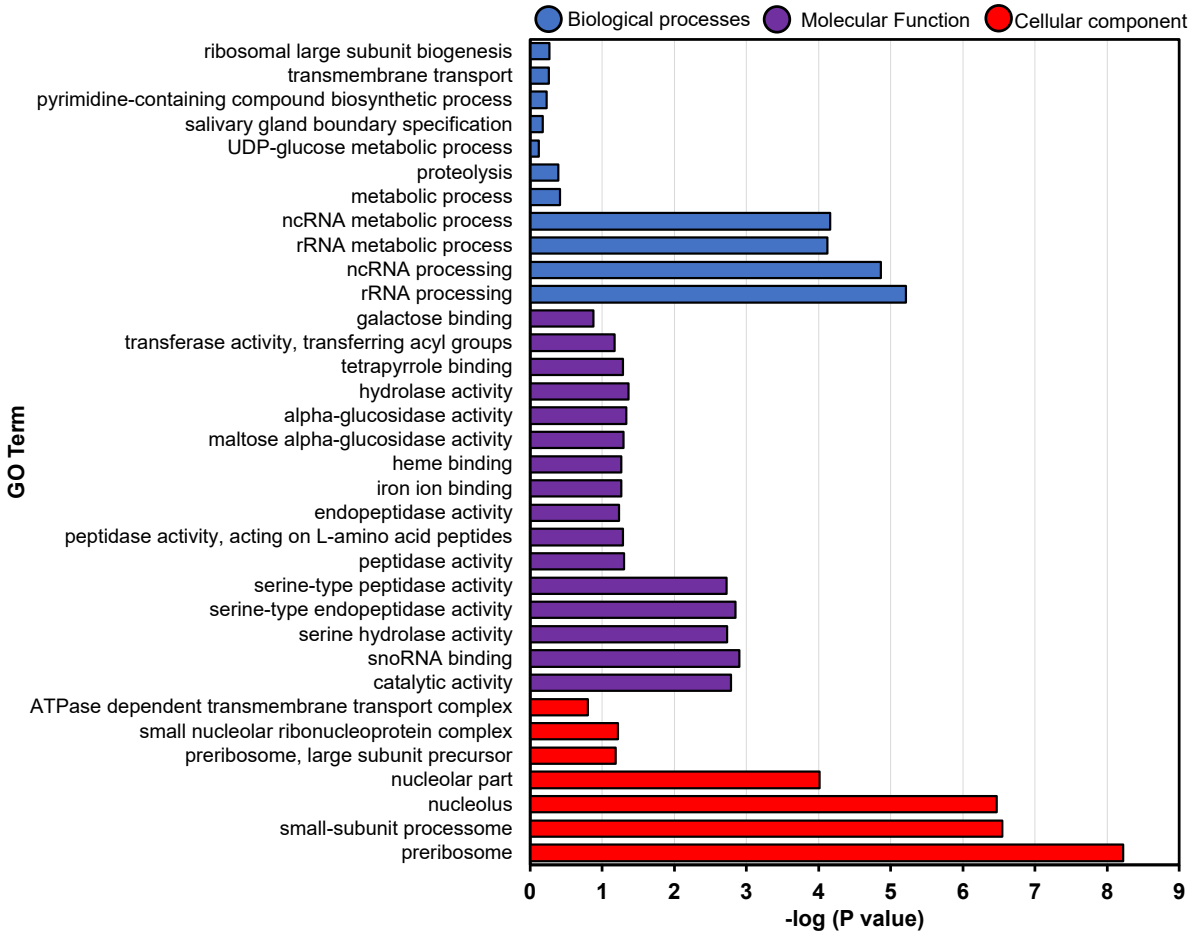

Figure 5.6 Gene ontology (GO) analysis of the differentially expressed genes in *DmGluRA[112b]* mutants.

Enriched GO terms associated the differentially expressed genes identified were identified using GOrilla (Eden et al., 2009) and grouped into biological process, molecular function and cellular component. GO terms are plotted according to the significance of their enrichment (-log p-value after Benjamani-Hochberg correction).

5.2.5 Analysis of nucleolar size in *DmGluRA* mutants

Based on the GO pathway analysis, the most significantly enriched GO terms associated with the DEGs were involved in ribosomal function and ribosomal RNA (rRNA) processing. This was of particular interest because several long-lived animals exhibit decreased expression of rRNA suggesting that decreased ribosome biogenesis is associated with longevity (Tiku et al., 2017). The nucleolus, a non-membrane bound organelle located within the nucleus, is the site of ribosome transcription and processing (Lam et al., 2005). The size of the nucleolus correlates with rRNA synthesis – reduced nucleolar size reflects reduced rRNA biogenesis (Tiku et al., 2017). Studies of various long-lived models in *Drosophila* and also other animals, have revealed that their longevity was linked to reduced nucleoli size and hence reduced ribosomal biogenesis (Tiku et al., 2017).

To study whether *DmGluRA* mutants showed a reduced nucleolar size, the nucleoli within the intestinal epithelial cells of both control and $DmGluRA^{[112b]}$ homozygous mutant 10-day old males and females were labelled by immunostaining using an anti-Fibrillarin antibody. Fibrillarin is a nucleolar protein, rRNA 2'-O-methyltransferase, required for pre-ribosomal RNA processing (Shubina et al., 2016). Nucleolar size was then measured as a ratio of nuclear size. Strikingly, the nucleolar/nuclear ratio of control males was much smaller than control females (Figure 5.7; $p<0.001$). However, no differences were observed in the nucleolar/nuclear ratios in $DmGluRA^{[112b]}$ mutants for either males or females (Figure 5.7; males $p=0.4544$, females $p=0.9617$).

Fibrillarin promotes ribosomal biogenesis and so a reduction in Fibrillarin results in reduced nucleolar size leading to longevity suggesting that levels of expression of Fibrillarin may regulate lifespan (Tiku et al., 2017). Quantifications of Fibrillarin protein levels in *DmGluRA* mutants were further investigated by Western blotting of whole fly protein extracts. Fibrillarin protein levels (normalised to actin) were reduced in control males compared to control females (figure 5.8, $p= 0.0354$) but again there were no differences in Fibrillarin protein levels observed between $DmGluRA^{[112b]}$ mutants and wildtype controls for either sex.

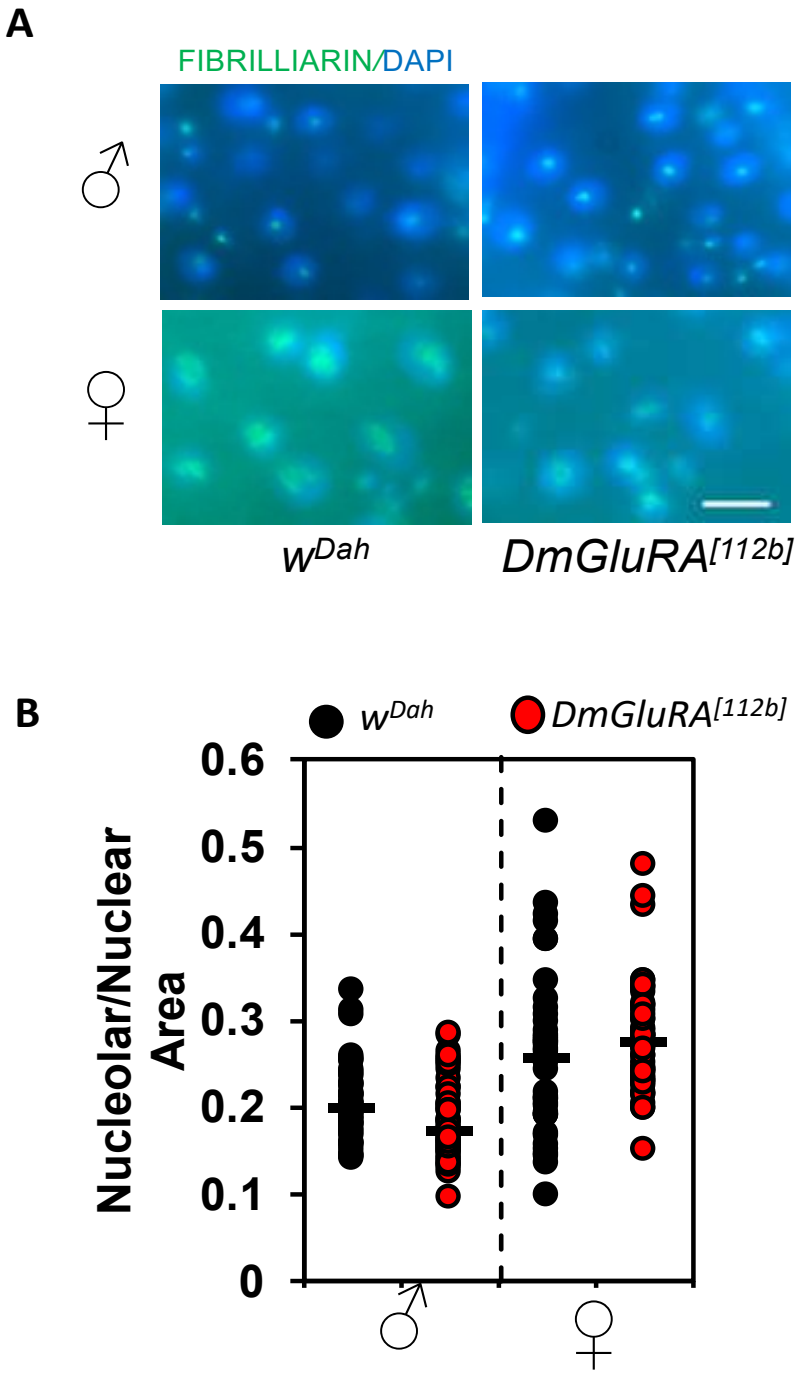

Figure 5.7 Nucleolar size in intestinal epithelium cells of *DmGluRA* mutants.
Nucleolar size was measured in intestinal epithelium cells from 10-day old flies of the indicated genotypes (5 isolated intestinal tracts were examined per condition) and calculated as the ratio of nucleolar area to nuclear area. 10 cells were measured from each dissected intestine.
(A) Images of intestinal epithelial cells stained with anti-Fibrillarin antibody to label the nucleoli (green) and DAPI to label the nuclei (blue). Scale bar represents 10μm.
(B) Quantification of the nucleolar/nuclear ratios. Bars represent the means. No significant differences were observed between *DmGluRA*$^{[112b]}$mutants and their wildtype controls (One-way ANOVA with Tukey's post hoc test).

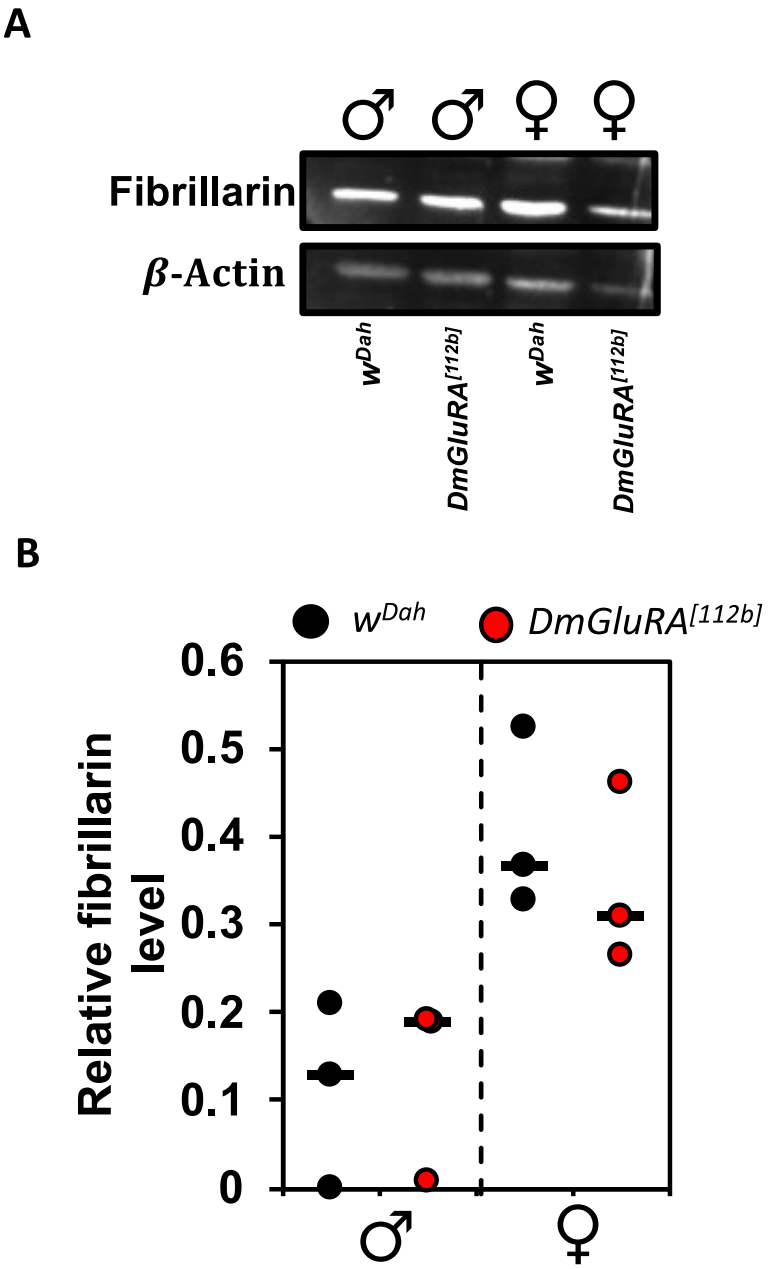

Figure 5.8 Western blot analysis of Fibrillarin expression in *DmGluRA* mutants

Protein extracts were prepared from whole 10-day old adult flies of the indicated genotypes. Blots were probed with anti-Fibrillarin antibody and anti-actin antibody was used as a loading control.

(A) Representative blots, showing levels of Fibrillarin and Actin from protein extracts of the indicated genotypes.

(B) Quantification of Fibrillarin protein expression by densitometry analysis of Western blots using ImageJ. Data represents relative Fibrillarin protein expression normalised to Actin from three biological replicates per condition. Bars represent the means (one-way ANOVA with Tukey's post hoc test).

5.3 Discussion

The data presented so far have described a novel role for DmGluRA during ageing and identified previously unreported physiological responses associated with *DmGluRA* loss of function. However, outstanding questions remained regarding the molecular responses to loss of DmGluRA activity that are associated with *DmGluRA* mutation and that may underlie the observed physiological effects, particularly in lifespan. In order to address this, RNA sequencing (RNA-seq) was used to describe the genomic-wide transcriptional response to loss of DmGluRA activity. The aim was to identify gene expression changes in key genes and/or biological processes that are associated with the phenotypes described earlier.

This transcriptomic analysis revealed that many genes showed altered expression in *DmGluRA* mutants compared to wildtype controls (2194 genes in total). Interestingly, more genes showed a decrease in their expression than increased expression. This suggests that signalling via DmGluRA would normally promote the expression of downstream target genes because loss of DmGluRA activity leads to downregulation of a host of genes. This imbalance in down- versus upregulated genes is not uncommon in *Drosophila* RNA-seq data sets. For example, flies lacking the deglycosylating enzyme *dNGLY1* (*Drosophila N-Glycanase 1*) show downregulation of 290 genes compared to upregulation of just 96 genes (Owings et al., 2018). dNGLY1 usually activates the transcription factor, Cap 'n' collar (Cnc), the fly ortholog of *NRF1* and *NRF2* and many of the downregulated genes identified were Cnc-regulated genes. This fits with the idea that DmGluRA would normally be involved in activating gene expression, as DmGluRA is upstream of transcription factors, such as FOXO and AOP, and may affect the expression of genes they regulate.

Several genes that showed differential expression in the RNA-Seq data were validated using qRT-PCR. Two genes that were identified as upregulated in *DmGluRA* mutants (*TotC* and *Hsp83*) and one gene that was downregulated (*mGluR*) were selected. *TotC* is a member of the *Turandot (Tot)* family of immune and stress response genes, which are induced in response to various stress conditions, including paraquat-induced oxidative stress, and may play a role in stress tolerance (Ekengren and Hultmark, 2001). It is noteworthy, however, that none of the other *Tot* family members (*TotM, TotX* or *TotA*) demonstrated altered expression. Hsp83 (83kDA heat-shock protein) is the *Drosophila* homologue of Hsp90, another stress-responsive protein that is required for Raf activation (van der Straten et al., 1997). Heat shock proteins protect cells from oxidative stress by functioning as molecular chaperones to help re-fold proteins that have

become misfolded or unfolded. These proteins would otherwise accumulate and lead to cell damage or death (Tower, 2011).

DmGluRA expression was downregulated in the RNA-seq data, generally supporting earlier findings on *DmGluRA* mRNA expression measured by qRT-PCR. Though it may be surprising that *DmGluRA* was not one of the top-most downregulated genes in these mutants, the RNA-seq expression analysis of *DmGluRA* correlates well with the previous expression analysis by qRT-PCR. Both techniques have identified that *DmGluRA* transcripts of some form are still expressed in these loss-of-function mutants albeit at reduced levels. The nature of these transcripts are as yet unknown and cannot be elucidated by either RNA-seq or qRT-PCR as both only capture small fragments of transcripts.

qRT-PCR is often used as a validation method for high-throughput techniques such as RNA-seq. It is important to confirm gene expression changes using an alternate method to ensure biological validity and reproducibility (Trost et al., 2015). Previous studies which have used qRT-PCR as a validation method, have shown close correlation between gene expression measures using qRT-PCR and RNA-seq (Hughes et al., 2012, Hall et al., 2017, Akkouche et al., 2017, Chen et al., 2018).

In this study, qRT-PCR validation of transcript expression demonstrated similar expression changes of both *TotC* and *DmGluRA* as had been observed using RNA-Seq. However, *Hsp83* did not show altered expression by qRT-PCR. This discrepancy could be due to a number of reasons. Genes which show inconsistent expression measurements between the two methods tend to show low expression and be smaller with fewer exons, compared to genes which show consistent expression (Everaert et al., 2017) and *Hsp83* is a small gene. Alternative mRNA-splicing is also a plausible reason for inconsistencies between the two techniques. Zhang et al. (2019) demonstrated that if a transcript undergoes post-transcriptional modifications, such as RNA splicing, then it is difficult to accurately measure expression of that gene by qRT-PCR. They compared the quantification of expression of several alternatively-spliced transcripts by qRT-PCR with other methods including RNA-seq, microarray, Northern blot and Serial Analysis of Gene Expression (SAGE). Over 50% of the selected RNA-spliced genes failed to be accurately quantified by qRT-PCR but were successfully quantified by RNA-seq. They suggested that this was because qRT-PCR products were produced from a mixture of different transcripts of the targeted gene whereas in the case of RNA-seq, individual alternatively-spliced transcripts can

be separately detected and so RNA-seq measures the expression of individual transcripts, not a mixture of transcript variants. It is therefore possible that increased gene expression of *hsp83* was not detectable by qRT-PCR because *hsp83* transcripts undergoes splicing. Expression of almost all heat shock protein transcripts are independent of splicing in *Drosophila*, because they are devoid of introns. However, *hsp83* is an exception to this, as the gene locus contains introns (Garbe and Pardue, 1986, Shen et al., 1993, Gatfield et al., 2001).

Despite this, the qRT-PCR data presented in this thesis is consistent with the RNA-seq data with gene expression changes showing changes in the same direction. This shows that there is biological reproducibility, as the RNA used for both experiments was isolated from two independent cohort of flies. Using the same cohort of flies, and hence the same RNA samples only verifies RNA-seq and qRT-PCR as technologies (Fang and Cui, 2011).

The identification of differentially expressed stress responsive genes associated with *DmGluRA* loss of function led to a more thorough examination of those stress responsive genes that would correlate with the increased resistance to oxidative stress induced by paraquat or hydrogen peroxide as described earlier. DJ-1β was identified as upregulated in the *DmGluRA* mutants. This gene is one of two DJ-1 orthologs in flies which encodes an antioxidant protein, associated with Parkinsonism (Meulener et al., 2005). DJ-1β loss-of-function mutants show motor impairments and reduced survival in response to oxidative stress induced by both hydrogen peroxide and paraquat, and also a third agent, rotenone, a mitochondrial complex I inhibitor. The other DJ-1 gene, DJ-1α is expressed only in the male testis (Menzies et al., 2005), which reflects its absence in the list of the DEGs in the presented data set which was derived from females. Studies have shown that DJ-1β, which in both males and females is expressed in almost all tissues, is the ortholog responsible for the DJ-1-dependent antioxidant phenotype (Meulener et al., 2005).

Several genes encoding heat shock proteins including *hsp26, hsp27* and *hsp83* (which was selected for qRT-PCR validation) were also upregulated in *DmGluRA* mutants. Studies have shown that overexpression of either *hsp26* or *hsp27* is sufficient to increase *Drosophila* lifespan as well as increasing resistance to paraquat-induced oxidative stress (Wang et al., 2004a). Interestingly, Wang et al. (2004a) also reported that increased expression of either of these two genes did not affect resistance to starvation, reflecting the observation that after backcrossing into w^{Dah}, *DmGluRA* mutant females were not starvation resistant.

Expression of *MEKK1*, encoding a MAP kinase kinase kinase (MAPKKK) was also upregulated in *DmGluRA* mutants. *MEKK1* expression is necessary for protection against oxidative stress induced by paraquat (Brun et al., 2006) but not by hydrogen peroxide (Craig et al., 2004). This supports the observations presented earlier that w^{Dah} *DmGluRA* mutant females are resistant to paraquat but not hydrogen peroxide. Interestingly, Girardot et al. (2004) suggested that the expression of different subsets of genes are specific to each of these oxidative stress-inducing compounds.

Two members of the SLC22 (solute carrier 22) family of membrane transporters were among the downregulated genes in *DmGluRA* mutants: *CG6006* and *CG16727.* Recently, the knockdown of *CG6006* and *CG16727* as well as other SLC22 transporters has been demonstrated to increase resistance to paraquat-induced oxidative stress (Engelhart et al., 2020). SLC22 transporters located in the Malpighian tubule are responsible for the uptake and excretion of small molecules such as antioxidants from the circulation. Therefore, knockdown of these transporters leads to an increase of these molecules within the haemolymph thereby increasing tolerance to oxidative stress (Engelhart et al., 2020).

Altogether, the analysis of stress responsive gene expression within the *DmGluRA* transcriptome has identified several genes that show altered expression and are therefore likely to contribute to the paraquat-induced oxidative stress resistance phenotypes observed in *DmGluRA* mutants.

Previous studies have identified several proteins that respond to changes in DmGluRA activation. For example, dFMRP shows increased protein expression in *DmGluRA* mutants (Pan et al., 2008), while the activities of both dFak and CaMKII are increased by DmGluRA activation (Chun-Jen Lin et al., 2011). Also, activity of the adenyl cyclase, *rut,* is thought to be decreased in response to DmGluRA activity (Kanellopoulos et al., 2012).

However, with the exception of *rut* which showed decreased expression in *DmGluRA* mutants, expression of the genes encoding these proteins was unchanged. This change in *rut* gene expression appeared to occur in the opposite direction to what might be expected – so where protein activity has been shown to increase in response to reduced *DmGluRA*, expression of the gene was increased instead. This could reflect transcriptional feedback onto *rut* – loss of DmGluRA activity leads to a reduction in *rut* activity which could be detected by the cell which responds by increasing expression of *rut* to try and compensate this.

An alternative method to confirm this reduction of *rut* in these mutants is to measure cyclic AMP activity. As *rut* affects cAMP synthesis by converting ATP to cAMP (Livingstone et al., 1984), if *rut* activity is decreased then there should be an increase in cAMP which can be measured in whole flies. This would be interesting to study as previous *in vitro* studies have shown cAMP directly binds sirtuins and increased cAMP in different mouse tissues leads to an increase in sirtuin levels and increases lifespan in mice (Wang et al., 2015c) .

DmGluRA can signal through two highly conserved signalling pathways that have well-established effects on lifespan – PI3K/AKT and RAS/MAPK. The DEGs associated with loss of DmGluRA function were therefore examined for any evidence at the transcriptional level that there were alterations in signalling through either of these pathways. Two key downstream effectors of PI3K/AKT and RAS/MAPK signalling are the transcription factors, FOXO and AOP. Evidence from the literature has shown that perturbations in signalling results in transcriptional feedback onto genes encoding core signalling molecules within these pathways because these genes are direct targets of FOXO and AOP (Alic et al., 2011a, Puig and Tjian, 2005). However, most genes encoding upstream signalling molecules within these pathways including *dilps, chico* and *PTEN,* showed no changes in expression. But expression of the *InR* gene was decreased in *DmGluRA* mutants while expression of both *Ras* and *Raf* increased which together may indicate altered signalling through these pathways.

Activation of FOXO or AOP via inhibition of PI3K/AKT or RAS/MAPK would lead to a wider change in expression of their target genes. The DEGs that show altered expression in response to loss of *DmGluRA* were therefore examined for any changes in the expression of known FOXO/AOP target genes. A significant overlap was identified between the genes differentially expressed in response to loss of DmGluRA and direct FOXO targets. A significant overlap was further identified between the genes upregulated by loss of *DmGluRA* and direct targets of FOXO, that are upregulated in response to FOXO activation. A similar significant overlap was identified between the genes that are differentially expressed in response to loss of DmGluRA and direct target genes of the Ras-responsive transcription factor, AOP. Together, this suggests that both FOXO and AOP may indeed be activated in *DmGluRA* mutants and so may mediate at least in part the transcriptional response to loss of DmGluRA activity and potentially the observed lifespan extension. However, it remains unclear from this analysis whether these effects on gene expression would be direct effects of reduced DmGluRA activity on the activation of these

transcription factors, or indirect effects. Interestingly, a similar overlap was not observed in the genes that were both downregulated by loss of *DmGluRA* and direct targets of FOXO, that are downregulated in response to FOXO activation. Thus, the genes that show reduced expression in the *DmGluRA* mutants may be regulated via FOXO-independent transcriptional mechanisms.

Further studies are needed in order to determine if the longevity phenotype of these mutants is dependent on FOXO and AOP. An epistasis approach could therefore be taken i.e. if the observed phenotypes are dependent on transcription factors FOXO and AOP and these transcription factors are removed in the *DmGluRA* mutant then the phenotype will be lost.

Alongside the analysis of individual gene expression changes, Gene Ontology (GO) enrichment analysis was also performed to group the differentially expressed genes identified in the *DmGluRA* mutants into functional categories. This type of analysis is useful especially because high-throughput methods such as RNA-seq usually identify a large number of genes that show differential expression, and this can be difficult to interpret. GO enrichment analyses helps to better understand the biological meaning of the data by grouping DEGs by their common properties or functions (Huang da et al., 2009). This is particularly more useful that studying DEGs on a gene-gene basis, as often biological processes are not regulated by a single gene but by groups of genes.

Here, GO enrichment analysis revealed several functional categories associated with ribosomal function and rRNA processing such as ribosomal large subunit biogenesis, rRNA processing, ncRNA processing. This was an interesting finding as existing evidence suggests that the disruption of ribosome biogenesis is linked to increased lifespan (Tiku et al., 2017). In *C. elegans,* the nucleolar protein, Fibrillarin, is reduced in various long-lived mutants, and loss of ring finger protein, ncl-1, which increases Fibrillarin expression in long-lived worms, abolishes the lifespan extension of various long-lived mutants.

rRNA synthesis correlates with the size of the nucleolus, and studies have shown that small nucleolar size and therefore reduced ribosomal biogenesis is a key hallmark of longevity across several animal models including *Drosophila* (Tiku et al., 2017). In addition, knockdown of key regulators of ribosome biogenesis including *Myc* or inhibition of enzymes involved in the transcription of rRNA, and therefore the initiation of ribosomal biogenesis, have all been reported to extend *Drosophila* lifespan (Greer et al., 2013, Martinez Corrales et al., 2020).

Similar effects have been seen in other animals, for example, mice heterozygous for the *Myc* gene show extended lifespan compared to their wild-type littermates (Hofmann et al., 2015). Moreover, inhibition of RNA Pol III activity, most likely linked to reduced ribosomal biogenesis, extends lifespan in yeast, flies and worms (Filer et al., 2017), highlighting that this is an evolutionary conserved mechanism.

Despite the identification of key transcriptional changes in genes regulating nucleolar function and rRNA production, examination of nucleolar size in *DmGluRA* mutants revealed no differences in either males and females when compared to their controls. It should be noted, however, that these studies only examined nucleolar size within the intestinal epithelium and not in other tissues. Nevertheless, other studies that have looked at nucleolar size in long-lived flies have all found size differences in these same cells irrespective of the manipulation. Thus, rapamycin treatment, IIS mutants and flies subjected to DR all possess smaller nucleoli within the intestinal epithelial cells (Tiku et al., 2017). Tiku et al. (2017) further anticipated that there may be some exceptions to this, that longevity and nucleoli size may be uncoupled since in wild-type worms, loss of *ncl-1*, which usually inhibits rRNA transcription by inhibiting Fibrillarin, is not associated with longevity but these worms have larger nucleoli. Perhaps a process downstream of loss of *DmGluRA* causes the uncoupling to nucleolar size from increased lifespan.

One interesting and unexpected observation from the examination of nucleolar size, was that differences were observed between wildtype males and females. Thus, in males nucleolar size was significantly smaller than in females. Furthermore, Western blot analysis of Fibrillarin expression confirmed that sex differences were also apparent at the level of expression of this key protein involved in rRNA processing. Sex-specific differences in nucleolar size in *Drosophila* have not been previously reported in the literature although there are studies which support sex differences in nucleolar function. For instance, lifespan extension by inhibition of RNA polymerase I activity, which transcribes ribosomal RNAs, was observed in females but not in males (Martinez Corrales et al., 2020) suggesting RNA polymerase I is usually life-limiting for females but not males. If males have smaller nucleoli, as suggested by the present findings, males are potentially less metabolically active. Ribosome biogenesis and protein synthesis are energetically costly and so perhaps for females this increased nucleolar size reflects a greater level of rRNA metabolism that is removed in RNA pol I mutants and so extends lifespan. Studies have also shown that the transcription factor, Myc, which induces expression of genes required for ribosome biogenesis, shows higher expression in females than males (Mathews et al., 2017). There is currently great interest in sex-specific lifespan responses within the field and it is

interesting to hypothesise that these may be linked, at least in part, to these sex differences in nucleolar size and/or function and so should be studied further.

Chapter 6 : Final Discussion

The primary aim of this thesis was to determine the role of metabotropic glutamate receptors (mGluRs) in biological ageing using the fruit fly, *Drosophila melanogaster,* as a model system. mGluR activity has been linked to several cell signalling pathways that play evolutionary conserved roles in animal ageing, including the PI3K/AKT and MAPK/ERK signalling pathways, and preliminary studies performed in the laboratory had shown that mutation of the single mGluR homologue in flies, *DmGluRA*, caused sex-specific effects on longevity.

The first objectives of the project were to characterise in more detail the molecular nature of the mutant line, *DmGluRA*$^{[112b]}$, used for these experiments and then to confirm the longevity response of the mutant across different backgrounds. It was also important to test whether other factors that are known to impact on longevity, such as the presence of the endosymbiotic bacteria, *Wolbachia pipientis*, influenced the lifespan of the *DmGluRA*$^{[112b]}$ mutants. The final objective of the first chapter, was to investigate the basis for the observed sex-specific differences of *DmGluRA* mutation on lifespan. It was hypothesised that sex-specific differences in longevity may be caused by differences in *DmGluRA* expression between males and females. Following this, the aim was then to compare *DmGluRA* expression across both sexes in both wild-type and *DmGluRA* mutant flies as well as to identify whether *DmGluRA* expression changes during ageing.

Next, this study aimed to characterise phenotypes that are commonly associated with increased lifespan. It was hypothesised that by identifying other DmGluRA-dependent phenotypes, this may highlight the key physiological responses that underlie the longevity effects of loss of DmGluRA activity. Alongside these experiments, the *DmGluRA* mutants were also examined for indicators of age-related functional decline, including locomotor activity and intestinal barrier function, to determine if loss of DmGluRA activity also leads to improved healthspan as well as lifespan.

The final part of this thesis aimed to describe the transcriptional response to loss of DmGluRA activity in *Drosophila*. As a member of the GPCR family of cell surface receptors, DmGluRA activation induces signalling through several downstream intracellular pathways that modulate the activity of downstream transcription factors, which in turn would affect the expression of the genes they regulate. For instance, the transcription factors FOXO and AOP are both downstream effectors of PI3K/AKT and Ras/MAPK signalling. As such, if DmGluRA signals

through either of these pathways then the activity of these transcription factors would be modulated, changing the expression of the genes that they control. It was therefore hypothesised that by performing differential gene expression (DEG) analysis using RNA sequencing, key transcriptional changes in the *DmGluRA* mutants would be described that would aid the identification of the biological mechanisms associated with the longevity response. These changes in gene expression could also explain some of the phenotypic responses observed in the *DmGluRA* mutants.

Together, these results would help to understand the functions of mGluR signalling in lifespan and age-related health, offering the potential to identify pharmacological interventions that target this signalling pathway in order to delay the adverse effects of ageing.

6.1 Discussion and summary of findings

Despite its use in previous studies (Bogdanik et al., 2004, Schoenfeld et al., 2013, Chun-Jen Lin et al., 2011), the precise endpoints of the $DmGluRA^{[112b]}$ deletion were not well described although the deletion is known to extend into the coding sequence to prevent DmGluRA protein expression (Bogdanik et al., 2004). As the $DmGluRA^{[112b]}$ deletion was generated by P-element excision, the deletion is not marked, and adult flies have no obvious visible defects. The presence of the deletion can therefore only be detected by PCR of genomic DNA. By characterising the end-points of the $DmGluRA^{[112b]}$ deletion, it was found that 4.8kb of the *DmGluRA* locus is deleted in these mutants which includes part of the promoter sequence and the translational start site. This allowed the design of primer sequences for PCR detection of the deletion even in heterozygous flies.

Interestingly, although the $DmGluRA^{[112b]}$ mutants are reported to produce no DmGluRA protein (Bogdanik et al., 2004), expression analysis by both qRT-PCR and RNA-seq revealed the presence of *DmGluRA* transcripts within this mutant line albeit at a much reduced level than in controls. However, the nature of these transcripts is unclear and the expression analysis performed here cannot determine whether these transcripts would produce functional DmGluRA protein or a truncated version of the protein.

Previous work in the laboratory using this $DmGluRA^{[112b]}$ deletion had found that after backcrossing into the w^{Dah} background, homozygous mutant females showed extended lifespan

but similar effects on longevity were not observed in homozygous mutant males. These preliminary findings were confirmed here suggesting that loss of DmGluRA activity has sex-specific effects on lifespan. This is not an uncommon observation as many long-lived models including loss of signalling via the IIS, DR and inhibition of TOR signalling via rapamycin treatment show lifespan effects that are specific to females or show a reduced lifespan response in males (Clancy et al., 2002, Giannakou et al., 2004, Magwere et al., 2004, Ikeya et al., 2009, Bjedov et al., 2010).

However, this sex-specific lifespan response to *DmGluRA* mutation was found to be dependent on genetic background. After backcrossing into the w^{1118} background, both males and females, homozygous mutant for the $DmGluRA^{[112b]}$ deletion, were long-lived. This suggests that the lifespan response to loss of DmGluRA activity in males is influenced by genetic background. While the significance of these genetic background effects in males is not fully understood, these results highlight the importance of using different backgrounds for longevity studies, particularly as some backgrounds are naturally short-lived and therefore pro-longevity interventions could be rescuing a physiological deficit in survival rather than affecting ageing (Spencer et al., 2003). These results also demonstrate the importance of conducting experiments in both sexes when studying effects on ageing. Burger and Promislow (2004) when comparing previous studies of life-extending genetic interventions, found only a small proportion of studies used both sexes. Furthermore, from this small number of studies using both sexes, the majority had not attempted to look for differences between male and female lifespan.

To investigate whether the observed sex differences in lifespan could be explained by differences in *DmGluRA* expression, *DmGluRA* mRNA levels were measured using qRT-PCR. Interestingly, it was found that mRNA levels were different between wild-type males and females, with female flies showing significantly lower levels of *DmGluRA* expression compared to males, a previously unreported finding. Furthermore, while both males and females homozygous mutant for the $DmGluRA^{[112b]}$ deletion showed reduced *DmGluRA* expression, the extent of this reduction in expression was more pronounced in females compared to males with a 61.2% reduction in *DmGluRA* mRNA expression in females compared to a 43.2% reduction in males. It is worth noting, that *DmGluRA* expression was not studied in the w^{1118} background, and so it is unclear whether these differences in expression are also observed within this strain and also if there are differences in expression in $DmGluRA^{[112b]}$ mutant males between the w^{1118}

and w^{Dah} strains that could account for the differential effects of genetic background of the lifespan response.

During normal ageing, *DmGluRA* expression was not observed to change over time, at least in females, suggesting that the loss of *DmGluRA* activity itself leads to a direct effect on ageing. This is opposed to loss of DmGluRA rescuing a detrimental effect caused by DmGluRA misregulation during ageing. This was a possibility because *DmGluRA* resides on the fourth chromosome, a highly heterochromatic region and not only is loss of heterochromatin associated with ageing (Wood et al., 2010), but genes within such regions become misregulated and expressed elsewhere (Wood et al., 2010, Brown et al., 2020). For example, a study by Wood et al. (2016) showed that with increased age, there was an increase in transcript levels of many of the genes within this region in the fly heads and fat bodies. It is therefore suggested that this inappropriate gene expression may contribute to ageing pathologies (Larson et al., 2012, Wood et al., 2016).

Interestingly, these effects of *DmGluRA* mutation on lifespan appear to contradict recent findings by Ly and Naidoo (2019) who reported that female $DmGluRA^{[112b]}$ mutants were short-lived. However, there are some important differences between the experiments reported in Ly and Naidoo (2019) and those presented here. As well as differences in the genetic background used between the two studies, there are differences in the diets used in both studies. In the lifespan experiments presented here, a diet optimised for lifespan experiments was used (Bass et al., 2007). The published data however used a standard dextrose diet, which may have different compositions of components such as sugar and yeast. There is evidence that different carbohydrates, particularly sugars, affect lifespan differently (Lushchak et al., 2014). For example, flies that eat fructose lived longer than flies fed with sucrose. Ly and Naidoo (2019) used dextrose whilst the Bass et al. (2007) diet uses sucrose, which may therefore have different effects.

Experiments presented here in which the nutritional composition of the diet was modulated to examine the effects of dietary restriction (DR) suggest that diet is likely to be important for the lifespan extension caused by loss of DmGluRA activity. Although $DmGluRA^{[112b]}$ mutant females were found to respond similarly to controls with increased egg-laying and decreased median lifespan as the yeast concentration of the diet was increased, the optimal yeast concentration for lifespan for the $DmGluRA^{[112b]}$ mutants was at a lower yeast concentration than for the

controls. Also, at higher dietary yeast concentrations, the *DmGluRA*$^{[112b]}$ mutants were found to be shorter-lived than controls which emphasises that diet, specifically protein concentration, is important for how this particular mutation influences lifespan.

The observed differences in the lifespan response of *DmGluRA*$^{[112b]}$ females across different yeast concentrations is somewhat unusual. Other interventions that promote longevity, for example rapamycin treatment, show increased lifespan extension across all yeast concentrations (Bjedov et al., 2010) or like *chico* mutants or DILP-producing mNSC-ablated flies, that show increased lifespan extension at concentrations above the food concentration of which they achieve peak lifespan (Clancy et al., 2002, Broughton et al., 2010).

This influence of dietary protein on the lifespan response of *DmGluRA*$^{[112b]}$ mutants may also account for the differential effects of this mutation on lifespan observed between experiments presented in this study. For instance, although the *DmGluRA*$^{[112b]}$ mutant females exhibited a normal DR response, their lifespan was not enhanced relative to wild-type controls at 1x SYA concentration, as observed in the earlier lifespan experiments. The media used for these two sets of experiments were made using two different batches of yeast. It has been shown that the nutritional composition of yeasts is highly variable between suppliers (Bass et al., 2007) and even between batches from the same supplier, which can lead to different effects on lifespan and egg-laying (Magwere et al., 2004).

It was next determined if loss of DmGluRA activity, like other pro-longevity interventions, is associated with phenotypes which could contribute, at least in part, to the effects on lifespan. Due to the differences observed in the lifespan studies, many of these phenotypic analyses were performed using both sexes and in different genetic backgrounds. As DmGluRA signals via known pro-longevity mediators including PI3K/AKT and Ras/MAPK, the phenotypes selected for study were consistent with down-regulation of these signalling pathways.

These studies identified several phenotypes associated with loss of DmGluRA activity that have not been previously reported such as increased body mass, differences in TAG utilisation during starvation (males only) and increase stress tolerance. Initially, it was hypothesised that these phenotypic changes may correlate with the observed lifespan effects and so could give some insight to the lifespan-extending mechanisms of this mutation. However, this appears to be more complex because *DmGluRA* mutants show several phenotypes which do not necessarily

correlate with the lifespan effects. For example, stress tolerance has been observed in several other long-lived fly models including loss-of function *Lnk* mutants, ablation of mNSCs in the brain that produce DILPs 2,3 and 5, over-expression of (tumour suppressor homolog) Insulin Growth Factor Binding Protein 7 (IGFBP7), Imaginal morphogenesis protein-Late 2 (IMP-L2) and *chico* heterozygotes females, to name a few (Broughton et al., 2005, Slack et al., 2010, Alic et al., 2011b, Bai et al., 2015). However, while *DmGluRA*$^{[112b]}$ mutant males and females show increased resistance to oxidative stress in both the w^{1118} and w^{Dah} genetic backgrounds, the longevity response in *DmGluRA*$^{[112b]}$ mutant males was dependent on genetic background. This suggests that it is unlikely that increased tolerance to oxidative stress is the mechanism of lifespan extension as mutant males can show increased resistance to oxidative stress without increasing lifespan.

The only phenotype which correlated with the observed lifespan effects across different genetic backgrounds was body weight. Thus, after backcrossing into the w^{1118} genetic background, long-lived males and females homozygous mutant for the *DmGluRA*$^{[112b]}$ deletion showed increased body weights while in the w^{Dah} genetic background, long-lived *DmGluRA*$^{[112b]}$ homozygous females showed increased body weights but *DmGluRA*$^{[112b]}$ homozygous males showed comparable lifespans and body weights to control flies.

Adult body mass alongside wing area are often used as read-outs for developmental growth in *Drosophila.* However, where *DmGluRA*$^{[112b]}$ mutants showed an increase in body weight compared to controls, they did not show any differences in wing size suggesting that DmGluRA activity is not required for normal growth. Adult body weight may also be affected by differences in food intake or metabolism. However, the increased body weight of *DmGluRA*$^{[112b]}$ mutants was not associated with increased feeding behaviour or food intake nor were any differences observed in the levels of key metabolic storage molecules including TAG, glycogen or trehalose under fully-fed conditions at the same age at which body weight was measured. As such, the underlying mechanisms that lead to increased body weight in long-lived *DmGluRA*$^{[112b]}$ mutants remain unclear.

Interestingly, in male *DmGluRA*$^{[112b]}$ mutants, higher levels of TAG were observed at day 7 but not at day 10. Furthermore, when *DmGluRA*$^{[112b]}$ mutant males were subjected to starvation from day 7, they appeared to retain their TAG stores for longer, suggesting that TAG breakdown during starvation may be dependent on DmGluRA activity. In contrast, female *DmGluRA*$^{[112b]}$ mutants did not show any differences in TAG levels at either of the two ages nor during

starvation. These findings are intriguing as recently Wat et al. (2020) identified that differential expression of the TAG lipase, *brummer* (*bmm),* regulates sex-specific differences in TAG storage and breakdown. They showed that during the early days of adulthood in wildtype flies, males and females start out with comparable amounts of stored TAG. However, due to higher levels of *bmm* expression in males, these TAG stores are broken down faster resulting in lower levels of TAG in males than females. As such, in *bmm* mutants, the sex-specific differences in TAG breakdown and storage are removed.

Differences in TAG breakdown and/or storage are often associated with differential survival under starvation (Bjedov et al., 2010, Slack et al., 2010, Wu et al., 2017, Ugrankar et al., 2019). In *DmGluRA*$^{[112b]}$ mutants, male flies showed improved survival during starvation in both w^{Dah} and w^{1118} genetic backgrounds while females showed no increase in survival under starvation in the w^{Dah} background and a reduced response to starvation in w^{1118}. In w^{1118} flies, these sex differences in the starvation response were associated with differential effects of starvation on TAG breakdown in the two sexes with *DmGluRA*$^{[112b]}$ mutant males showing higher levels of TAG than controls during 48 hours of starvation while *DmGluRA*$^{[112b]}$ mutant females did not show any differences in TAG levels compared to controls over the same time-course of starvation.

Based on the sex-specific differences in *bmm* expression reported by Wat et al. (2020), it would be interesting to examine *bmm* expression in *DmGluRA*$^{[112b]}$ mutants. DmGluRA activity may somehow be involved in the sex-specific regulation of *bmm* expression. Thus, removal of *DmGluRA* activity in males may lead to lower levels of *bmm* resulting in less rapid breakdown of TAG during starvation in *DmGluRA*$^{[112b]}$ mutant males. This may also result in differences in the dynamics of TAG breakdown during early adulthood in males which is why elevated TAG levels were observed in fully-fed *DmGluRA*$^{[112b]}$ mutant males at day 7 but not day 10. A more refined analysis of TAG levels during early adulthood may help to address this. Expression of *bmm* would be expected to be comparable between *DmGluRA*$^{[112b]}$ mutant females and their controls. Interestingly, RNA-seq analysis of differential gene expression in *DmGluRA*$^{[112b]}$ mutant females did not identify *bmm* among the significantly differentially expressed genes supporting this hypothesis.

As well as showing that loss of DmGluRA activity extends lifespan, it was important to determine whether the longevity response of *DmGluRA*$^{[112b]}$ mutants was also associated with improvements in any age-related physiological parameters to show an increase in healthspan.

Two well-characterised age-related phenotypes were therefore examined in *DmGluRA*$^{[112b]}$ mutants: loss of intestinal epithelial barrier function and neuromuscular function. However, the age-related loss of intestinal epithelial barrier function was still observed in long-lived *DmGluRA*$^{[112b]}$ mutants using the Smurf assay while loss of *DmGluRA* expression did not prevent or reduce the age-related decline in neuromuscular function as detected using the negative geotaxis climbing assay. Indeed, long-lived *DmGluRA*$^{[112b]}$ males actually showed a faster rate of decline in neuromuscular function during ageing compared to controls whereas long-lived *DmGluRA*$^{[112b]}$ females generally performed better than controls across the time-course of the assay although the age-related decline in neuromuscular function occurred at a comparable rate to controls. It may be useful to test these functions beyond the time-points presented here, particularly for intestinal epithelial barrier function, as differences between *DmGluRA* mutants and controls may be more pronounced at even older ages, for example after 60 days. However, at this stage, many of the animals were already dead and therefore the sample size was severely reduced. Nevertheless, taken together, these results suggest that the pro-longevity effects of the *DmGluRA*$^{[112b]}$ mutation occur through alternative mechanisms other than age-related improvements in these physiological parameters.

Increased lifespan is not always associated with improved physiological function during ageing. For example, not all pro-longevity interventions ameliorate the age-related decline in intestinal epithelial barrier function. Feeding flies with the MEK inhibitor, trametinib, extends lifespan but does not prevent the age-related leakiness in the intestinal epithelia(Slack et al., 2015). Also, female flies treated with the marine carotenoid, fucoxanthin are long-lived but treatment did not prevent age-related decline of the intestinal barrier as the proportion of Smurfs produced was similar to controls at 70 days of age (Moskalev et al., 2018). Similarly, despite its positive effects on lifespan, flies subjected to DR do not show improvements in neuromuscular function with age as assayed in the negative geotaxis assay (Bhandari et al., 2007). Also, most recently, Hurley and Staveley (2021) have shown that inhibition of *Ref(2)P* expression, which encodes a p62 orthologue, in motor neurons increases lifespan in male flies but this is associated with a sharp decline in locomotor function. Mutations in the human p62 orthologue are associated with neurodegenerative disorders such as Parkinson's disease and amyotrophic lateral sclerosis. The authors therefore suggest the existence of a possible trade-off between longevity and the decline in locomotor activity. It is therefore possible that a similar trade-off may also occur in *DmGluRA* mutants.

One major question still unanswered is the precise mechanism(s) by which the *DmGluRA*$^{[112b]}$ mutation extends lifespan although based on the data presented in this thesis some potential mechanisms, such as improvements in intestinal epithelial physiology, can be excluded. Transcriptomic analysis using RNA-seq was performed to try and identify transcriptional changes within the *DmGluRA*$^{[112b]}$ mutants that could account for their observed phenotypic differences. For example, several stress responsive genes showed differential expression in *DmGluRA*$^{[112b]}$ mutants including the heat shock protein genes *hsp26, hsp27* and *hsp83*, all of which were up-regulated in *DmGluRA*$^{[112b]}$ mutants. Increased expression of these heat shock protein genes has been shown to increase resistance to paraquat-induced oxidative stress (Wang et al., 2004a) and so their elevated expression in *DmGluRA*$^{[112b]}$ mutant flies may contribute to the increased survival of *DmGluRA*$^{[112b]}$ mutants in the presence of paraquat.

Gene Ontology (GO) analysis of the differentially expressed genes associated with loss of *DmGluRA* expression also revealed some interesting possible mechanisms for the longevity response of the *DmGluRA*$^{[112b]}$ mutants, including rRNA processing and metabolism. Previous studies have shown an association between rRNA biogenesis and ageing (Tiku et al., 2017, Tiku and Antebi, 2018). As the primary site of rRNA biogenesis, nucleolus size is often used as a direct read-out for rRNA synthesis (Tiku et al., 2017). However, no differences in nucleolus size were observed between *DmGluRA*$^{[112b]}$ mutants and controls. It should be noted that in this study nucleolus size measurements were only performed within the intestinal enterocytes of the midgut and so any tissue-specific effects of *DmGluRA* loss of function that are mediated by processes outside of the intestine would not have been detected. Nevertheless, other studies that have looked at nucleolar size in long-lived flies have found size differences in the nucleoli of these intestinal enterocyte cells irrespective of the manipulation used, including after rapamycin treatment, DR and genetic reductions in IIS (Tiku et al., 2017). However, measurements of nucleolar size in other tissues may be necessary to confirm this lack of response.

It is possible that the longevity effects of the *DmGluRA*$^{[112b]}$ mutation are mediated via the activation of the key longevity regulators and transcription factors, FOXO and AOP. The differentially expressed genes within the *DmGluRA*$^{[112b]}$ mutants identified by RNA-seq included many target genes of these two transcription factors. Genetic epistasis experiments using FOXO and AOP loss of function manipulations will determine whether the longevity of *DmGluRA*$^{[112b]}$ mutants is dependent upon the activity of either or both of these transcription factors. Hence, such studies will determine if the changes in expression of AOP/FOXO are indirectly or directly

mediated by changes in either transcription factor expression/activity. This is important as FOXO is a key regulator of other physiological functions apart from lifespan such as stress resistance and growth (Mattila et al., 2009). These results were somewhat unexpected as DmGluRA do not display typical phenotypes associated with reduced IIS such as reduced body size or fecundity and do not show many gene changes in the upstream IIS components.

6.2 Limitations and future work

One of the primary limitations of the work presented here is that despite the data supporting a role for DmGluRA in biological ageing, as a genetic deletion the effects of the *DmGluRA*$^{[112b]}$ mutation are widespread. It therefore remains unclear whether the lifespan effects observed are due to loss of DmGluRA activity in a specific tissue or group of tissues. In addition, all the expression analysis studies and transcriptomic analyses were carried out on mRNA isolated from whole adult flies. While this approach provides a global view of gene expression changes, the data is not representative of expression changes within individual fly tissues and so any tissue specific transcriptional changes may have been missed. Future studies should therefore examine gene expression changes in isolated tissues.

Several lifespan-extending interventions cause their effects via specific tissues. For example, overexpression of dTsc1 and dTSc2 (tuberous sclerosis complex genes 1 and 2), which encode key inhibitors of the TOR pathway, fail to promote longevity when expressed in the eye or nervous system but do extend lifespan when overexpressed specifically in the fat body (Kapahi et al., 2004). Similarly, RNAi-mediated knockdown of expression of *dAhcyL1* and *dAhcyL2*, encoding components of the methionine pathway, extends lifespan when targeted to the brain and intestine but not in other tissues such as the fat body (Parkhitko et al., 2016). More recently, RNAi knockdown of the receptor tyrosine kinase, anaplastic lymphoma kinase (Alk), which similarly to DmGluRA, is expressed mostly in the nervous system, was shown to extend lifespan with both widespread knockdown or knockdown specifically within the neurons (Woodling et al., 2020). Future work should therefore include targeting *DmGluRA* loss-of-function to specific tissues within the adult fly to deduce those tissues in which DmGluRA activity is important for lifespan extension. This may therefore provide additional insights into the underlying mechanisms.

Another main limitation of this project is that it remains unclear when exactly during the life-course that DmGluRA activity influences ageing. Many of the phenotypic assays were performed

at a single time-point early in adult life e.g. in 10-day old adults. As such, the results may not be indicative of the impact of loss of DmGluRA function during ageing as the effects may occur later on in the life-course after the time-points studied. Longitudinal analysis of some of these traits over the life-course of the fly will therefore be important to understand how these changes relate to differences in lifespan. One suggestion for longitudinal analysis is the effects of *DmGluRA* mutation on feeding behaviour. Both feeding assays performed in this study, the CAFÉ assay and the proboscis extension assay, were essentially performed at one time-point in young adults. It may be more informative to examine feeding behaviour at later ages to see if differences in food intake arise between the *DmGluRA* mutants and controls.

Determining the timing requirements for loss of DmGluRA activity to extend lifespan will be particularly important when investigating possible pharmacological interventions that mimic loss of DmGluRA function in flies. Due to limitations of time, drug studies were not carried out as part of this study. However, several chemical inhibitors of mammalian mGluR signalling have been characterised some of which have been shown to be effective inhibitors of DmGluRA in *Drosophila,* such as the selective antagonist, 2-Methyl-6-(phenylethynyl)pyridine (MPEP). Future investigations using these compounds will determine whether pharmacological inhibition of DmGluRA produces similar effects on lifespan as *DmGluRA* genetic mutation. These experiments will be an important first step into possible pharmacological interventions that target this signalling pathway as a means to delay the adverse effects of ageing. Knowing the timing requirements for administration of the drug to extend lifespan will be beneficial for these experiments and varies between different interventions. For example, work has been carried out on 2,5-dimethyl-celecoxib (DMC) a derivative of an anti-inflammatory drug celecoxib, to test its effect on *Drosophila* lifespan (Wu et al., 2017). The drug was introduced into the fly food throughout the fly's lifespan, only during the first 20 days of the lifespan, switching the flies back to normal media for the rest of the duration of the study or they administered the drug later in life, so the flies were switched onto drug-containing food at 42 days of age. They found that administration of DMC could extend lifespan to a similar extent at all stages of the life-course.

Lastly, a very important part of this study is the effect of this pro-longevity intervention on healthspan. As mentioned earlier, the ultimate aim for any intervention is not to just extend lifespan but to also improve health at older ages. One of the key markers for healthspan in *Drosophila* is neuromuscular function as measured using the negative geotaxis climbing assay. However, as discussed earlier, not all long-lived flies (including $DmGluRA^{[112b]}$ mutants) show improved performance in the negative geotaxis climbing assay with age.

Importantly, it has been suggested that the uncoupling of age-related changes in locomotor activity and longevity is dependent on which behavioural function is measured (Ismail et al. (2015). The negative geotaxis climbing assay is based more on muscle function rather than improved cognitive function. For example, Ismail et al. (2015) showed using long-lived IIS mutants that improvements in performance in the negative geotaxis climbing assay is due to beneficial effects of IIS outside the nervous system i.e. in peripheral tissues.

Therefore, to measure functional healthspan in *DmGluRA*$^{[112b]}$ mutants, it may be more informative to include multiple behavioural parameters of neural function as loss of DmGluRA function may not prevent the decline in muscle function with age but may have a specific effect on the ageing CNS, particularly as DmGluRA protein is expressed predominantly within the brain. This also reinforces the importance of more tissue-specific studies in which loss of *DmGluRA* expression specifically within the brain should be examined for lifespan effects. The brain should be an important tissue to study, since mGluR is highly expressed in the CNS in flies and also loss of DmGluRA affects learning and memory (Schoenfeld et al., 2013) highlighting the role these receptors play in these functions. Such tissue specific studies in the brain may confirm if there is a disconnection between healthspan and lifespan in these long-lived mutants. It may therefore be useful to include exploratory walking as a second locomotor behaviour to measure in future studies. Exploratory walking is thought to provide a better indication of cognitive function as it is involves more complex behaviours such as decision making, in addition to peripheral functions such as walking speed (Ismail et al., 2015). Studies have shown healthspan and lifespan can be disconnected (Fischer et al., 2016) therefore to establish if mGluR plays a role in healthy ageing, these further functional studies are required. As emphasised throughout this thesis, enhancing healthspan is the overall goal as this is what causes a decrease in the quality of life and increase cost to healthcare.

6.3 Conclusions

The main aim of this thesis was to understand the role of metabotropic glutamate receptors in healthy ageing and to determine the mechanism whereby loss of DmGluRA activity in *Drosophila* leads to longer lifespan. It can be concluded based on the investigations presented here that the effects of DmGluRA activity on lifespan depend on several intrinsic and extrinsic parameters including sex, genetic background and diet.

Molecular characterisation of a widely used deletion mutant for *DmGluRA*, *DmGluRA*$^{[112b]}$ identified the precise end-points of the deleted region within the *DmGluRA* locus and showed that homozygous mutants for this deletion still produce *DmGluRA* transcripts although at a much reduced level compared to controls. Sex-specific differences in *DmGluRA* expression were identified with higher levels of *DmGluRA* transcripts seen in male flies compared to female flies. Furthermore, the extent of reduction of *DmGluRA* transcript expression in *DmGluRA* mutants was more pronounced in female flies than male flies. However, no changes in *DmGluRA* transcript expression were observed during normal ageing in either sex.

Several additional phenotypes were also observed in *DmGluRA* mutants, including increased resistance to both oxidative and starvation stress, demonstrating that the physiological response to loss of DmGluRA activity is complex. However, many of the phenotypes identified did not correlate with the longevity effects of *DmGluRA* mutation except increased body weight which was observed under all conditions that promoted lifespan extension.

Transcriptomic analysis using RNA-seq identified genes which were differentially expressed in *DmGluRA* mutants relative to controls. Several of the gene expression changes identified could be aligned to phenotypic differences observed in the *DmGluRA* mutants. Among the differentially expressed genes were several encoding key components of the ribosomal RNA biogenesis and metabolism pathways suggesting that ribosomal RNA processing may be a differentially regulated in *DmGluRA* mutants. However, further molecular and cellular characterisation of this process within *DmGluRA* mutants was inconclusive.

As such, the precise mechanisms by which loss of DmGluRA activity extends lifespan remains unclear but further investigations are warranted. Understanding the functions of metabotropic glutamate receptor signalling on lifespan and age-related health offers the potential to identify pharmacological interventions that target this signalling pathway in order to delay the adverse effects of ageing.

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